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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Commercial - In Confidence

Grower Summary

FV 189a

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

Annual report 2006

Project title: **Development of detection systems for conidia of**

***Peronospora destructor* (downy mildew) in onion crops**

Project number:	**FV 189a**
Project Leader:	**Dr Roy Kennedy**
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Key workers:	**Dr R Kennedy** **Ms A. Wakeham**
Location of project:	**Warwick HRI, University of Warwick, Wellesbourne, Warwick** **Growers' holdings in Lincolnshire**
Project co-ordinators:	**Mr Philip Langley**
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Key words:	Downy mildew, onions, air-borne spores, disease, disease control

Signed on behalf of: Warwick HRI

Signature:……..…… ***Date***:…….
Name: Professor Simon Bright
Director and Head of Department

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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 improve onion downy mildew control in bulb and salad onion crops and prevent crop to crop transfer.
- Kits will be made available to growers in 2007. Contact HDC office for further information.

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. 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.

- 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 onion downy mildew spores are thought to be related to the times when disease spreads.

The expected deliverables from this project are:

- Better detection of onion downy mildew in the field before disease is visible in the crop.
- Detection tests which can be used “in field” to determine the level of risk to the onion crop posed by onion downy mildew.
- Less reliance on eradicant fungicide applications for onion downy mildew control.
- More effective use of fungicides with protectant modes of activity.

1.3 Summary of the project and main conclusions

Development of antibodies for detection of onion downy mildew spores

In year one and two of the work, specific antibodies which recognise downy mildew were developed using onion downy mildew spore washings (Fig 1). Two antibodies which recognised onion downy mildew spores were raised. Both antibodies (did not react when tested with other downy mildew species notably downy mildew on lettuce and downy mildew on Brassicas. Both antibodies 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).

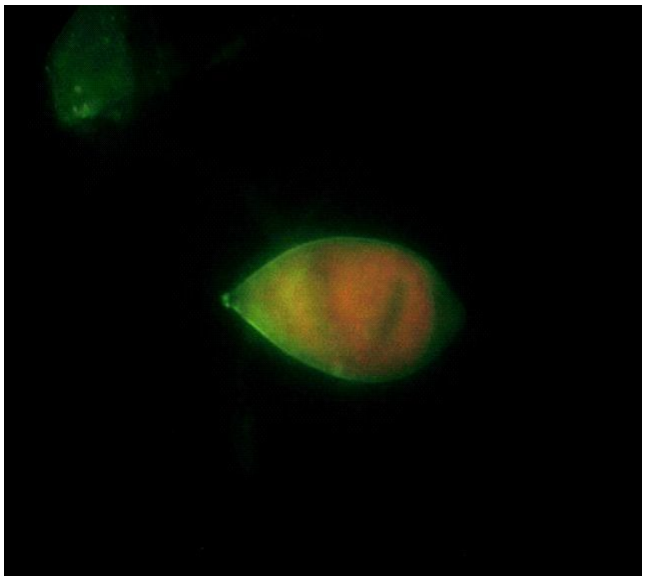


Fig 1. Immunofluorescence of onion downy mildew spore.

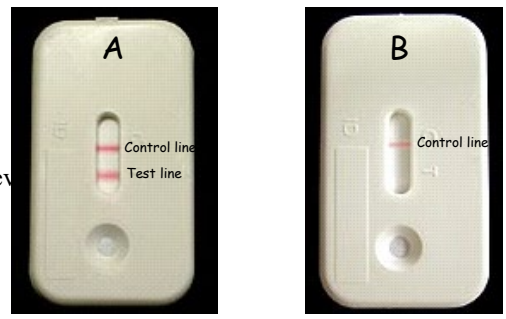
Development of a trapping system for airborne spores of onion downy mildew

Spores of onion downy mildew were trapped using an air sampling device (Fig 2). This device samples particles in the air and deposits them into tubes. Tubes containing air samples can be checked to determine the presence of onion downy mildew spores using rapid field based tests (Fig 3). The retention of spores in the sample tubes requires the development of suitable well coatings. Spore trapping studies were used to ascertain environmental factors required for spore dispersal and to assess spore trapping formats for detecting onion downy mildew spores. 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 spores in comparison to an untreated control well. Neither of these well coatings affected the detection process. The results of the trial show that the predictions of onion downy mildew spore production were accurate particularly those from the MILIONCAST system. The results indicated that when downy mildew spores 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 spores of onion downy mildew could be trapped within the vials in the air sampler and give accurate estimates of spore number when assessed by antibody methods (calledELISA).

Fig 2 Portable air sampling device for onion downy mildew conidia

Development of an in field test for onion downy mildew spores (lateral flow device)

A lateral flow test can be used to detect conidia of onion downy mildew. A competitive lateral flow (LFD) format was developed using antibodies developed within this project (Fig 3). The lateral flow test works on similar principals to the human pregnancy test. The competitive lateral flow device detected onion downy mildew spores in the vials in the air sampler. Trials in seeding crops of onions demonstrated that onion downy mildew spores could be detected within the crop for many weeks before symptoms were visible using the device.



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

Fig 3. Competitive lateral flow assay showing a negative sample result (A) and (B) a positive sample result.

Further tests with the prototype lateral flow device during 2006

Tests carried out with the onion downy mildew prototype lateral flow device during 2006 were not satisfactory. Due to extremely hot conditions which occurred in June and July 2006 no onion downy mildew was observed in the commercial trial crops or detected in the air sample tubes visually. An additional problem was the sampling regime used within commercial crops. Three day periods or even 24 H periods were too long to obtain samples which could be used with the current onion downy mildew prototype lateral flow device. Sampling times of 12 H used in trials at Warwick HRI were more successful in giving accurate positive results. These trials had the added benefit of having onion downy mildew development at the sampling site however this resulted in only four positive sample days which was still too few to properly test the device. Further testing with the device maybe required to ascertain the correct air sampling times and the need for any pre filtration steps with the sample before it is used in the lateral flow device.

1.4 Financial benefits

The main financial benefits will be in the use of the device to reduce unnecessary fungicidal applications to the crop. Fungicide usage is costly and is one of the major inputs in crop production. Using the lateral flow device the grower/consultant will be able to check for the presence of onion downy mildew in the air at sufficient quantities to cause disease development. For example in 2006 fungicide applications were applied to many crops when the risk of downy mildew was zero (using the test in conjunction with weather criteria for onion mildew development). Many applications were therefore not necessary. However the grower consultant had no means of determining what the real risk to the crop was. While weather conditions were shown to be marginally conducive there was no onion downy mildew inoculum present in the cropping system. There will be some initial outlay in purchasing the equipment necessary for the air sampling. However lateral flow tests are expected to cost approximately £ 4 – 5 per test. This cost must be compared with £15 – 20 per hectare for fungicide treatment. Saving will be variable between years and depend on the numbers of reduced sprays that can be achieved. The use of this approach as a means of forecasting onion downy mildew will be more reliable when compared to more conventional disease forecasting approaches based on weather conditions alone.

Expected financial deliverables are therefore:

- The usage of the “in field “ test for onion downy mildew will improve the timing of the first application of fungicide for controlling this pathogen in onion crops.
- There will be less 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.

1.5 Action points for growers

Specific action points for growers/consultants are further testing of the system within onion crops. Further lateral flow tests will be required to be manufactured. Additional tests of the system in different localities and regions and on different crops will be required. The use of the tests may be more applicable at certain times of year. Checking the early activity of onion downy mildew in bulb and over-wintered salad onions could be a useful starting point for the further use of the tests in the field.

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 a serious disease in bulb and salad onions and in onion seed production. Actual yield losses in bulb onions of 60 to 75% have been recorded (Cook, 1932, Cruickshank, 1958). These losses mainly result from severe infections in bulb onion crops causing early defoliation, reduced bulb sizes and poor storage quality of bulbs (Rondomanski, 1967). In salad onions, yield losses can be as high as 100% with whole crops being discarded as downy mildew symptoms on the plant make them unmarketable. Losses to seed production are frequently caused by the collapse of infected seed stalks and poor germination of seeds collected from infected stalks (Virányi, 1981). Fungicidal control of onion 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 onion downy mildew is dependent on the survival of spore populations until conditions become favourable for germination (Sutton & Hildebrand, 1985). However viability is affected by the prevailing temperature and humidity conditions. At 10^{o}C, spore viability is unaffected by relative humidity. However, at temperatures of 30^{o}C, viability declines rapidly at relative 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 downy mildew are produced are thought to be related to the times when disease spreads. Such direct relationship between airborne spore numbers and disease spread has been proposed for airborne fungal pathogens (Campbell and Madden, 1990), and have been found for *Mycosphaerella brassicicola* on Brussels sprouts (Kennedy *et al*., 2000). 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 onion diseases (BOTCAST/DOWNCAST/ONION$_{SPOT}$) (Gilles, *et al*., 2004) which can predict the early development of both downy mildew and *Botrytis* in onion crops. This system incorporates the DOWNCAST model (Jesperson & Sutton, 1987), which predicts sporulation and infection events of *P. destructor*. DOWNCAST predicts sporulation, but cannot predict the quantity of sporangia produced. Tests of the model in Canada suggested it gave positive predictions of sporulation for 38 out of 45 nights when sporulation was observed. However, in field tests in the Netherlands, DOWNCAST gave positive predictions of sporulation for only 11 out of 24 nights when sporulation was observed (de Visser, 1998). Thus, the model often failed to predict sporulation events in a north-west European maritime climate in which the weather conditions are highly variable. Battilani and colleagues developed ONIMIL, a forecaster, which is also based on DOWNCAST, and which gives a quantitative prediction for sporulation (Battilani, 1996).

A new model, named MILIONCAST (an acronym for 'MILdew on onION foreCAST'), was developed based on the data from controlled-environment studies investigating the effect of temperature and humidity on downy mildew sporulation. The rate of sporulation was predicted using controlled environmental data. This model is not yet available within ONION*SPOT* (the Warwick HRI modelling system for onion diseases) 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. Unnecessary fungicide applications, which are based on weather information alone, will be reduced especially during periods of high risk.

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 these criteria, in combination with disease forecasts based on weather information the number of fungicide applications could be reduced with no impact on disease development. Similar results were obtained for *Botrytis* blight (*Botrytis squamosa*) on onion

crops where thresholds of 15 - 20 conidia/m^3 could be used to reduce fungicide application by up to 100% (Carisse *et al*., 2003). Thresholds of inoculum required for disease establishment have also been reported for *M*. *brassicicola* which is the fungal pathogen responsible for ringspot on vegetable brassicas (Kennedy *et al*., 1999, 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.

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 begin to flow across the membrane. If the target antigen (onion downy mildew spore component) is present in the sample extract, antibody binding will occur to produce a coloured particulate conjugated antibody -antigen complex. Any antibody conjugated coloured particles that fail to bind to target antigen will attach to the immobilised test line as they traverse the membrane. If present at a high enough concentration, a visible line of deposited coloured particulates will form at the test line. The anti-species antibody will capture excess sensitised antibody / coloured particles to produce an internal control line, providing a visible confirmation of antibody / particulate flow. Sufficient antigen target presence (onion downy mildew spores), would induce complete inhibition of antibody attachment to the test line, a result that is indicated by a single line of coloured particle deposition (the control line). Two lines of equal colour intensity indicate a negative result.

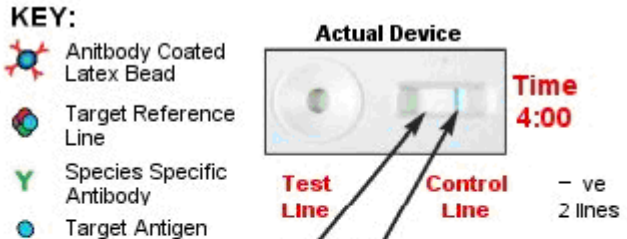


Figure 1. The Competitive lateral flow assay format.

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 (onion downy mildew), antibody binding will occur to produce a coloured particulate conjugated antibody -antigen complex. As this target complex passes over the test line capture of the antigen can occur, immobilising the antibody coated coloured particulates to produce a visible line of deposited coloured particulates at the test line. Excess coloured particulate material is captured at the control line, providing a visible confirmation of the success of the test. Two lines of equal intensity indicate a positive result.

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

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 onion downy mildew spores. No specific antibodies for onion downy mildew exist. This

report details the raising of specific antibodies to onion downy mildew and their incorporation within lateral flow tests which detect conidia of the onion downy mildew pathogen. Successful lateral flow test formats have been used to determine their accuracy. Cross-reaction of the test with spores of other pathogenic and non pathogenic species have been ascertained. These tests included 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. They should also remain non reactive to other biological and non biological particles. The level of reactivity of the antibody to onion downy mildew conidia is also important as this will affect the sensitivity of the test and how it can be used to quantify the number of onion downy mildew conidia present in samples. The third year of the work on this project will test "in field" lateral flow devices for onion downy mildew conidia which were developed in year two of the project. The comparison of air sampling systems for trapping onion downy mildew in crops of onions was also investigated. This needs to be accurate if quantification of onion downy mildew is to be achieved.

3. SUMMARY OF YEAR ONE WORK (2003/4)

3.1 Production of antisera to *Peronospora destructor*

3.1.1 Production of monoclonal antisera to *Peronospora destructor*

3.1.1.1 Introduction

The onion downy mildew pathogen (*Peronospora destructor*) is an obligate parasite which cannot be cultured on agar and will only grow and develop directly 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

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 3 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 48hrs. 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

A hand held Burkard surface cyclone sampler (Burkard Manufacturing Co., Rickmansworth, Herts, UK) was used to collect spores of *P. destructor* from the surface of the infected leaf material. The collection vessel containing the collected spores was removed and to it one ml of chilled sterile distilled water was added. Using a Gallenkamp Spin Mix the collected *P. destructor* spores were suspended in to the aqueous phase and a further volume of chilled water added. The spore suspension was filtered through a stainless steel membrane (47µm pore size) to remove any large contaminating material. The liquid phase was collected and bacterial and other small leaf contaminants removed by filtering using a polyester membrane (10 µm pore size). The filtrate was collected and resuspended in 1ml Phosphate Buffered Saline solution, pH 7.0 (PBS). Bright field microscopy was used to determine the presence of *P. destructor* conidia which was adjusted to a concentration of $2.5x10^4$ conidia ml^{-1}.

3.1.2.3 Immunization of mice with *P. destructor* spores

Five Balb C mice (coded PAS 7341…7345) were immunised (by intraperitoneal injection) each with 50µl of the *P. destructor* spore preparation mixed with an equal volume of Titermax adjuvant (Sigma T-2684). This process was repeated four weeks later. To determine the immune response of each mouse to the homologous immunogen preparation, tail bleeds were taken seven days later. Employing a plate trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) and an immunofluorescence assay (IF) the immune response of each

mouse to *P. destructor* was then determined. For each mouse the immune response was determined to be too low for monoclonal antibody development. To induce a high immune response it was decided to initiate a new set of mice and use an alternative immunogen format.

Freshly sporulating spore material of *P.destructor* was collected as described previously from bulb onions taken from the field. The collected spore material was suspended in 1ml of chilled sterile distilled water and held at 0- 4ºC while conidial counts were determined ($3.5x10^5$ *P. destructor* ml^{-1}). Following a further three hours at 0-4ºC the spore suspension was removed and, using a Gallenkamp spinmix, agitated continuously for a period of 5 minutes. To separate particulate spore material from the soluble spore fraction of the sample a microfuge (MSE Microcentaur) was employed at 13 r.p.m for 5 minutes. The soluble fraction of the sample was retained and concentrated at first by freeze-drying (Modulyo 4k, Edwards) and then rehydrating to a final volume of 100µl PBS. Two Balb C female mice (coded 7996, 7997) were immunized (by intraperitoneal injection) each with 50µl of the concentrated soluble *P. destructor* spore preparation mixed with an equal volume of Titermax adjuvant. All further immunizations were as described above. Tail bleeds were taken seven days after the second immunization procedure and a PTA-ELISA was carried out to determine whether the mice had produced an immune response to *P.destructor*.

The mice received a final pre-fusion boost of the *P. destructor* soluble spore immunogen mixed with adjuvant (100µl). The spleen of mouse 7996 was removed four days later (Mouse 7997 died shortly after the final fusion boost and could not be used for spleen fusion). The fusion was carried out according Warwick HRI standard protocol and the hybrids were fed on days 3, 6, and 10 days. Cell culture supernatants were screened by PTA ELISA and immunofluorescence 14 days after cell fusion for the presence of antibodies which recognised conidial spore components of *Peronospora destructor*.

3.1.2.4 Results

The immune responses from the five Balb C mice immunized with *P. destructor* spores collected from glasshouse cultivated onion sets, were not observed to their homologous immunogen (*P. destructor* whole conidia) when tested by PTA-ELISA. This was 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 (Figure 2). Positive hybridoma cell lines selected from post fusion screening of mouse 7996 are currently undergoing further evaluation for inclusion within rapid assay detection formats for *P. destructor* (downy mildew).

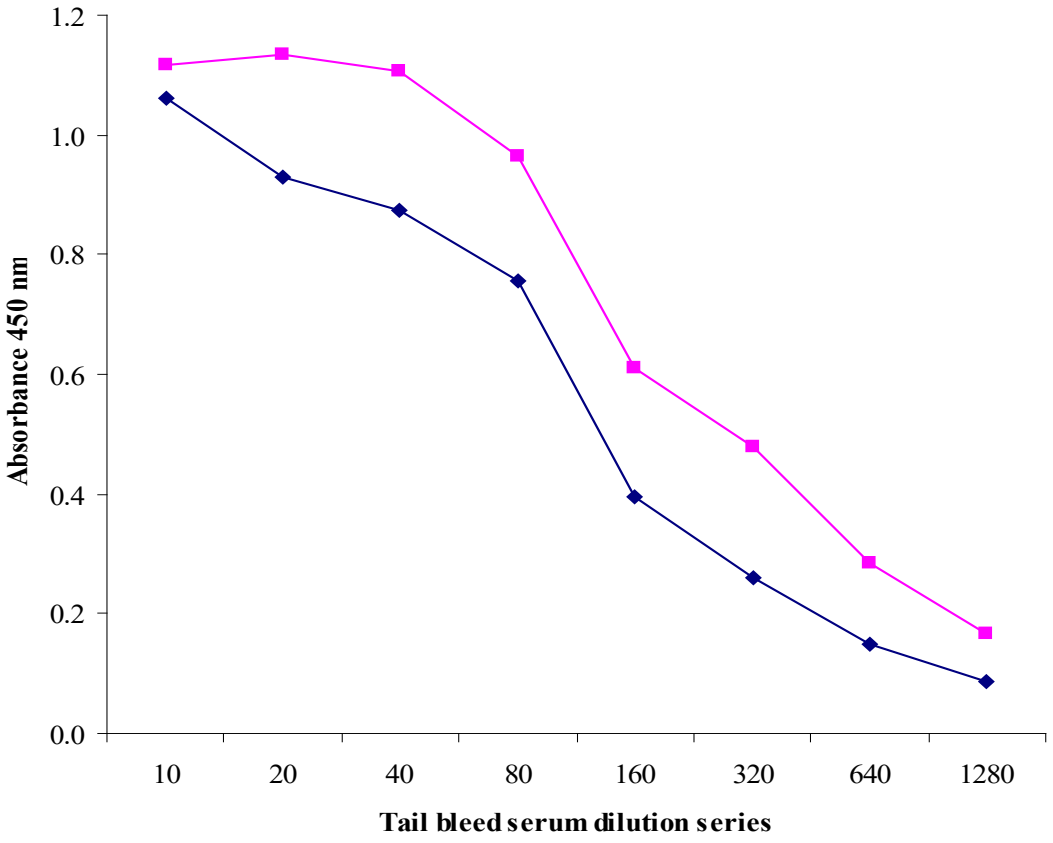


Figure 2. **Titration of mice tail bleeds (PAS 7996 and 7997) to freshly collected sporulating spores of *P. destructor* in Phosphate buffered saline (PBS) by PTA-ELISA**

3.1.2.5 Conclusions

Using A soluble concentrate (onion downy mildew spore washings) triggered an immune response by the immunised animals to *Peronospora destructor*. This would suggest soluble components released by the spore are highly immunogenic and that these may have been absent from the initial set of immunizations (mice 7341 - 7345) resulting from the pre-filtration of the immunogen that was used in these immunizations. Concentration of the soluble spore fraction, in the absence of whole spores, may have invoked a more targeted immune response in the immunized animal. The ability of selected monoclonal antisera to recognise soluble determinants of the *Peronospora destructor* spores should prove useful in the development of a lateral flow assay where antigen solubility is critical for movement during the aqueous stage of the test.

3.2 Production of polyclonal antisera to *Peronospora destructor*

3.2.1 Materials and Methods

3.2.1.1 Immunization of rabbit

A female White New Zealand rabbit (coded PAS 7346) received an initial immunization of a 500µl *P. destructor* spore suspension (2.6×10^4 conidia ml^{-1}) mixed with 500µl Freunds adjuvant. Over a six week period, the rabbit received a further three immune boosts at two weekly intervals, as described above. Ten days later a test bleed was taken and a PTA-ELISA was carried out to determine whether an immune response to the homologous immunogen had occurred. One week after the ELISA test a concentrate solublised *P. destructor* spore preparation (prepared as described in section 3.1.2.3) was mixed with an equal volume of Freund's adjuvant and injected into the rabbit. A test bleed was taken 7 days later and a PTA-ELISA carried out on it. Following confirmation of an increased immune response to the homologous immunogen (onion downy mildew spores) a final immune boost (solublised downy mildew spore components mixed with an equal volume of Freund's adjuvant) was given to the rabbit as previously described. Seven days later a terminal bleed was carried out and the serum collected (coded PAS 7346).

Using a standard protein A extraction protocol 20 ml of the collected blood serum was IgG purified and coded PAS 7346p. The IgG pufied serum was titrated against collected *P. destructor* spores by PTA-ELISA and its reactivity to downy mildew conidia determined. The whole blood serum and the IgG purified fraction was stored at -20ºC prior to use in 250µl aliquots.

3.2.1.2 Results

The initial immune response to the homologous immunogen of whole *P. destructor* conidia was low when tested by PTA-ELISA. Using solublized components of the *P. destructor* spore at the final stage of the immunization protocol increased the immune response of the rabbit. Nevertheless for each of the antisera tested (pre-immune and immune bleeds) a high level of background signal was observed when *P. destructor* conidia, collected from plant host material, was assayed by PTA-ELISA. Purification of the final immune serum bleed (coded PAS 7346p) to retain only IgG antibodies removed this problem while retaining a good level of reactivity to the downy mildew spores (Figure 3).

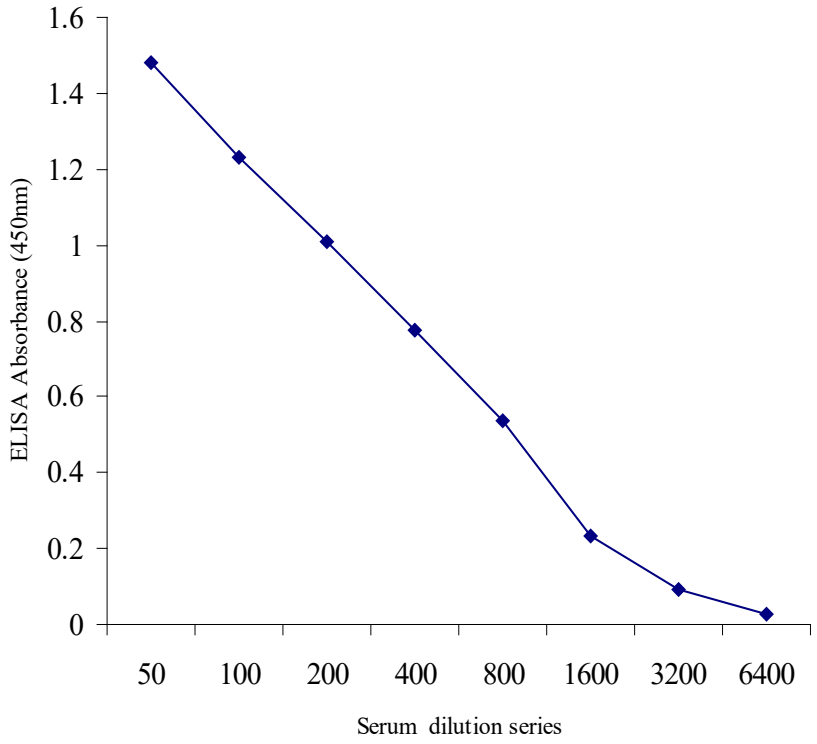


Figure 3. Titration of rabbit IgG purified polyclonal antiserum (coded PAS 7346p) to freshly collected downy mildew spores (*P. destructor*) in Phosphate buffered saline (PBS) by PTA-Protein A Horseradish peroxidase ELISA.

3.2.1.3 Conclusions

The collected antisera titration (assessed by PTA ELISA) indicated that the concentration of the soluble spore fraction, in the absence of whole spores, enhanced the final immune response (as observed with mice (see Section 3.1.2.4) of the rabbit to spore components of *P. destructor*. However employing a conventional PTA-ELISA a high level of background signal/binding was observed using both non-immune and immune serum. Employing a

Protein A Horseradish peroxidase secondary conjugate, in place of the secondary antibody phase, removed this problem. Protein A binds readily to the IgG antibody classes of rabbit but not to IgM type antibodies. IgM antibodies are produced predominantly during the early stage of the immune response and are generally associated with lower affinity and specificity to the target immunogen. IgG antibodies are produced in the second wave of the immune response and are considered generally to be of higher affinity for their target antigen and of increased specificity. The results confirmed this with background levels reduced to zero employing the Protein A PTA ELISA. Nevertheless the high background level observed in the initial ELISA format was unusually high. Onion downy mildew is however an obligate parasite and it is probable that host material is retained during the PTA ELISA assay process increasing the potential for interference within the assay format. In addition a host/antigen complex may exist which facilitates common binding sites for targeted antibodies. Employing an IgG purified serum may have enabled the removal of potential reactive antibody classes.

3.3 Cross reactivity tests with polyclonal antiserum (PAS 7346P)

3.3.1 Method

To determine specificity of PAS 7346p (purified IgG polyclonal) the serum was screened, by PTA-ELISA against a range of fungal species (Table 1).

Table 1. Fungal species used in a PTA-ELISA to assess reactivity of developed antisera PAS 7346p

Fungal species tested	Spores and mycelia present
Ascochyta rabiei	✓
Botrytis allii	✓
Pyrenophora teres	✓
Stemphyllium sp.	✓
Paecilomyces variotii	✓
Phoma lingum	✓
Botrytis cinerea	✓
Fusarium culmorum	✓
Penicillium roquefortii	✓
Peronospora parasitica	✓
Peronospora destructor	✓

Each of the fungal species used in cross-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 8 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 microtitre wells were blocked with 200 µl of 1 % casein buffer (1 % (w/v) casein PBS) and incubated at 37 º C for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200 µl PBS Tinc 0.05 % Tween 20 (PBSTincTw). Following this procedure wells received 100 µl per well of polyclonal antibody PAS 7346p (diluted 1:150 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 microtitre stips were incubated for 45 minutes 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).

3.3.2 Results

A level of reactivity was observed to a number of the fungal species tested (Figure 4). However no reactivity was observed to *Hyaloperonospora parasitica* which is the causative agent of 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).

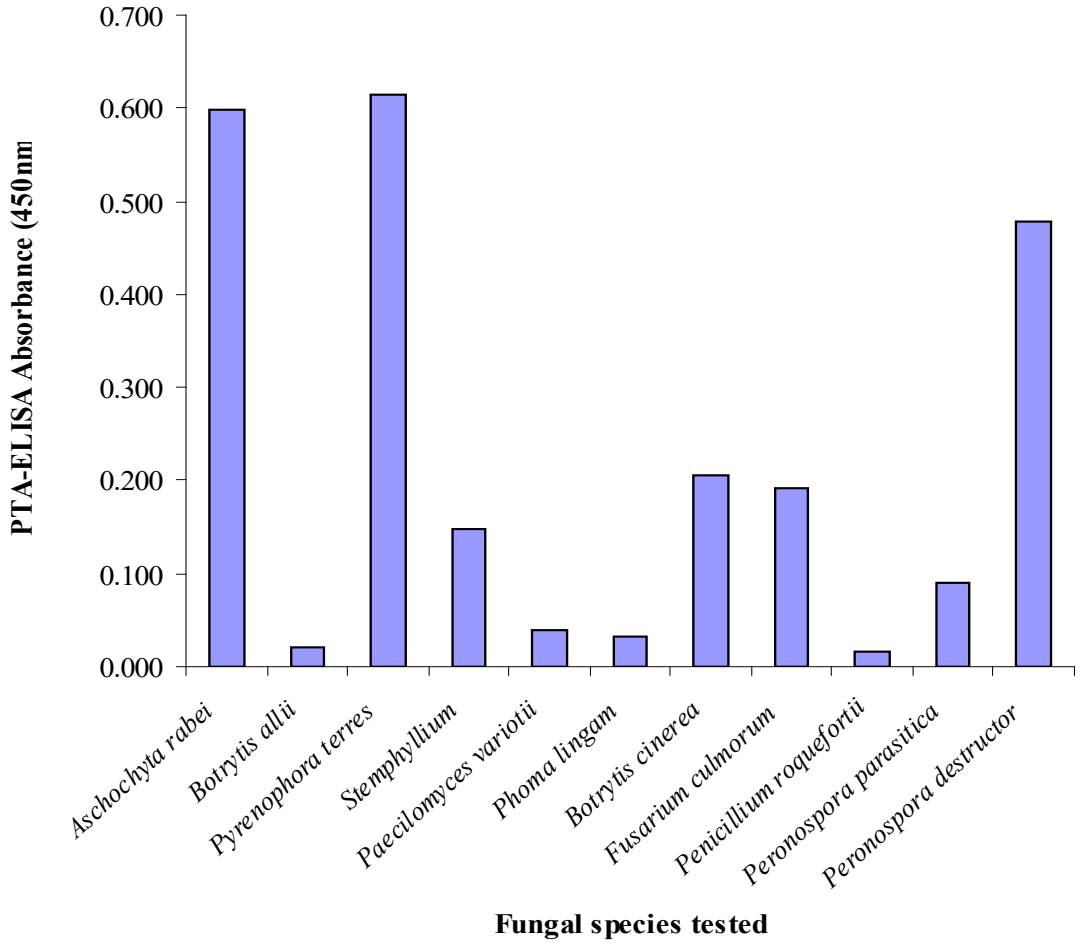


Figure 4. Cross-reactivity of polyclonal antibody PAS 7346p to a range of fungal organisms found in the field

3.3.3 Conclusions

The development of specific polyclonal antibodies which provide the required specificity for fungal diagnostics can prove problematical with levels of reactivity to other 'non-target' fungal species often observed. It was of interest that the IgG purified fraction of the antiserum (PAS 7346) did not react to *Hyaloperonospora parasitica*, an associated downy mildew found on vegetable brassicas. Nevertheless the affinity and sensitivity of the produced antiserum may be useful in the development of a lateral flow device for *Peronospora destructor* when used in conjunction with a specific monoclonal antiserum.

3.4 Determination of spore trapping periods and trapping formats for downy mildew on onion

3.4.1 Measuring the diurnal periodicity of downy mildew in air samples

3.4.1.1 Introduction

Relationships between weather and downy mildew sporulation and release in the air within a diseased crop indicates that warm conditions following periods of wetness are required. Measurement of sporulation under field conditions has been difficult as this will be affected by the degree and intensity of factors influencing spore release. Environmental factors governing the degree of spore release have not been measured for downy mildew on onion. For many diseases spore release during periods when there is no rainfall is inhibited by high humidity and triggered by falling humidity (Humpherson-Jones, 1991). For other diseases the effect of light and wetness are critical. The factors influencing spore release are often dependant on the mechanism of spore release. The mechanism (active or passive) of spore release also determines which environmental parameters will be important. Onion downy mildew spores are released passively. Detection of spores in the field using spore traps therefore depends on the time period when spores are usually found in the air and when the air-borne concentration is at a maximum.

3.4.1.2 Materials and Methods

3.4.1.2.1 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 2003 at HRI Wellesbourne. In June 2003 potted salad onion plants (two pots) infected with downy mildew weres 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 and August 2003. The crop was over-wintered to produce a seeding crop in spring 2004.

3.4.1.2.2 Spore trapping measurements

A 24 H volumetric spore traps (Burkard Scientific Ltd., Rickmansworth, Hertfordshire, England) were operated continuously (Hirst, 1952) within the plot to sample the air at 10 l min^{-1}. The trap was placed in the crop at first signs of disease and operated over 24 H periods when disease was visible on many of the seeded onion stalks. The trap was placed in a 2m diameter clearing in the centre of the crops with the orifice 40cm above the ground. The spores were directly impacted on to glass slides which were replaced in the trap at 24 H intervals. The date and time that slides were positioned in the trap was recorded. After each 24 H period slides were removed and spore numbers counted using a microscope. The numbers of spores trapped during each hour could be ascertained by the position of the

spores on the slide in relation to the initial impact point of the spore at the beginning of the period.

3.4.1.2.3 Micro-climate measurements

Measurements of temperature, humidity, leaf surface wetness and rainfall were collected at 30 min intervals from crop emergence using a SKYE Datahog II 7 channel data 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 spores in the air could be directly compared with corresponding environmental conditions.

3.4.1.2.4 Prediction of spore production in the field

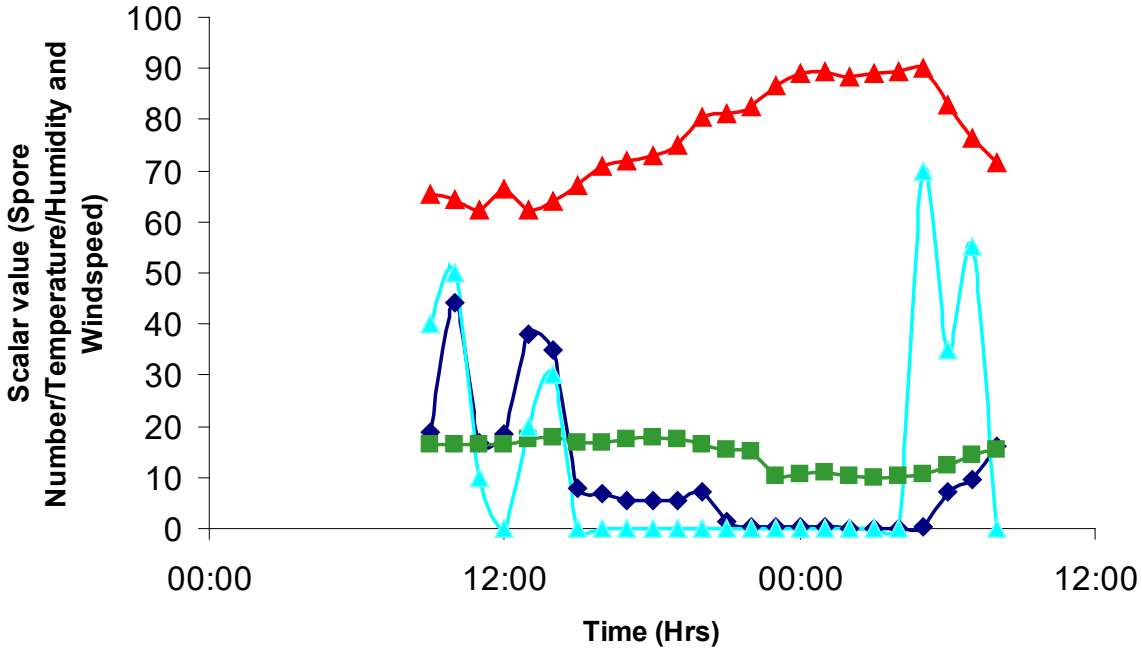
Disease forecasting models were used to predict when onion downy mildew conidia were produced under prevailing environmental conditions. The forecasting models used were DACOM and the Warwick HRI downy mildew sporulation model MILIONCAST. Both models use environmental data to predict if downy mildew conidia have been produced. Differences between the DACOM model and MILIONCAST are summarised in Gilles Phelps, Clarkson & Kennedy, 2004. Downcast predicts downy mildew conidial production when the air temperature was < 24 C from 08:00 – 20:00 on the preceding day and the temperature at night (20:00 – 08:00) was between 4 and 24 C with no rainfall. The relative humidity at night was greater or equal to 95 %. The output of the model is expressed as a plus or minus for downy mildew conidial production. There are specific variations to this model which have been produced (de Visser, 1998). The MILIONCAST model differs from all DACOM model types by being quantitative. Controlled environmental data was used to determine sporulation at temperatures of 92 – 100 % relative humidity and 5.4 C – 24.1 C.

3.4.1.2.5 Results

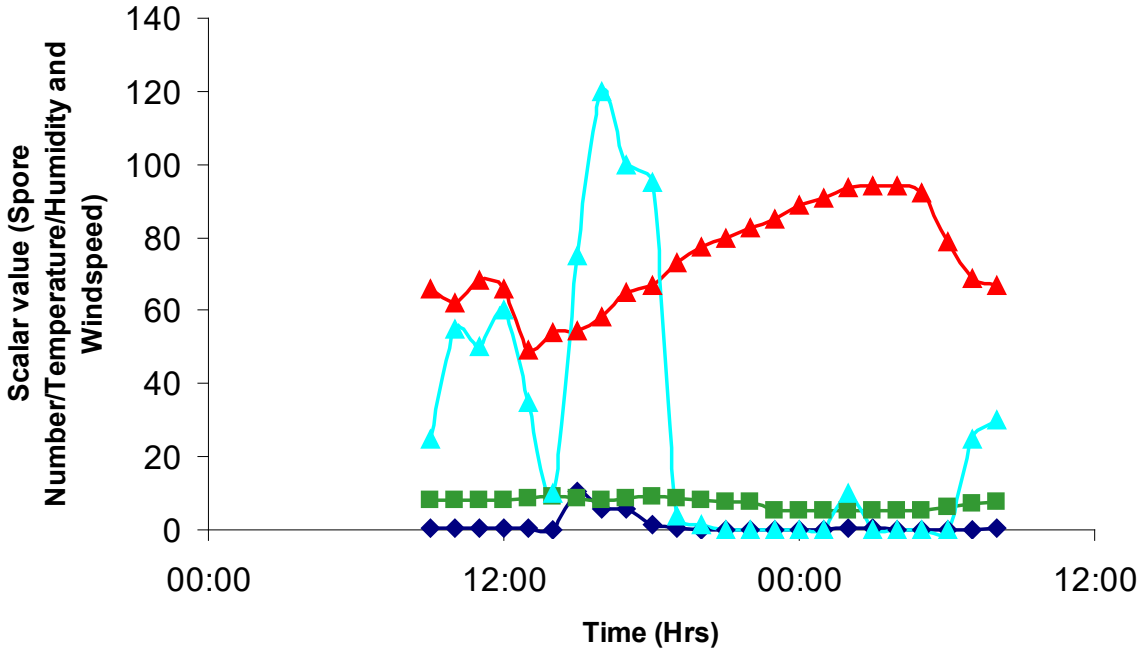
3.4.1.2.6 Environmental factors affecting periodicity of spore release by onion downy mildew

The effect of major environmental parameters on onion downy mildew release is shown in Figures 5 a, b and c. These figure show three days where spore production by downy mildew was predicted. The numbers of trapped downy mildew conidia are shown in relation to the diural variation in weather conditions over 24 h trapping periods. No data was presented for the effect of rainfall as none occurred during the trapping periods presented and in other trapping periods which are not presented. Additionally leaf wetness did not vary significantly throughout any trapping period. The environmental parameters presented in Figures 5 a, b, c were temperature, humidity and maximum windspeeds above 5 km/h for 5 sec periods. The results show that spores of the downy mildew pathogen were trapped after periods where windspeed increased to 5 km/h. There was no association between the trapping of onion downy mildew conidia and either leaf wetness or rainfall events. However these environmental parameters were more important in the production of downy mildew conidia. The results show that numbers of trapped downy mildew conidia increased during periods of increased wind.

a)



b)



c)

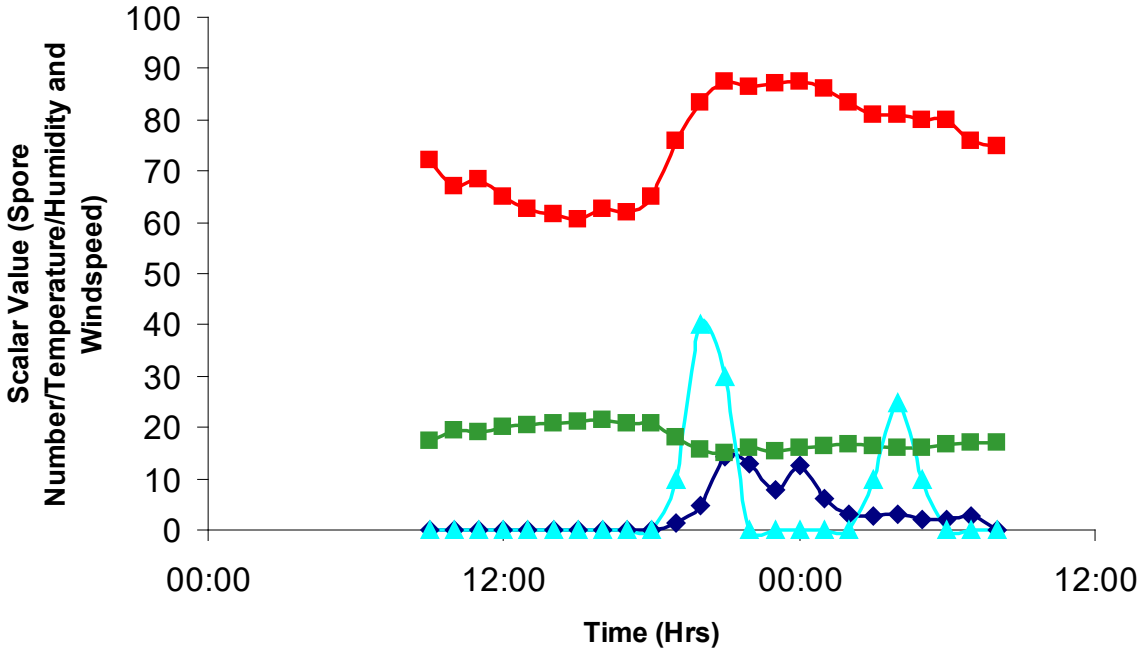


Figure 5. **Numbers of airborne conidia of downy mildew trapped x 10 $^{-2}$ (♦) over a) 3 June 2004, b) 4 June 2004 and c) 5 June 2004 and corresponding temperature (■), humidity (■) and x10 windspeed at 5 km h for 5 sec (▲) recorded at the monitoring site.**

However numbers of trapped downy mildew did not increase during all periods of higher windspeeds. Relative humidity also increased during periods of downy mildew dispersal.

3.4.1.2.7 Predictions of spore production and spore release by onion downy mildew

The predictions of downy mildew sporulation according to DACOM and MILIONCAST are shown in year one Annual Report FV189a. The two models used different environmental criteria to determine if sporulation by onion downy mildew had occurred in the crop. In this study the DACOM system predicted that there would be no sporulation by downy mildew in the crop over the trapping period (June 2004). Trapping did not commence prior to this period because there were very few signs of downy mildew infection on the crop. The MILIONCAST system predicted sporulation by downy mildew on the 1 June 2004, 22 June 2004 and 23 June 2004. MILIONCAST distinguishes between days where abundant sporulation is predicted and those with sporadic sporulation. Lower numbers of downy mildew conidia were predicted by MILIONCAST as being produced on the 22 June 2004.

3.4.1.2.8 Conclusions

All spore trapping studies for ascertaining environmental factors for spore dispersal were conducted in a seeding crop of bulb onions which was extremely susceptible to downy mildew infection and sporulation. The crop was checked regularly for downy mildew symptoms to ascertain when to begin the spore trapping study. However no downy mildew symptoms were observed until the 30 May 2004 at which point spore trapping studies commenced. 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. The results of the trial show that the predictions of onion downy mildew sporulation were accurate particularly those from the MILIONCAST system. The environmental requirements for downy mildew spore dispersal were particularly difficult to ascertain. Over a month long trapping period in a downy mildew infected crop there were few days when enough downy mildew conidia were trapped for comparisons with weather requirements to be made. One reason for this is that downy mildew conidia must be present if they are to be dispersed. However the environmental conditions required for spore production by downy mildew do not occur every night. Therefore even if environmental criteria are present for spore dispersal no spores are dispersed. The results show that when downy mildew conidia are produced, windspeeds of 5 km (over 5 sec bursts) are required for their dispersal. There is also potentially a requirement for increasing humidity. However many of these environmental conditions are related to each other for example humidity will change when temperature changes. Temperatures will be affected by windspeeds. Further data sets will be required to ascertain all the critical factors necessary for downy mildew dispersal. However other factors may also be involved. Other studies have indicated that alternation of light and darkness may also determine the time course of sporulation (Yarwood, 1937).

3.5 Evaluation of trapping formats for onion downy mildew in the field

3.5.1 Monitoring downy mildew conidia in air samples in a commercial crop of bulb onions

The available antibodies were evaluated for their ability to detect downy mildew in the air in a commercial crop of bulb onions. Only one trapping format was used in this trial.

3.5.1.1 Materials and Methods

Trapping inoculum of Peronospora destructor in a commercial crop of bulb onions
A field modified microtitre immunospore trap (MTIST), (Burkard Scientific Ltd., Rickmansworth, Hertfordshire, England) was operated continuously within a commercial crop of bulb onions sampling air at 57 l min^{-1}. The onion crop was grown from sets (cv.) and was sited near Sleaford and the trap was operated with the kind permission of the Allium and Brassica Centre. A description of the spore trap and its characteristics have been reported by Kennedy et al., (2000). Air borne spores were channelled through delivery trumpet nozzles and collected by impaction across the base of a microtitre strip (4 by 8 well microtitre strips). The trap was operated from 06:00 H to 16:00 H daily during the trial. The microtitre strips in the trap were changed twice weekly and sent to Warwick HRI for evaluation of onion downy mildew conidial presence.

Detection and quantification of collected spore samples
For each sampling period the total number of *P. destructor* spores in selected wells was determined using a Nikon model TMS inverted binocular microscope. Rapid quantification of trapped airborne inoculum of *P. destructor* spores was by PTA-ELISA employing PAb PAS 7346p as previously described in Section 3.2 Absorbance values (colour changes) could then be compared with the numbers counted by light microscopy.

Micro-climate measurements
See section 3.4.1.2.3 for details of environmental monitoring.

Prediction of spore production in the field
See section 3.4.1.2.4 for details of spore production prediction systems used in the trial.

3.5.1.2 Results

Prediction of onion downy mildew conidial presence
The predictions from the MILIONCAST and DACOM disease forecasting systems at Sleaford are shown in Table 2. There were differences in the predicted sporulation by onion downy mildew at the Sleaford site between the two systems. Predictions were calculated daily. There were consecutive periods of predicted sporulation (high risk) by both forecasting systems from the 8 May 2004 – 14 May 2004, 30 May 2004 – 6 June 2004 and the 20 June – 23 June 2004. Other individual days were sporulation by onion downy mildew may have occurred however according to both systems these may not have been significant.

Table 2. Predictions of onion downy mildew conidial production at Sleaford

Date (2004)	Predicted Sporulation	
	MILIONCAST	DACOM
25 April	+	-
26 April	-	-
27 April	-	-
28 April	+	-
29 April	-	-
30 April	+	-
01 May	+	-
02 May	+	-
03 May	-	-
04 May	-	-
05 May	+	-
06 May	-	+
07 May	+	-
08 May	+	-
09 May	+	-
10 May	+	+
11 May	+	+
12 May	-	+
13 May	+	-
14 May	+	-
15 May	-	-
16 May	-	-
17 May	-	-
18 May	-	-
19 May	-	-
20 May	-	-
21 May	-	-
22 May	-	-
23 May	-	-
24 May	-	+
25 May	-	-
26 May	+	-
27 May	+	+
28 May	-	-
29 May	-	+
30 May	+	-
31 May	+	-
01 June	+	-
02 June	+	+
03 June	+	+
04 June	+	+

05 June	+	+
06 June	+	-
07 June	-	-
08 June	+	-
09 June	-	-
10 June	-	-
11 June	-	-
12 June	-	-
13 June	+	+
14 June	-	-
15 June	-	-
16 June	+	+
17 June	-	-
18 June	-	-
19 June	-	+
20 June	+	-
21 June	+	+
22 June	+	+
23 June	+	-
24 June	-	-
25 June	-	-
26 June	+	+
27 June	+	+
28 June	-	+

Estimated numbers of downy mildew conidia using ELISA employing polyclonal antibody PAS 6346p

The higher the absorbance value, the greater the colour change indicating an increased reaction in the sample which may denote onion downy mildew conidial presence (for detailed daily absorbance values see year one Annual Report FV189a). The results show that generally higher values were recorded over periods when downy mildew conidia were predicted as having been produced. However no direct counts could be made in the wells.

3.5.1.3 Conclusions

The results indicate that there was a poor relationship between ELISA values and predictions of sporulation at the Sleaford site. However it is important to realise that the crop was sprayed with fungicides and no mildew was observed at the site during the trial (although it was reported in the area). The values obtained in the ELISA may therefore not be high enough to indicate a positive test for onion downy mildew. Further microscopic evaluation of the samples would help confirm this. Additionally the antibody reaction in the sample vessels may result from cross-reactivity. Polyclonal antibody PAS 6346p has not been extensively tested for its cross reactivity although it does strongly react with onion downy mildew conidia. These tests ideally should use monoclonal antiserum which as yet is not available. The trap contains four individual test strips for each sampling period. One strip has been used to generate the results which leaves 3 strips available for further evaluation with other antibodies.

3.6 Monitoring airborne inoculum of the downy mildew pathogen in an inoculated overwintered seeding onion crop

3.6.1 Materials and Methods

Monitoring downy mildew in air samples in relation to plant infection

An over-wintered bulb onion field plot (15 m x 15 m) heavily infected with downy mildew (*P. destructor*) was monitored continuously for the presence of downy mildew spores in the air using a conventional Burkard 24hr glass sampler and a reverse Burkard cyclone sampler (Burkard Scientific Ltd., Rickmansworth, Hertfordshire). The traps were was placed in a 2m diameter clearing in the centre of the crops with the orifice 40cm above the ground. The spores collected by the 24hr sampler were directly impacted on to glass slides which were replaced in the trap at 24 H intervals. The date and time that slides were positioned in the trap was recorded. After each 24 H period slides were removed and spore numbers counted directly using a microscope. A glass slide which exhibited a high level of downy mildew spores was identified and further processed by immunoflourescence (IF). The spores sampled by the reverse cyclone trap were directly impacted on to the sides of an eppindorf collection tube. The eppindorf tube in the cyclone sampler was replaced at the same time as the glass slide in the 24hr glass slide sampler. The field exposed tubes were stored at -20ºC prior to analysis by PTA ELISA.

Detection of onion downy mildew conidia in air samples using Immunofluorescence

An exposed glass slide exhibiting a high downy mildew spore concentration was submerged in a volume of antiserum (PAS 7346p) diluted in Phosphate buffered saline solution, 0.05% Tween 20 and 0.1% Casein (PBS TC). Following an incubation period of 45 min at 37ºC the slide was removed and washed three times in PBS TC. After which the glass slide was placed in a solution containing an anti-rabbit fluorocein conjugate (Sigma F-0382) mixed with counterstains Evan's blue (Sigma E-0133) and eriochrome black (Stigna E-2377) and diluted in PBS TC. The slide was incubated as described above in darkness. The slide was washed as described above, air-dried in darkness and mounted in DAKO fluorescence mounting oil. Using an excitation filter of 450-490nm and a barrier filter of 520-560nm, the slide was viewed by a Nikon Optiphot-2 microscope with episcopic-fluorescence.

Detection of downy mildew conidia in air samples by PTA ELISA

To each eppindorf tube 400ul PBS was added and, using a Gallenkamp Spinmix set at high, the samples were agitated for a period of 2 minutes. After which a 100µl sample was aliquoted into each well in each microtitre strip. Using an inverted microscope the total number of downy mildew spores (*P. destructor*) was determined. Following an overnight incubation at 4ºC any unbound contents of the microtiter wells were removed by gently flicking the polysorp plate against absorbent towelling. To block unbound sites each well received 200ul 1% Casein in PBS. The remainder of the ELISA was as described above in Section 3.3.1

3.6.2 Results

Detection of downy mildew conidia in air samples using Immunofluorescence

The antiserum PAS 7346p labelled components of the spore wall of *Peronospora destructor* which, with the addition of an anti-species fluorescein conjugate, enabled quick identification and quantification of the target downy mildew spore (Plate 5). Reactivity of the antiserum was noted however to a number of other trapped air spora but was at a low level.

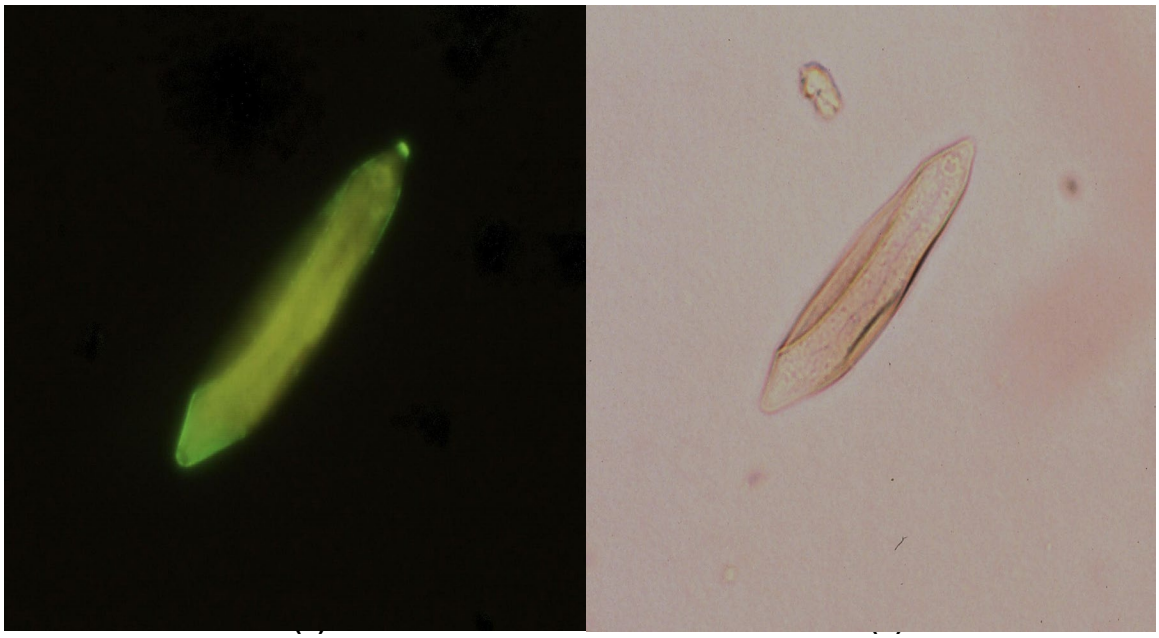


Plate 5 Onion downy mildew conidia as visualised by (a) immunofluorescence using polyclonal antibody PAS 7346p and (b) using bright field microscopy

Detection of downy mildew conidia in air samples using ELISA

In the initial three weeks of the field monitoring period a good correlation was observed between the observed number of downy mildew (*Peronospora destructor*) spores trapped (glass slide observations) and the results of rapid quantitative test (MTIST PTA-ELISA (Figure 6). However in the final week of the trial this relationship changed.

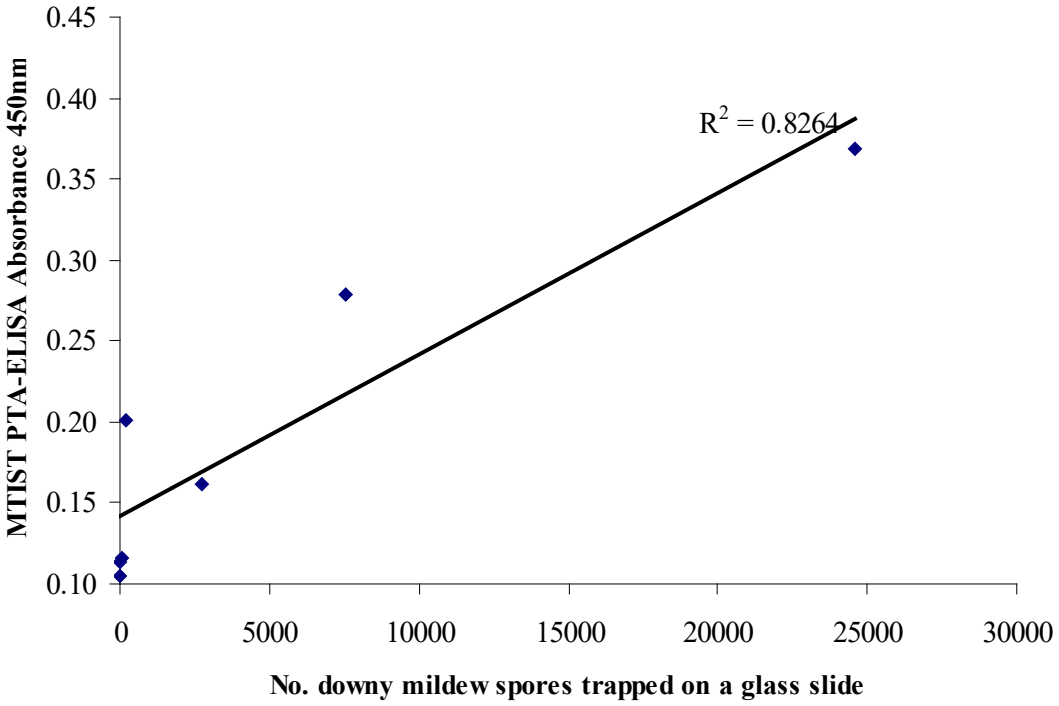


Figure 6. The relationship between the number of onion downy mildew conidia and absorbance from PTA ELISA.

3.6.3 Conclusions

Preliminary field studies, employing the MTIST spore trap, demonstrated the potential for a rapid quantitative test for the measurement of airborne downy mildew spores. Nevertheless the immunoflourescence field results (glass slide) indicated that the antiserum used (PAS 7346p) reacted to a number of other spore types present. Towards the end of the field trial a high level of *Stemphyllium* spora was observed. Reactivity tests show an interaction between PAb 7346p and this spore type (Section 3.3.2). Incorporation of a monoclonal antibody in to the test format which, recognises specifically *Peronospora destructor* (onion downy mildew) conidia, should remove this problem and enable the development of a rapid, reliable quantitative assay.

4. SUMMARY OF YEAR TWO WORK (2004/5)

4.1 Production of monoclonal antisera to *Peronospora destructor*

4.1.1 Materials and Methods

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

See section 3.1.2.1

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

See section 3.1.2.2

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

See section 3.1.2.3

4.1.1.4 Monoclonal Antibody Screening

4.1.1.4.1 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 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 incubator (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).

4.1.1.4.2 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 selected hybridomas were cloned twice to achieve monoclonal antibody status.

4.1.1.4.3 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. parastica*, *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 microtitre 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 microtitre wells were blocked with 200 µl of 1 % casein buffer (1 % (w/v) casein PBS) and incubated at 37 º C for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200 µl PBS Tinc 0.05 % Tween 20 (PBSTincTw). Following this procedure wells received 100 µl per well of 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 microtitre 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).

4.1.2 Results

4.1.2.1 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

7). The Immunofluorescence test determined that antibodies were present in the initial test bleed of each mouse to components of *P. destructor* conidia (Plate 6).

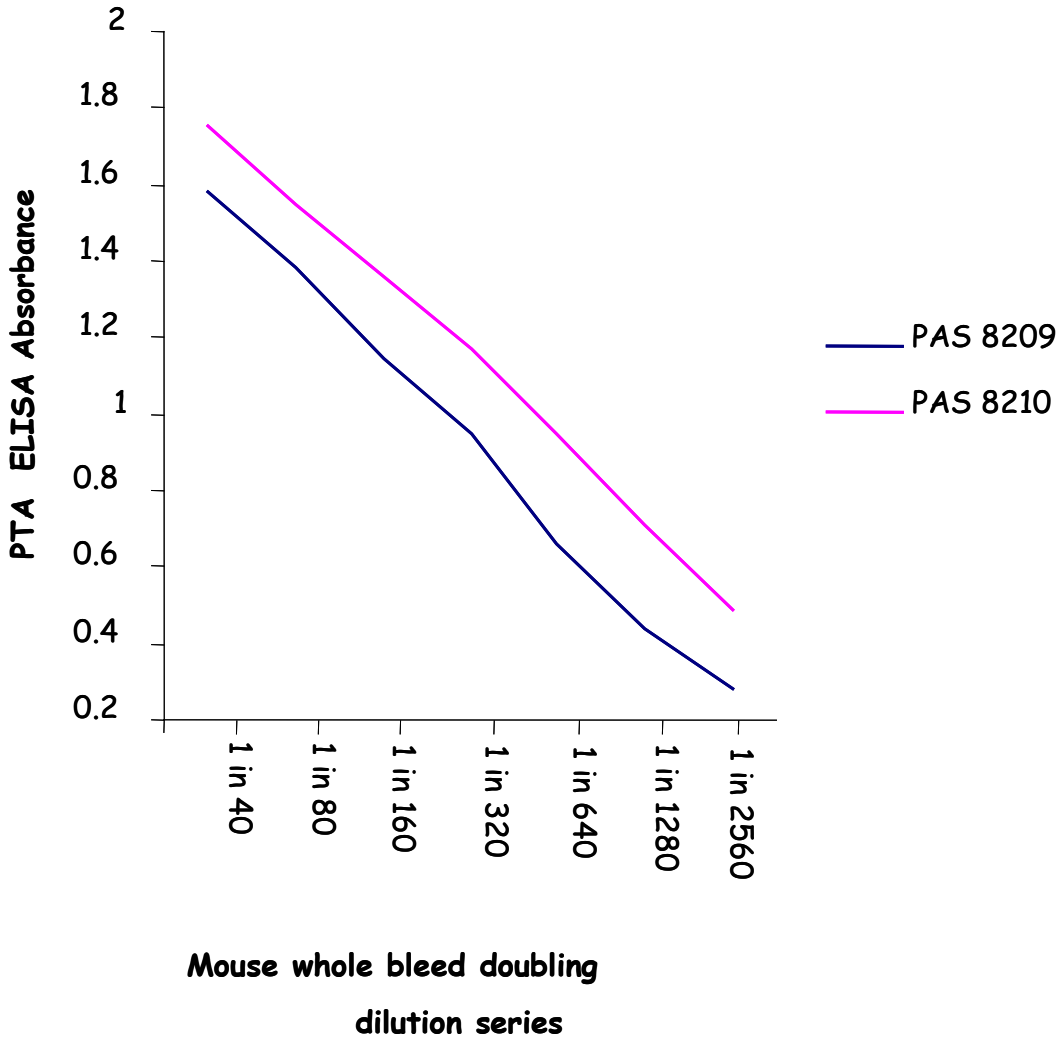


Figure 7 disrupted conidial spore soluble fraction of *P. destructor* to homologous antigen by PTA ELISA.

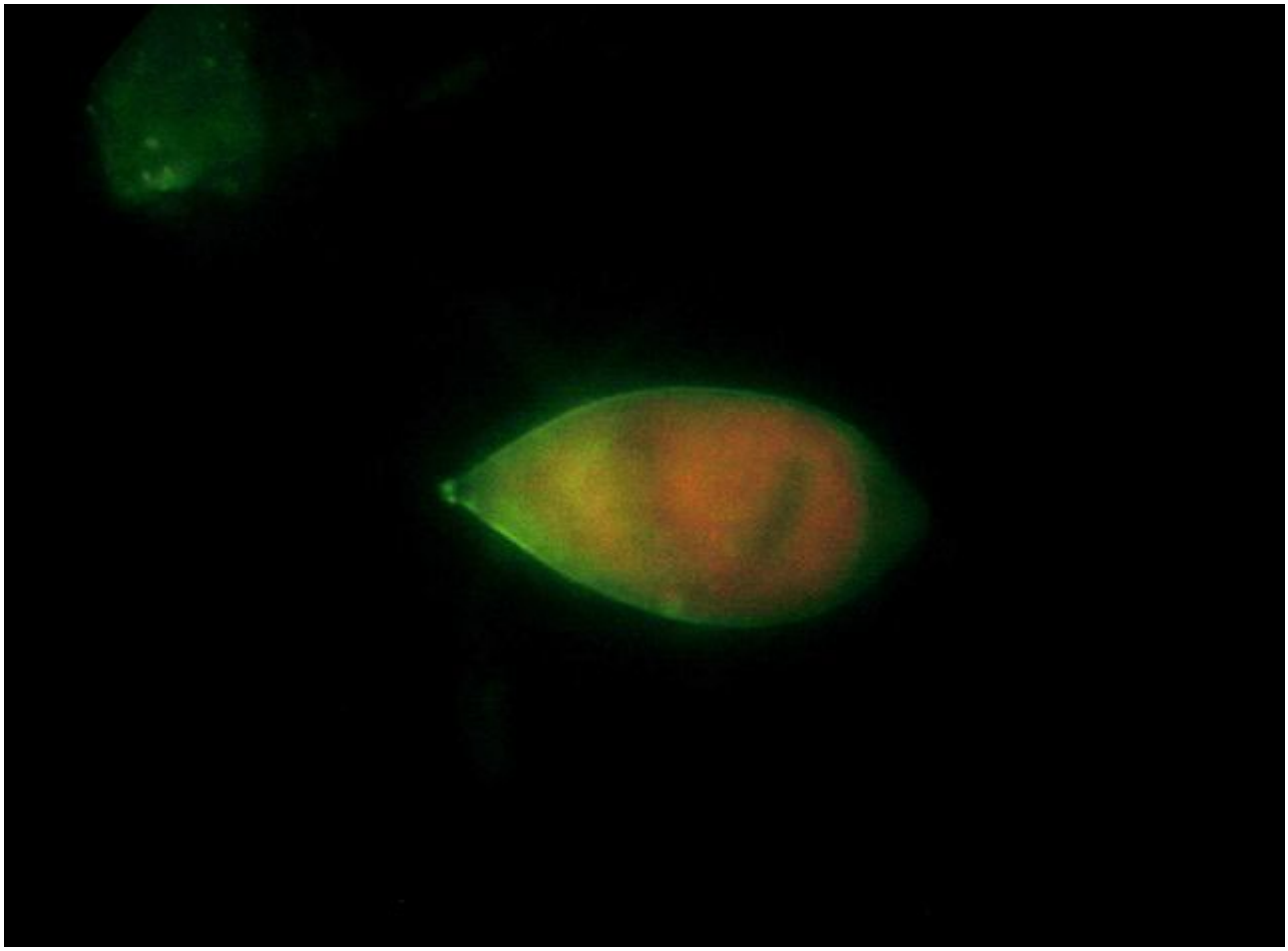


Plate 6. Immunofluorescence of onion downy mildew conidia using fluorescein tagged monoclonal antibodies .

4.1.2.2 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). Monoclonal antibodies EMA 242 and 243, with the exception of their homologous antigen (*P. destructor*), demonstrated a high level of specificity to all fungal species tested (Figure 8).

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 6) 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.

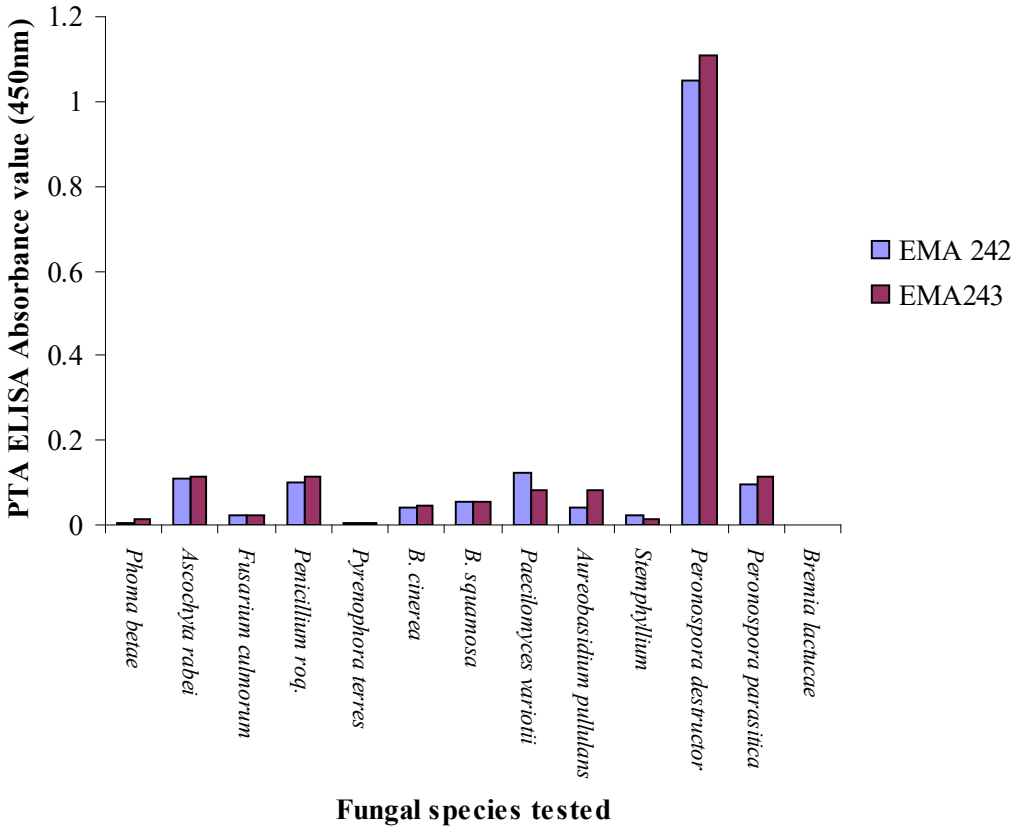


Figure 8 Reactivity of monoclonal antibodies EMA 242 and 243 to a range of airborne fungal species as tested by PTA ELISA.

Table 3. 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

4.1.3 Conclusions

A high 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 (Figure 2). 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*.

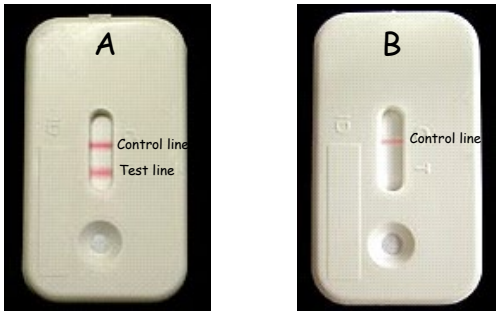
4.2 Development and optimisation of lateral flow for detection of *Peronospora destructor* (downy mildew on onions).

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 (onion 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.

4.2.1 Development and optimisation of lateral flow device for detection of conidia of *P. destructor*

4.2.1.1 Competitive assay test format and procedure

The absence of a test line represents a positive result a competitive lateral flow test format. As in the DAS format a control line is observed to demonstrate successful test operation (Plate 7).



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

Plate 7. Competitve lateral flow assay showing a positive sample result (B) and (A) a negative sample result.

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.

4.2.1.2 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 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 membranes were air dried at 35ºC for a period of 4 hours. The test and control line labelled lateral flows were cut in to 4 mm strips and each strip housed within a plastic case (Schleicer and Schuell, Germany). 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 4).

Table 4 Antibody dilutions in the sample pad and corresponding *P. destructor* test line

concentration

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**

4.2.1.3 Results

At a test line application was at a 500ug ml^{-l} 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 competitive lateral flow device. However at an antibody dilution of 1: 640 line development was barely visible.

At a test line application of 250ug ml^{-l} 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^{-l} 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.

4.2.1.4 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 lateral flow device format where test development proved unsuccessful. The competitive assay is used most often when testing for small molecules with single antigenic determinants, which cannot bind two antibodies simultaneously. 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 a *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.

4.2.2 Detection threshold of a competitive lateral flow device 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 5a 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.

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 5a and 6b). The mixture was applied to the competitive lateral flow device sample pad of each competitive lateral flow device 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.

4.2.2.1 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 competitive lateral flow device (Plate 8). 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 competitive lateral flow device 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 6). 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 8).

Table 5a 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 5b 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)

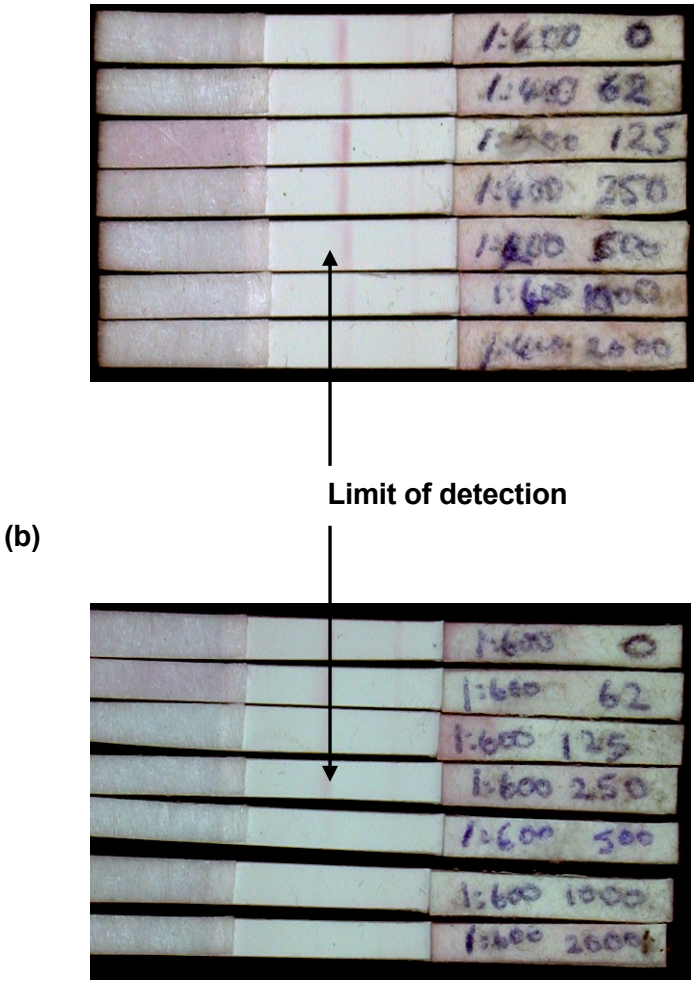


Plate 8 Development of a competitive lateral flow for onion downy mildew conidia (a) competitive lateral flow test employing detection antibody concentration of 1: 400 (b) competitive lateral flow test employing detection antibody concentration of 1 : 600

4.2.2.2 Conclusions

In the tests carried out above two membrane types were examined for their use in a competitive lateral flow device 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 has 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 competitive lateral flow device 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.

4.3 Evaluation of microtitre well coatings for optimisation of onion downy mildew trapping

4.3.1 Materials and Methods

4.3.1.1 Microtiter immunospore trap (MTIST)

The spore trapping equipment used in this study (MTIST) is manufactured by Burkard Manufacturing Company (Rickmansworth, Herts, UK) (Plate 9). 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 9). The collected impacted target particulates may, if appropriate antibodies are available, be immunoquantified by PTA ELISA.

Plate 9 Outdoor Microtiter Immunospore Trap (MTIST)

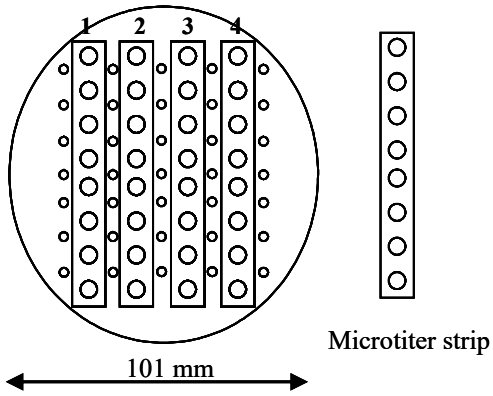


Figure 9 Schematic drawing of MTIST base plate and microtiter strips.

4.3.1.2 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 (catalogue 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.

4.3.1.3 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.

4.3.1.4 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

4.3.1.5 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).

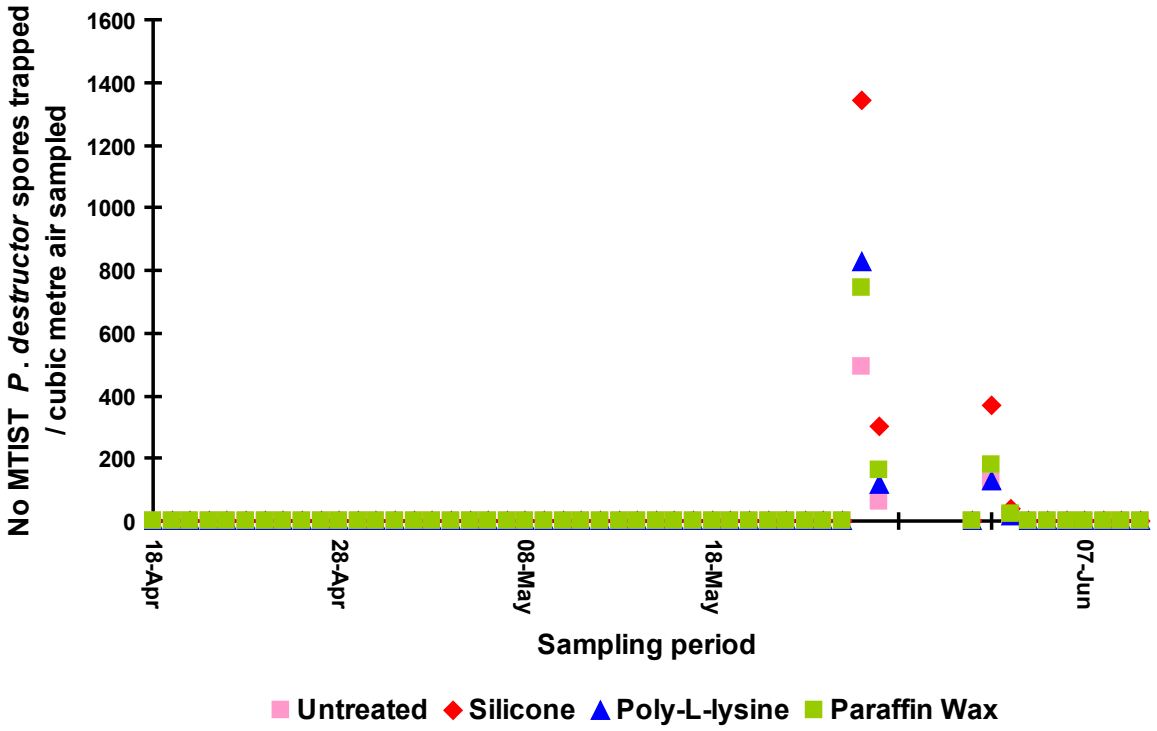
4.3.2 Results

4.3.2.1 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 10). In all instances the use of silicone proved superior, collecting 60 % more spores than the untreated and approx. 50% more than both the Poly-L-Lysine or Paraffin wax coated wells.

Figure 10 Number of onion downy mildew conidia trapped per cubic metre of air in the

field using the MTIST trap with varying micotitre well coatings.



4.3.2.2 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 11). 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 12, 13) however greater differentiation was observed between the upper and lower detection limit of the test employing the silicone coated wells (Figure 12). Poly-L-Lysine proved inhibitory to the PTA ELISA test and had a correlation with the PTA ELISA of $r^2 = 0.0465$. (Figure 14).

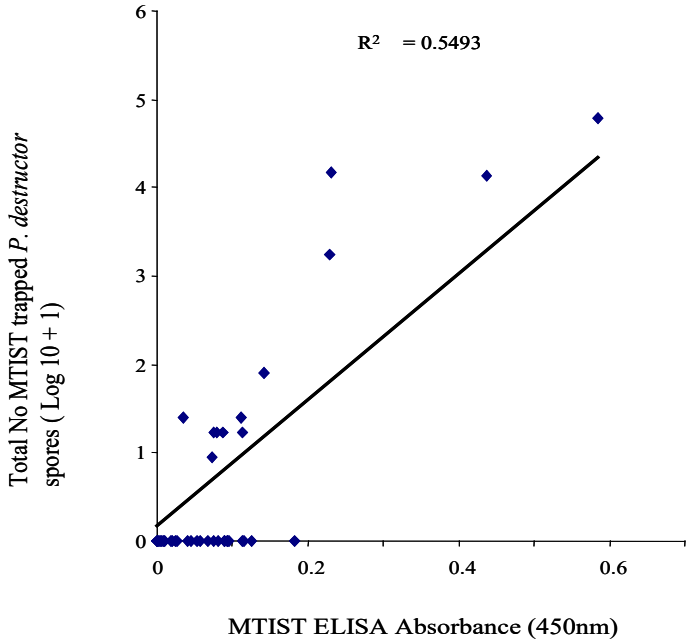


Figure 11 Relationship of MTIST trapped spores per m^3 of air sampled and the absorbance value generated post PTA ELISA using paraffin coated microtitre wells

Figure 12 Relationship of MTIST trapped spores per m^3 of air sampled and the absorbance value generated post PTA ELISA using silicone coated microtitre wells

Figure 13 Relationship of MTIST trapped spores per m^3 of air sampled and the absorbance value generated post PTA ELISA using distilled water to coat microtitre wells

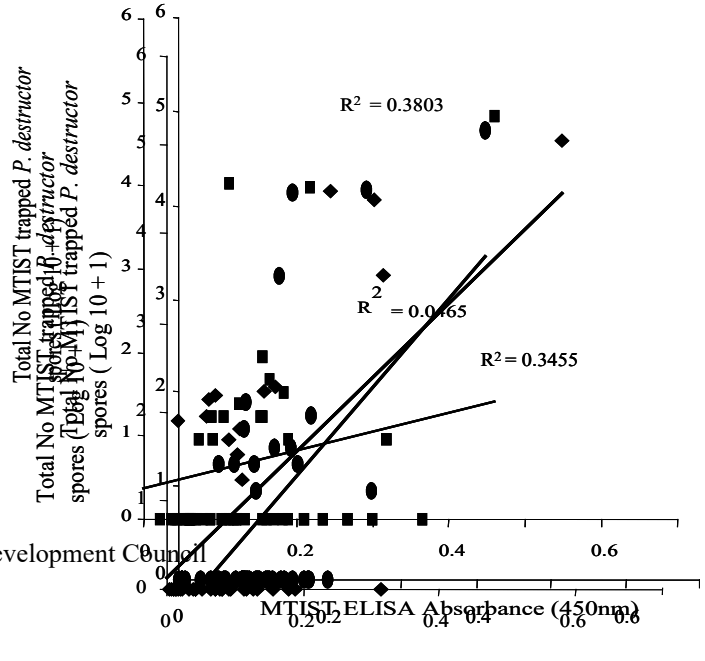


Figure 14 Relationship of MTIST trapped spores per m^3 of air sampled and the absorbance value generated post PTA ELISA using poly-L-lysine coated microtitre wells

4.3.3 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.

4.4 Comparison of trapping formats and weather based disease forecasts for onion downy mildew in the field

4.4.1 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 .

4.4.1.1 Materials and Methods

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

4.4.1.1.1 Infected bulb onion crop production

See section 3.4.1.2.1 for details

4.4.1.1.2 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 microtitre strips (catalogue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, Paisley, Scotland) was changed daily after 18:00 H. 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 (Basilidon Chemical Company Ltd, Abingdon, Oxfordshire). 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 microtitre well. The sampler contains four microtitre strips (catalogue 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.

4.4.1.1.3 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.

4.4.1.1.4 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).

4.4.1.1.5 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.

4.4.1.1.6 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.

4.4.1.1.7 Micro-climate measurements

See section 3.4.1.2.3 for details of environmental monitoring.

4.4.1.1.8 Prediction of spore production in the field

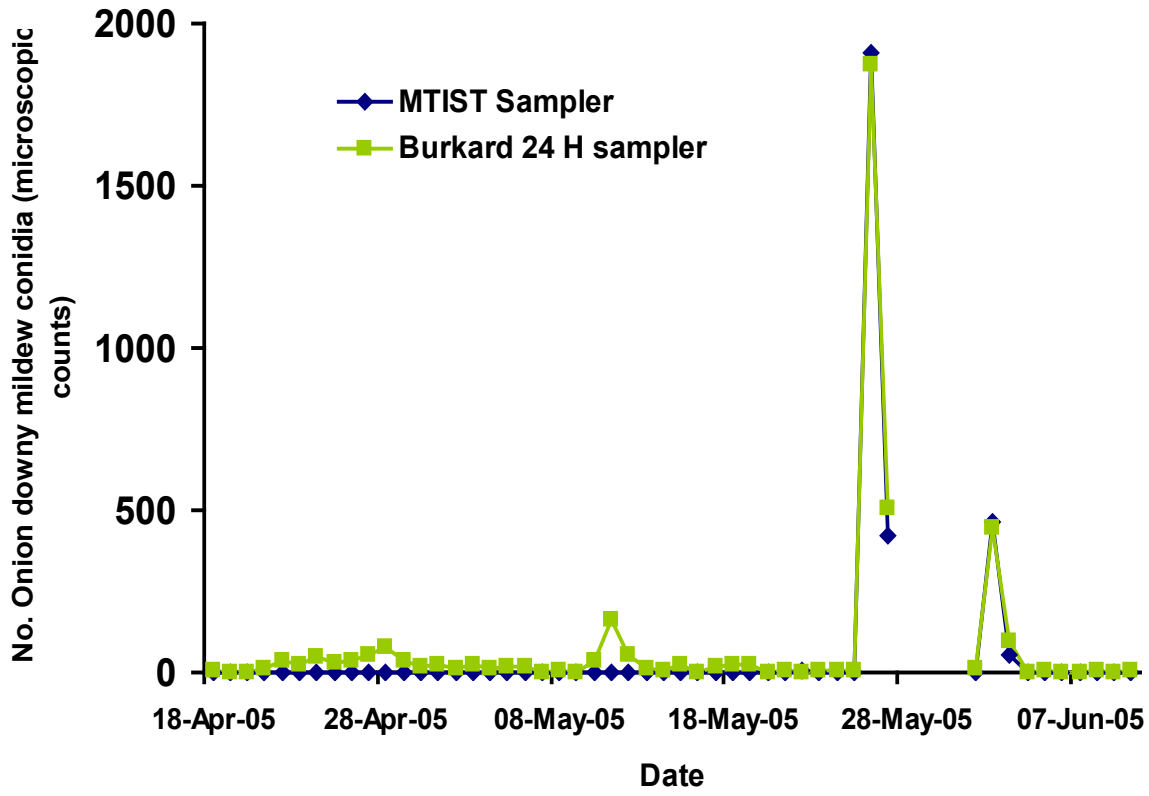
See section 3.4.1.2.4 for details of spore production prediction systems used in the trial.

4.4.1.2 Results

4.4.1.2.1 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 15) 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 15 Comparison of the numbers of trapped onion downy mildew conidia in the field using a Burkard 24 H volumetric sampler and an MTIST sampler

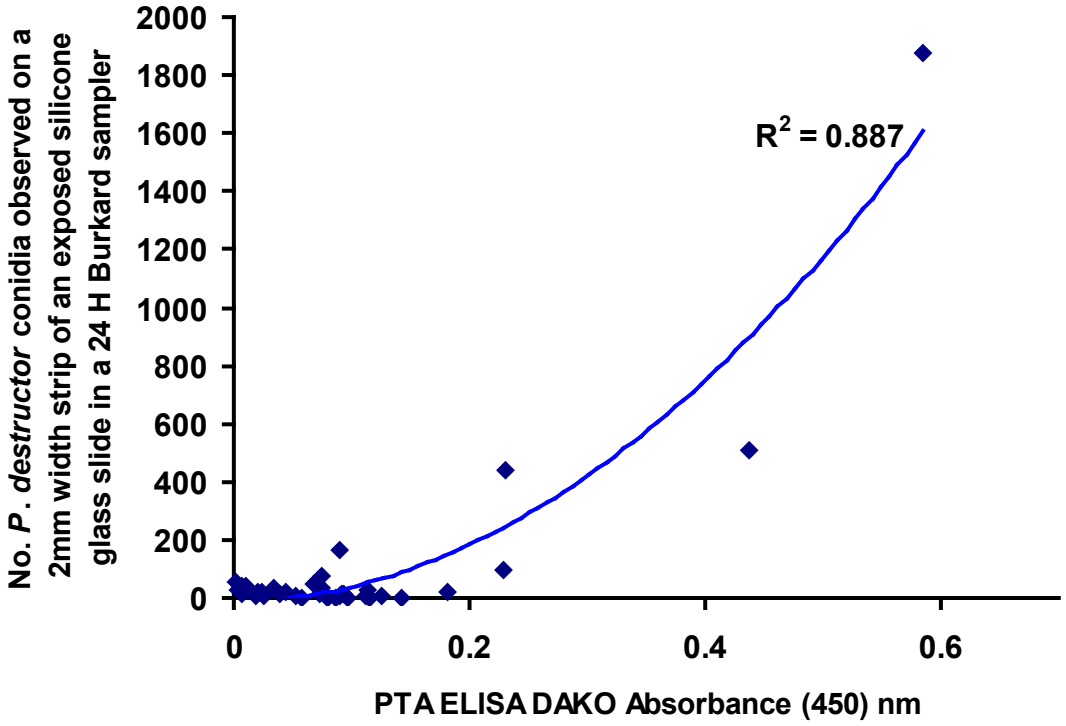


4.4.1.2.2 Comparison of onion downy mildew conidial counts using 24 H volumetric and MTIST samplers

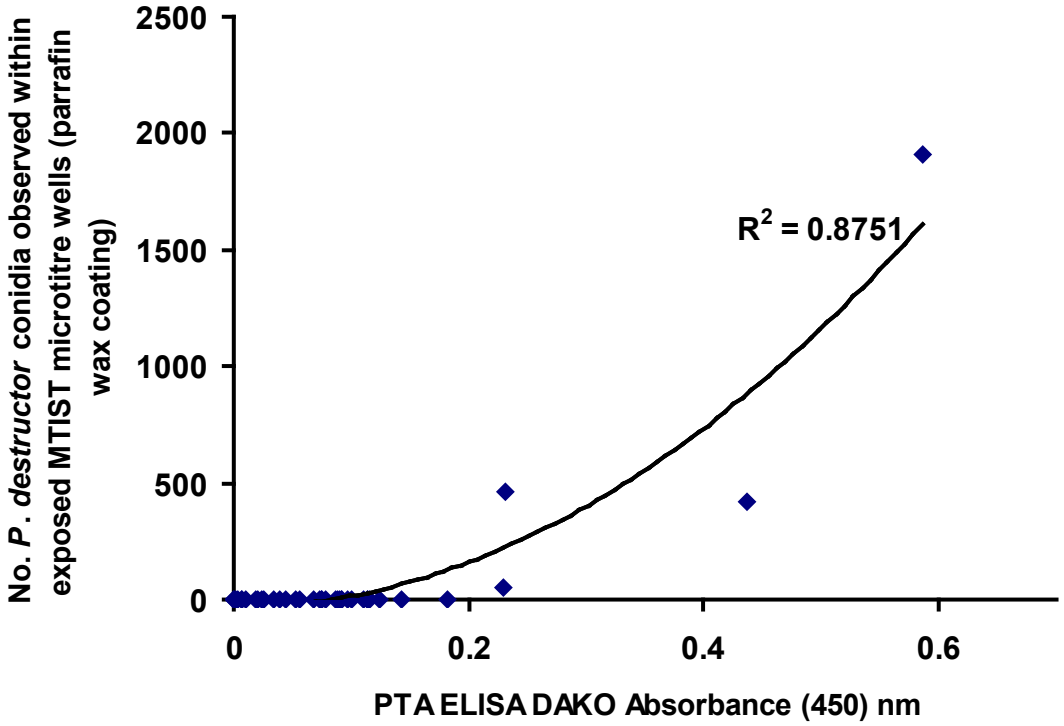
Comparison of the numbers of spores observed in each sampler from microscope counts against MTIST PTA ELISA of microtitre strips coated with paraffin wax hexane is shown in Figures 16a and 16b. Paraffin wax was used in this comparison because it proved to be the most effective well coating for retaining onion downy mildew conidia (see section 3.4) in comparison poly-l-lysine and silicone well coatings and an untreated control.

Figure 16 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 16a). 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

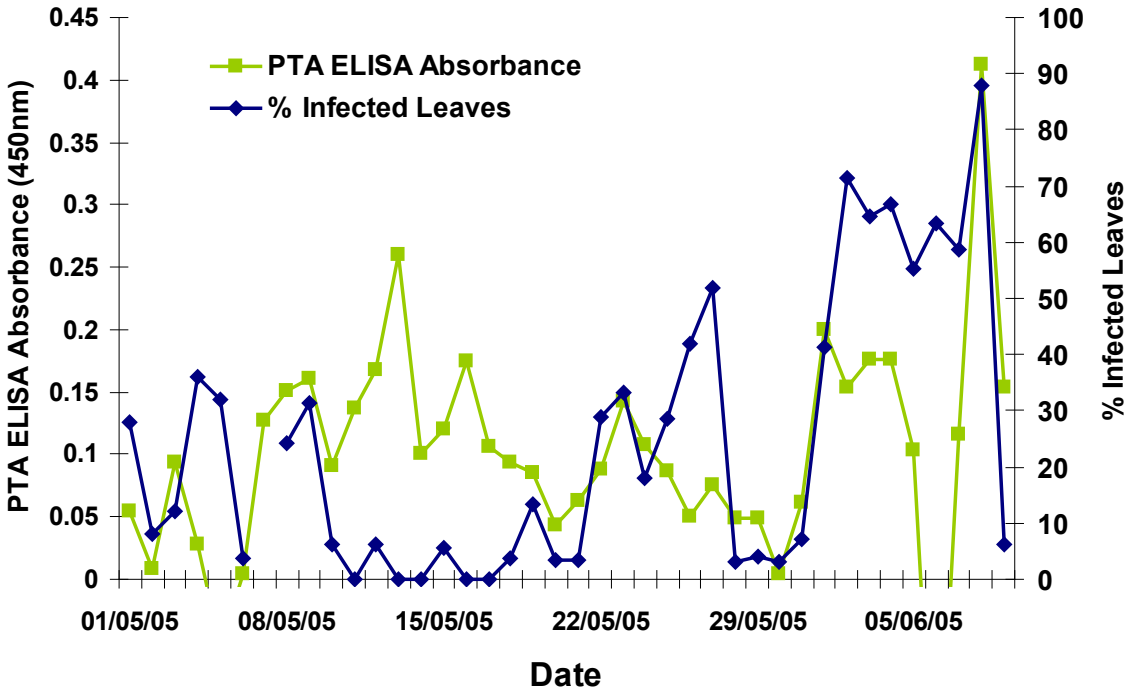
well coated with paraffin wax/hexane using an MTIST air-sampler.(Figure 16). Absorbance values of 0.2 indicated that there were high numbers of onion downy mildew conidia present in the slide or in the microtitre well.

4.4.1.2.3 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 17. 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 17)

Figure 17. Relationship between PTA ELISA and % Infected leaves of plants exposed for 24

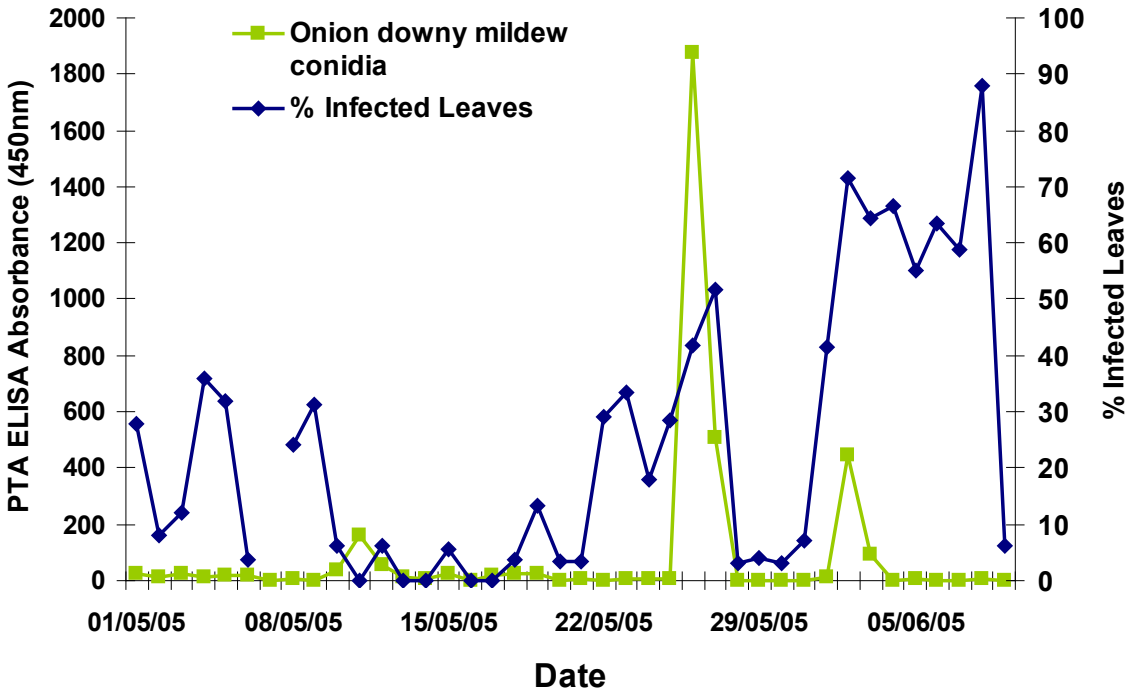
H periods within the infected plot (before 48 H leaf wetness).



Comparisons between % infected leaf number and onion downy mildew conidial counts are shown in Figure 18. 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 18). 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 3.4.1.2.6). For this reason the number of onion downy mildew required to initiate infection could not be ascertained.

Figure 18. 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).

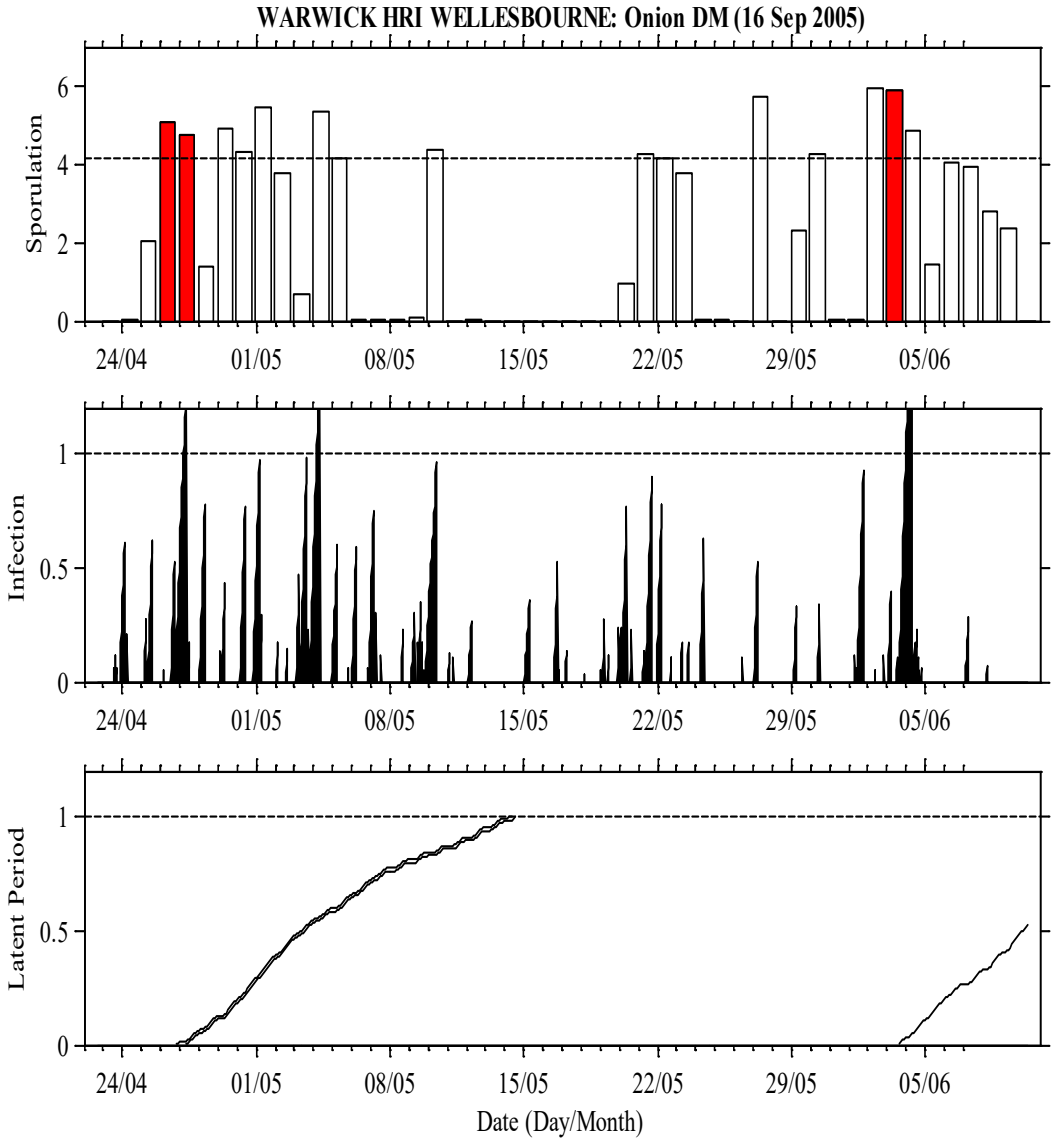


4.4.1.2.4 Prediction of onion downy mildew conidial presence

The results of using the Warwick HRI onion downy mildew forecasting system are shown in Figure 19. There were many periods where onion downy mildew sporulation was predicted. Sporulation *P. destructor* was predicted if the bar representing each day (Figure 19) reached the threshold value of 4.15 as represented by the dotted line in Figure 19 (upper bar chart) 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 19. 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

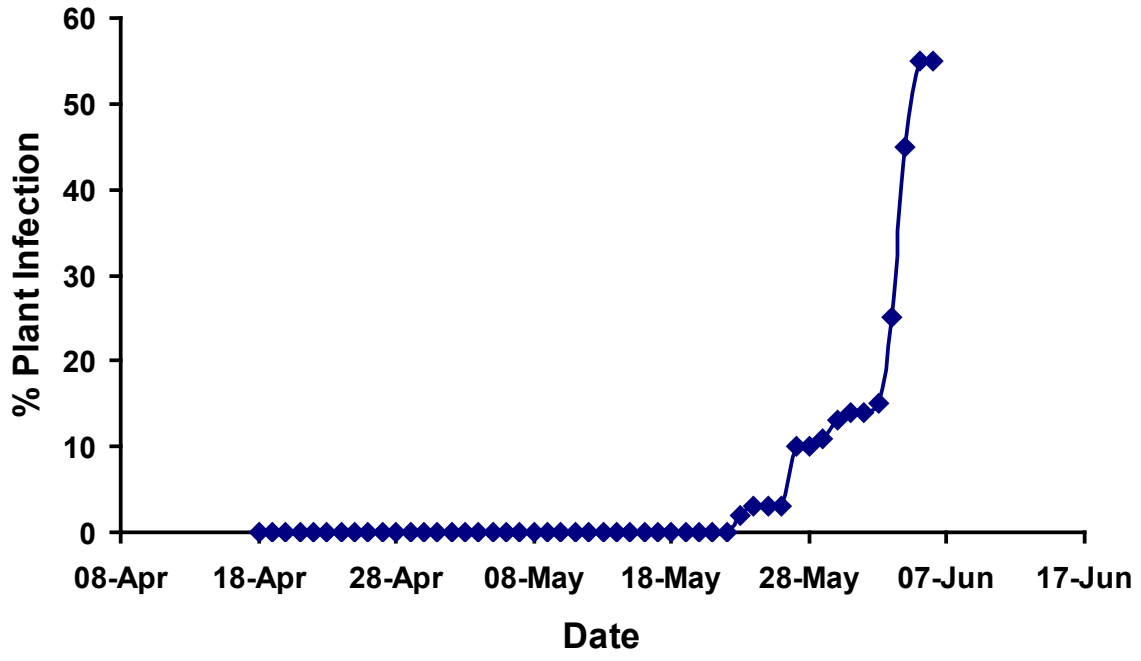


4.4.1.2.5 Onion downy mildew development within the field plot

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 section 4.4). There was no onion downy mildew observed on plants within the plot until the 23 May 2005 Figure 20). 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 20).

Figure 20. Onion downy mildew development (% plant infection) within an over-wintered crop of bulb onions at Warwick HRI in 2005.



4.4.1.3 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.

5.0 COMPARISON OF TRAPPING FORMATS AND WEATHER BASED DISEASE FORECASTS FOR ONION DOWNY MILDEW IN A SEEDING CROP OF BULB ONIONS

5.1 Monitoring onion downy mildew in air samples in a seeding crop of bulb onions

A major component of “in field tests” for onion downy mildew is the trapping system used to sample onion downy mildew conidia in the air. The available trapping formats investigated within year two of the project were further investigated in year three. Trapping formats were evaluated for their ability to detect onion downy mildew conidia in air samples in a seeding crop of bulb onions at Warwick HRI, Wellesbourne during April, May and June 2006. Two trapping formats were compared in this trial with two types of vessel coating. These were compared to disease observations on crop plants.

5.1.1 Materials and Methods

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

5.1.1.1 Production of a bulb onion seeding crop.

See section 3.4.1.2.1 for details

5.1.1.2 Air samplers used in the trial

Two types of air sampler were used in a field trial to compare different trap types in their accuracy in trapping onion downy mildew spores. An MTIST sampler was operated for 12 H periods form 06:00 H to 18:00 daily. This was compared to a Burkard 24 H volumetric trap which ran continuously over each 24 H period. The MIST trap containing microtiter strips (catalogue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, Paisley, Scotland) was changed daily after 18:00H. The Burkard 24 H volumetric trap used a glass slide which had been coated with silicone (Basilidon Chemical Company Ltd, Abingdon, Oxfordshire). In this trap air flow is 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 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 (catalogue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, Paisley, Scotland) each containing eight wells. Two types of well coating preparations were used (on each of 2 strips): 0.05 % Sodium Azide and silicone which following melting was mixed with hexane as previously described and, a well coating of distilled water. One hundred µl of each single coating solution was applied to each well of 60 microtitre strips (catalogue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, Paisley, Scotland). This process was repeated for both the coating preparations. After treatment the coated microtitre well strips were secured within ELISA multiframes (Catalogue No. 9503060, Life Technologies Ltd, Paisley, Scotland) and incubated at 20 ºC for 1 hour, after which any unbound material was removed by inverting the microtitre strips and tapping them down on to absorbent towelling. An inverted binocular microscope (Nikon model TMS) was used to check that the well coatings had been applied evenly. Prior to field exposure the microstrips were stored at 4ºC in a sealed container. Air flow through the sampler was estimated in still air by measuring the air speed at different points across the inlet manifold using a hot film anemometer (Air velocity transducer model number 8460, TSI Incorporated, St Paul, MN, USA) and integrating over the area of the inlet. In the tests reported here, the volume flow rate through the device was measured at 57-litre min^{-1}. The MTIST sampler was operated daily for 12 H periods (06:00H – 18:00H) as previous studies had shown that conidia of onion downy mildew were present in air samples only during daylight hours (See section 3.4.1.3.1).

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). Slides used in this trap were given a coating of silicone applied using a glass edge coated with silicone drawn over the surface of the slide exposed to the air stream in the trap. This was used to enhance numbers of conidia of onion downy mildew on the slide.

5.1.1.3 Enumeration of trapped spores in air samplers

Spore collecting tapes were removed from the Burkard 24 H volumetric trap and permanently mounted on glass microscope slides using "Mowoil" (supplied by Burkard Manufacturing Co., Rickmansworth, UK) and glass coverslips. The numbers of conidia of onion downy mildew was determined by bright field microscopy using a Zeiss binocular microscope (x 400) and scanning an 8 mm^2 area of the tape by counting spores in transverse sections across the tape. Spore concentrations (spores per m^3 air sampled) were calculated from the spore counts using the trap flow rate, the time of exposure and, the fraction of the area counted for each tape (British Aerobiology Federation, 1995). The total number of individual spores of each spore type deposited on the base of each microtitre well was counted by using a Nikon model TMS inverted binocular microscope (x 200). Onion downy mildew conidia in the MTIST microtitre wells were also determined using ELISA (see section 4.3.1.5). Spore concentrations were calculated from the numbers of spores trapped in each of the microtitre wells and the volume of air sampled by the MTIST spore trap.

5.1.1.4 Detection of 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 wells of each strip each received 100 µl of monoclonal Ab EMA 242 (raised at Warwick HRI to *P. destructor*), 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).

5.1.1.5 Monitoring onion downy mildew incidence within the field plot

A total of 7 plants were assessed in each plot for disease incidence (presence or absence of onion downy mildew sporulation) on a leaf basis. At each assessment time the presence of onion downy mildew sporulation on each plant was recorded. Crops to be examined at daily intervals. The presence or absence of onion downy mildew sporulation was recorded at each assessment time.

5.1.1.6 Micro-climate measurements

See section 3.4.1.2.3 for details of environmental monitoring.

5.1.1.7 Prediction of onion downy mildew infection, sporulation and latency in the field

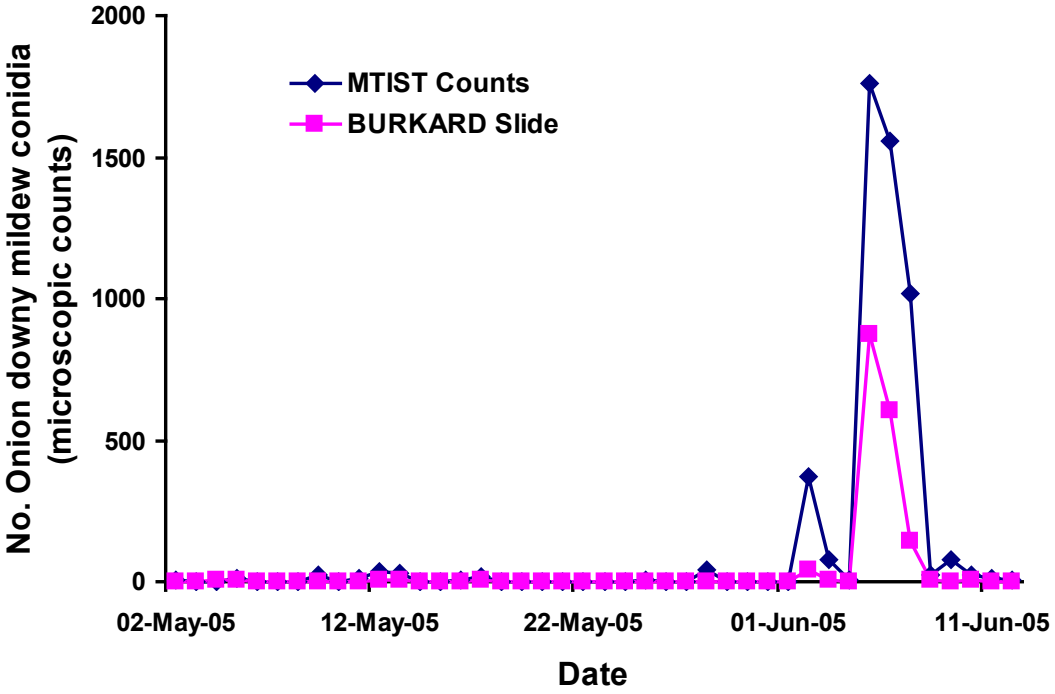
See section 3.4.1.2.4 for details of onion downy mildew spore production prediction systems used in these trials.

5.1.3 Results

5.1.3.1 Comparison of onion downy mildew conidial counts using 24 H volumetric and MTIST samplers in 2006

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 as in 2005. 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 21) that there was higher numbers of onion downy mildew conidia trapped in the MTIST sampler on days when onion downy mildew was present. There were 5 – 6 peaks in onion downy mildew conidial numbers were observed during the trial period (2 May to the 12 June 2005). These occurred on the 9, 12, 13, 17 and 28 May 2006, and the 2, 6 and 9 June 200 using the MTIST sampler. The Burkard 24 H sampler gave only significant peaks in onion downy mildew during early June 2006. Significantly lower numbers of onion downy mildew conidia were observed during the early part of the trial period using the Burkard 24 H sampler.

Figure 21 Comparison of the numbers of trapped onion downy mildew conidia in the field using a Burkard 24 H volumetric sampler and an MTIST sampler

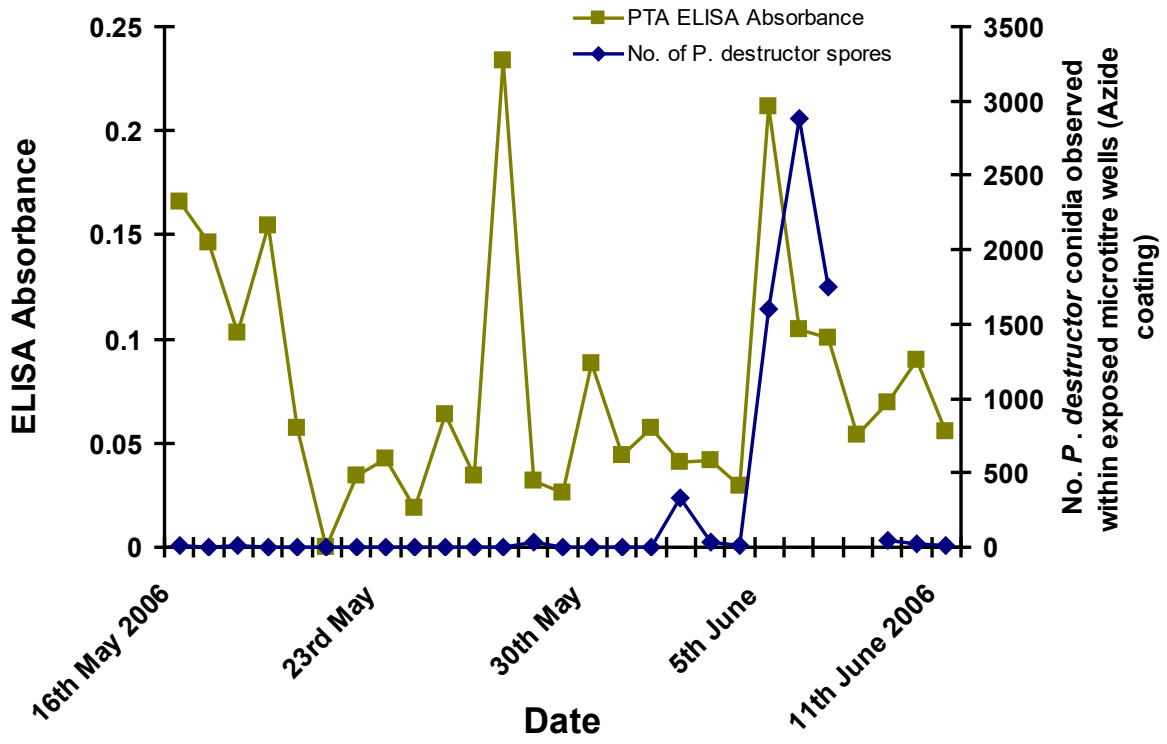


5.1.3.2 Comparison of onion downy mildew conidial counts using 24 H volumetric and MTIST samplers and MTIST microtitre well PTA ELISA

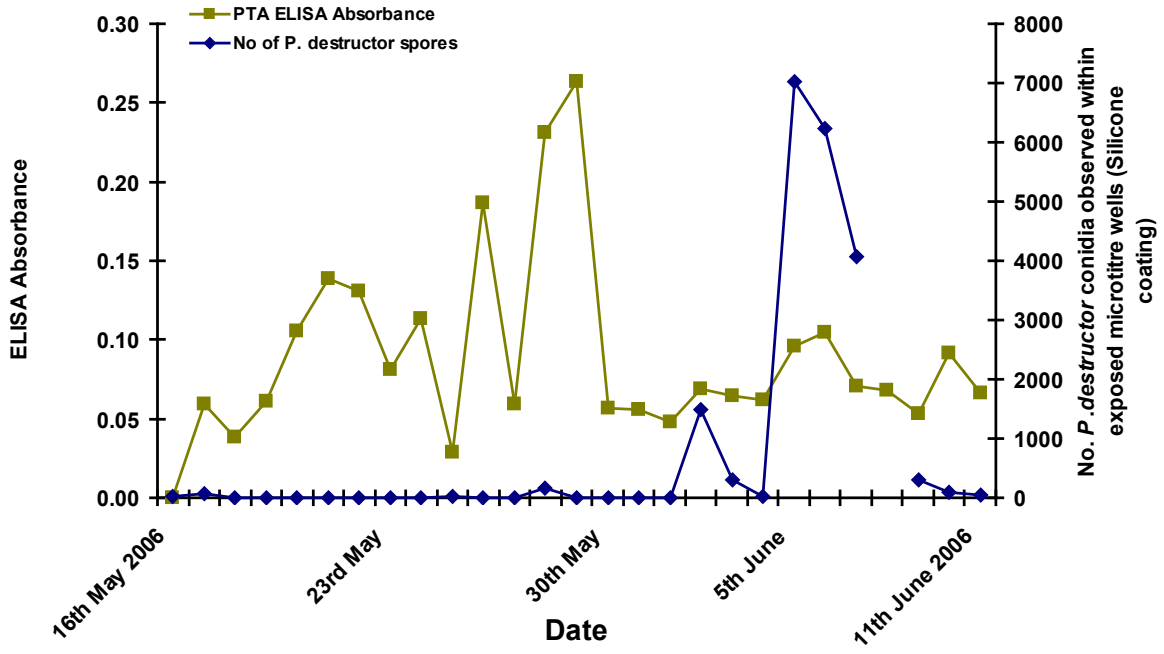
Comparison of the numbers of spores observed in each sampler from microscope counts against MTIST PTA ELISA of microtitre strips coated with sodium azide and silicone is shown in Figures 22a and 22b respectively. Both coatings were used in these comparisons because of their contrasting action. Sodium azide reduces onion downy mildew conidial germination. However silicone in previous studies was more efficient at retaining trapped onion downy mildew conidia.

Figure 22 Relationship between PTA ELISA and number of onion downy mildew conidia trapped using (a) Sodium Azide coated micro titre wells (b) Silicone coated microtitre wells

(a)



(b)



There was a better relationship between PTA ELISA absorbance and number of conidia of *P. destructor* trapped on sodium azide coated microtitre wells (Figure 22a). There was little clear

relationship between PTA ELISA absorbance values and onion downy mildew conidial numbers except during early June when high conidial numbers were recorded in the wells regardless of well coating. Absorbance values of 0.2 indicated that there were high numbers of onion downy mildew conidia present in the slide or in the microtitre well.

5.1.3.3 Comparison of PTA ELISA and pollen counts (MTIST sampler)

The PTA ELISA values for each day were compared to the numbers of pollen spores within microtitre wells to determine if their presence was affecting the accuracy of the ELISA values. Large numbers of pollen spores were observed within wells although the type of pollen could not be identified. Pollen of grasses and Betula were present in varying quantities. The results are shown in Figure 23.

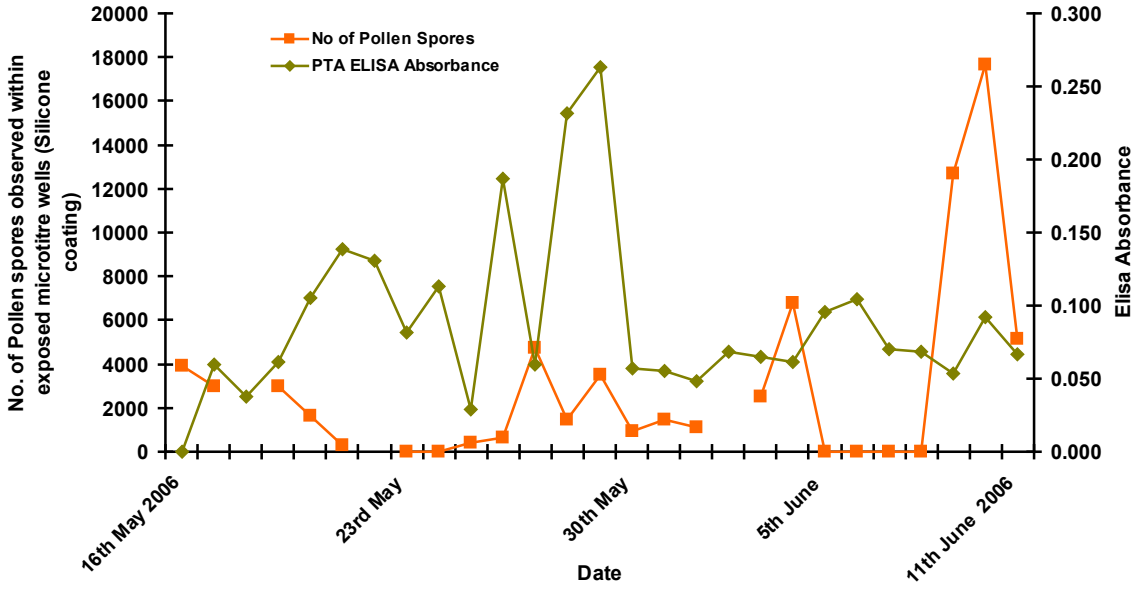


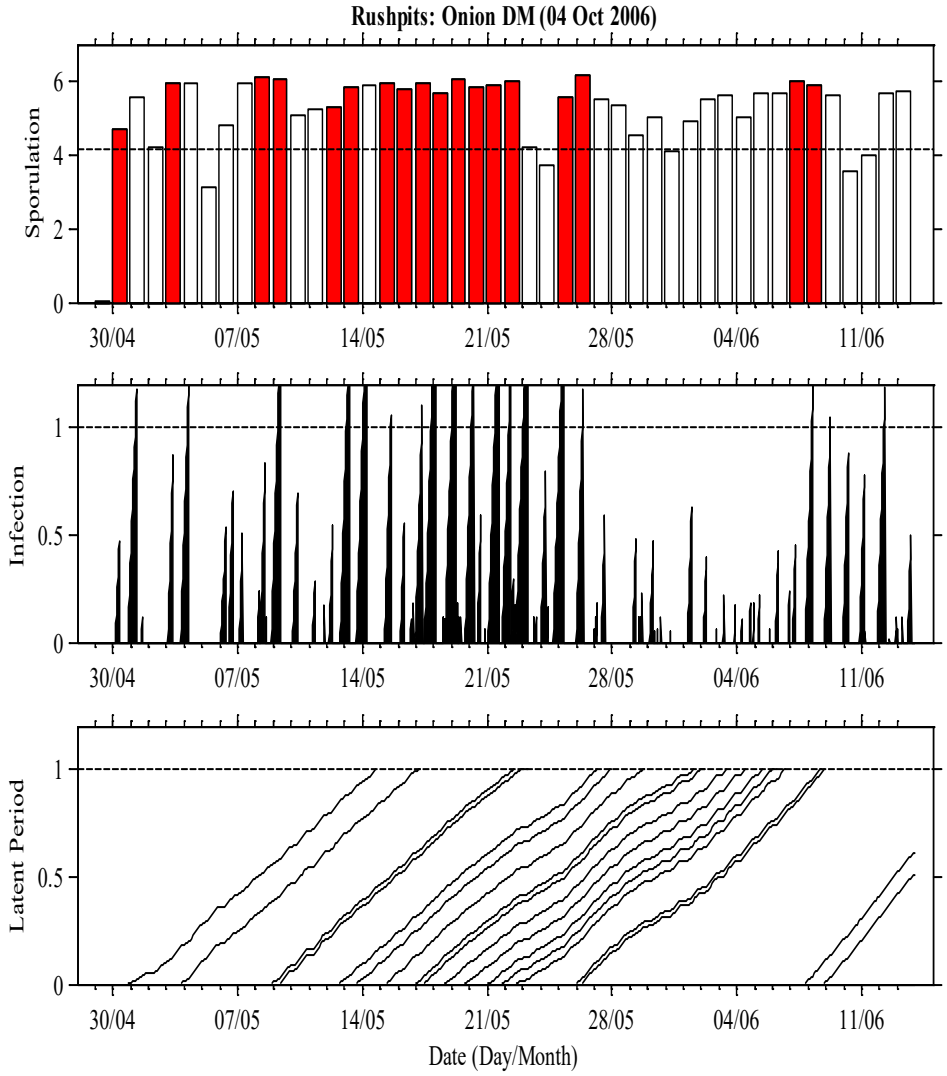
Figure 23. Relationship between PTA ELISA and numbers of pollen spores trapped using in Silicone coated microtitre wells exposed in downy mildew infected field plots.

Very high levels of pollen were recorded in air samples taken during the middle of May and in early June 2006. There was a poor relationship between PTA ELISA absorbance and pollen numbers however increases in pollen numbers were noted during periods when PTA ELISA absorbance fluctuated suggesting some relationship between the two variables. There appeared to be some connection between pollen levels in microtitre wells and conidial counts of onion downy mildew.

5.1.3.4 Prediction of onion downy mildew conidial presence

The results of using the Warwick HRI onion downy mildew forecasting system are shown in Figure 24. There were many periods where onion downy mildew sporulation was predicted. Sporulation *P*. *destructor* was predicted if the bar representing each day (Figure 24) reached the threshold value of 4.15 as represented by the dotted line in Figure 24 in the bar graph. 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 (the colour of the bar changes to red). There were five general periods (30 April 2006, 3 May 2006, 8 and 9 May 2006 12 May to the 22 May 2006 25 and 26 May 2006 and the 7 and 8 June 2006) when significant risk of downy mildew development occurred in the crop. Sporulation by onion downy mildew was predicted on most days over the monitoring period. Sporulation was not predicted on the 5, 23, 24 31 May 2006 and the 10 11 June 2006 according to the Warwick HRI onion downy mildew forecasting system (Figure 24). Latent period for onion downy mildew infections was approximately 14 days over the same period.

Figure 24. 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



5.1.3.5 Observed sporulation by onion downy mildew plants within the field plot

Sporulation by onion downy mildew on marked plants 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 period (see section 5). Onion downy mildew was observed on plants within the plot in early May 2006 Figure 25). 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 25). Visual estimates of onion downy mildew sporulation tended to be inaccurate as old mildew spores were present on the leaves but mildew was not produced on each day.

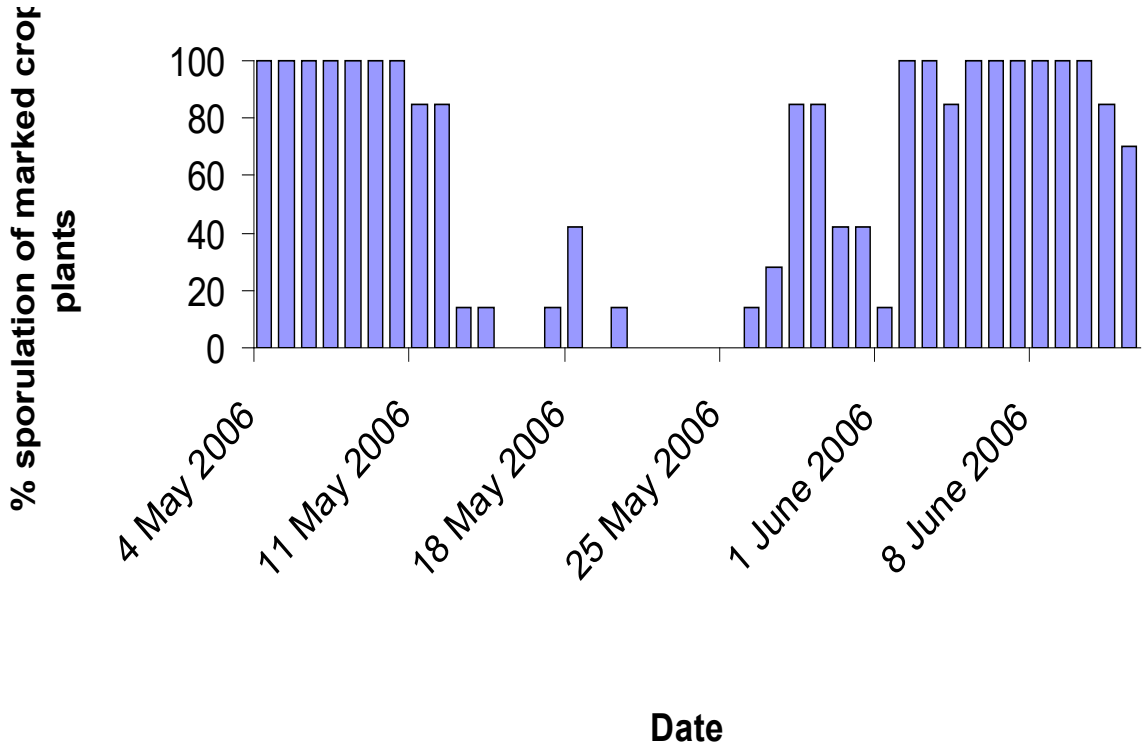


Figure 25. Onion downy mildew sporulation on crop plants within an over-wintered crop of bulb onions at Warwick HRI in 2006.

5.1.4 Conclusion

Optimisation of trapping formats which can collect and retain onion downy mildew conidia is important if detection systems for this disease are to be deployed accurately. Over-wintered onion crops frequently carry onion downy mildew infection as latent infections which can occur within bulbs as oospores or infected tissues. Crops of over-wintered bulb onions can be used in these studies because the crop is often symptomless but develops mildew very quickly and reliably due to the presence of latent infections. Seeding heads are very susceptible to onion downy mildew infection. The results of this trial demonstrate that onion downy mildew conidia could be detected within the crop before symptoms were largely visible however there was inaccuracy due to the presence of another cross reacting particle or spore.

Results show that the ELISA test was not a good indicator of days when onion downy mildew was present in samples. This is at variance with the year two results (Figure 16) where there was a close relationship between PTA ELISA absorbance value and numbers of trapped conidia of onion downy mildew. The results suggest that there is likely to be the presence of another cross reacting spore (pollen or another fungal organism). It was unclear if this cross reacting particle was pollen. However, high numbers of pollen spores were observed in microtitre wells. In subsequent immunoflorescence tests pollen spores were observed to react

with the monoclonal antibody used (EMA242) used within the ELISA test. If the test is to be used in the current format it maybe accurate only during times of the season when pollen spores are not present or in some locations. This could assume to be early spring and autumn and by using the trap located in the centre of the field not in the grassy margins. The test accuracy could be improved for all locations by incorporating a step which removes the pollen from the sample. This could be in the form of pre-filtration step before the sample was run within the lateral flow device. The results of the trial also confirmed the accuracy of the Warwick HRI onion downy mildew forecasting system.

6.0 Development of a Competitive lateral flow system for semi-quantitative detection of field trapped inoculum of *Peronospora destructor*

6.1 Development of lateral flow device for detection of field inoculum of onion downy mildew

6.1.1 The competitive lateral flow device

In a competitive lateral flow assay it is the absence of a test line which represents a positive result (Plate, 10). A control line is observed when the test is complete and demonstrates successful test operation.

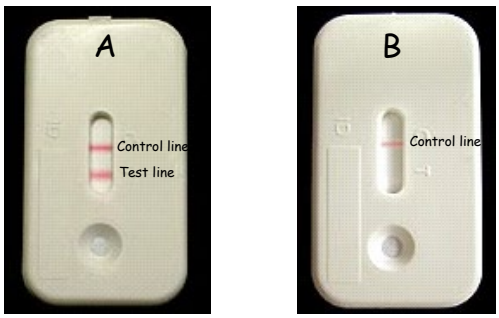


Plate 10. Competitive lateral flow assay showing a positive sample result (B) and (A) a negative sample result.

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

The sample extract is applied to the release pad, facilitating the 'immobilised' specific antibody bound gold spheres to flow on the nitrocellulose membrane. The test line is composed of soluble components of disrupted *P. destructor* conidia (downy mildew on onions). As the sample extract and the specific antibody bound gold spheres flow laterally though the membrane there is potential for binding between the target antigen (downy mildew (if present) and the specific gold labelled antibodies. The formation of a target antigen / gold conjugated antibody complex inhibits capture at the test line. With sufficient target antigen present (downy mildew condia) complete inhibition occurs and no test line is observed. In a negative sample (target *P. destructor* conidia absent) the antibody conjugated gold spheres remain unbound and are captured at the test line to produce a visible line. 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. The competitive lateral flow developed previously (FV 189a Second Year Annual Report 2005, Section 5) proved useful as a prototype for the final field version.

6.1.2 Materials and Methods

6.1.2.1 Competitive lateral flow components

Tests were carried out using lateral flows comprised of a Millipore 240 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing (Cat No. SHF2400225, Millipore Corp, USA.), an absorbent pad (Cat No. GBOO4, Schleicer and Schuell, Germany) and a sample pad (Cat No. T5NM, Millipore Corp., USA). Following lateral flow construction (Figure 26) control lines of an anti-mouse serum in PBS was sprayed directly on to the membrane surface using a flat bed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK). A collected *P. destructor* downy mildew conidial preparation was prepared (as described in Year 2 Annual report Section 3.1.2.1) in PBS and following disruption in a Fast Prep device (Q Biogene) was adjusted to a protein concentration of 500µg ml^{-1} in PBS. The onion downy mildew spore soluble fraction was retained and applied directly, using a flat bed air jet dispenser, to the lateral flow membrane to produced a test line. Membranes were air dried at 18° C for a period of 24 hours. The test and control line labelled lateral flow membrane cards were cut in to 5 mm strips.

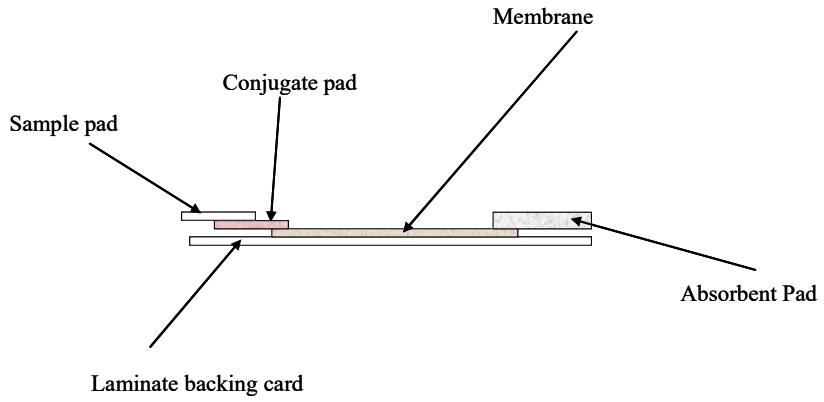


Figure 26 . Schematic overview of a competitive lateral flow device.

6.1.2.2 Antibody conjugation

A British Biocell gold anti-mouse IgM solution was pre-mixed (conjugated) with EMA 242 and then applied to the sample pad drop wise and air-dried. Variable antibody concentrations of EMA 242 were used to determine the optimal antibody activity required to provide a quantitative test within the disease detection threshold required. Variable

concentrations were applied to different sample pads to investigate the antibody conjugate concentration which gave optional test line formation on the lateral flow device.

6.1.2.3 Application of onion downy mildew to the competive lateral flow device

Conidial spores of downy mildew in sample buffer were applied dropwise (70µl) to the sample pads of pre-prepared lateral flows. Condial concentrations ranged from 240 to 960 conidia applied. The lateral flow devices were viewed 20 minutes post sample application for the formation of a test and control line and test line optical density values were generated using a BioDot lateral flow reader. A negative control of lateral flow running buffer alone (0 downy mildew conidia) was also included within these tests.

6.2 Results

6.2.1 Optimisation of antibody conjugate concentration within the onion downy mildew lateral flow prototype

A 6µl British Biocell gold anti-mouse IgM solution was pre-mixed with 12µl EMA 242 and then applied dropwise to lateral flow sample pads at a test volume of 18µl each. Variable antibody volumes of EMA 242 were tested (Table 6) to investigate the antibody activity required (EMA 242, 1 in 115) which would give a lateral flow test line when there was no risk of downy mildew to exposed field crops. In excess of 500 trapped conidia was considered to place exposed field onion crops at risk of disease under designated environmental risk periods.

Table 6 Test line formation at different antibody conjugate concentrations

	Onion Downy Mildew Conidial Number								
Antibody/Conjugate EMA 242 at 1:115 (µl)	12800	6400	3200	1300	650	325	162	81	0
25	-	t	T	T	T	T	T	T	T

21	-	-	t	t	T	T	T	T	T
17	-	-	t	t	T	T	T	T	T
15	-	-	-	-	t	T	T	T	T
13	-	-	-	-	t	T	T	T	T
11	-	-	-	-	-	t	T	T	T
9	-	-	-	-	-	-	t	T	T
7	-	-	-	-	-	-	-	t	t
5	-	-	-	-	-	-	-	-	-

T – strong test line, t – weak test line, - no test line

6.2.1.1 Lateral flow prototype tests with onion downy mildew

When a negative sample (0 conidia) was applied to a lateral flow device strong test line development was observed (Plate 11a). As spore concentrations increased the

Plate 11a Lateral flow prototype development (negative test)

test line colour formation decreased. When a downy mildew conidial concentration of 960 was applied to a lateral flow device no test line development was observed (Plate 11b).

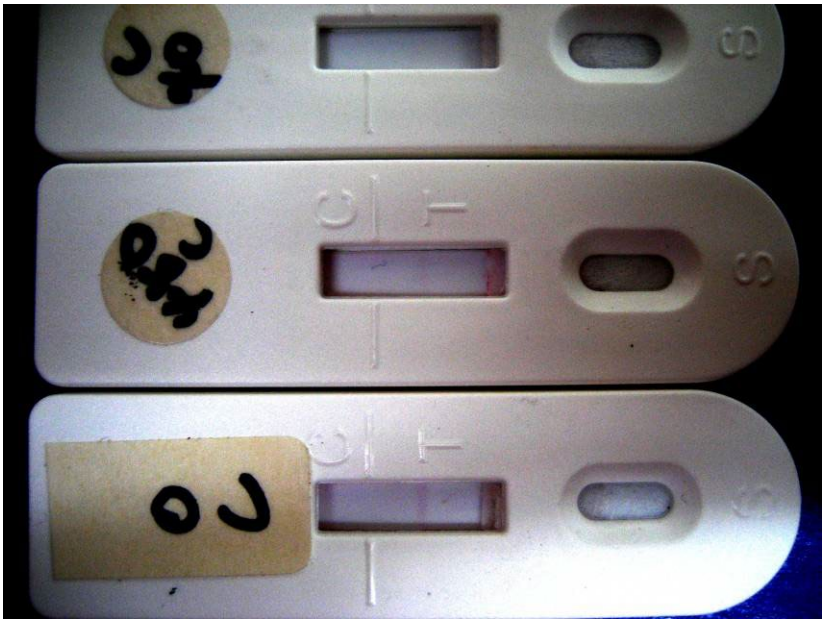


Plate 11b Lateral flow prototype development (positive test)

Using a Bio-dot lateral flow reader the potential for semi-quantitative analysis exists as optical density values are calculated (Table 7). A linear relationship (0.893) was observed between the OD value determined and the number of condia applied to each lateral flow device.

Onion downy mildew conidial number	Optical Density value
0	2.2
240	1.8
480	0.7
960	0.3

Table 7. Optical density values of the test line at varying onion downy mildew conidial concentrations

6.3 Conclusions

The results show that by using the immunogold antibody conjugate a competitive lateral flow device could be constructed which had a visual sensitivity at above 240 onion downy mildew conidia. It was unclear if this was the required sensitivity for onion downy mildew conidial detection in the field. However using a lateral flow reader device where the amount of gold conjugate on the test line could be quantified the sensitivity of the device could be made semi-quantitative. The competitive lateral flow format shows great promise as a rapid semi-quantitative test for onion downy mildew spore detection. The format was clearly able to detect target onion downy mildew inoculum at significant levels. Further studies will be required to address two points. Firstly assessment of the test under field conditions and, in

conjunction with air sampling technology. Secondly the cost of the test will need to be examined i.e. reagent costs (detection antibody) and required run time by the end user so that it can be used commercially.

7.0 TESTS WITH LATERAL FLOWS FOR ONION DOWNY MILDEW DETECTION IN COMMERCIAL TRIALS

7.1 Monitoring airborne inoculum of the onion downy mildew (*P. destructor*) in a seeding crop of bulb onions at Warwick HRI using onion downy mildew prototype devices

Trapping formats and the onion downy mildew lateral flow device were evaluated for their ability to detect onion downy mildew conidia in air samples in a seeding crop of bulb onions at Warwick HRI, Wellesbourne during April, May and June 2006. The 7 day cyclone sampler was used in this trial. This trap contains epindorf tubes for which a fresh tube was used automatically for each days sampling. Sample tube sets were changed at weekly interval.

7.1.1 Materials and Methods

See section 5.1.1 for details of the trial plot

7.1.1.1 Production of a bulb onion seeding crop.

See section 3.2.1.2.1 for details

7.1.1.2 Air samplers used in the trial

The characteristics of the Burkard 7 Day cyclone sampler, has been described by Ogawa & English (1995). Air is drawn through this sampler using a vacuum pump in the form of a cyclone. The characteristics of the trap namely the height of the cyclone, height of the air inlet, width of the air inlet, air exhaust diameter and the diameter of the cyclone with the length of the exhaust pipe influence the relative efficiency of the trap. These characteristics have been drawn together and standardised within the Burkard cyclone sampler. A separate sample container which is rotated automatically can be used for each sampling day. The sampling container was coated with 0.05 % Sodium Azide (as described in the previous section with microtitre strips). The trap can also be adjusted to sample for set durations each day. The quantitative efficiency of this type of trap is high as the sample volumes can be much higher than other types of trap. This type of trap is suitable for use with “in field” detection kits. This sampler was used to sample air for 12 H periods for 06:00 H to 18:00 H daily. This was the time observed from pervious studies (see Year one Annual report FV189a) when maximum numbers of onion downy mildew conidia were present in air samples.

7.1.1.3 Enumeration of trapped spores in air samplers

See section 5.1.1.3

7.1.2 Results

7.1.2.1 Detection of onion downy mildew conidia in air samples using lateral flow devices

The results of using the prototype lateral flow device are shown in Figure 27 (Quadscan reading for each sampling period) and Table 8 (visual assessment of the presence or absence of a test line). The Quadscan device gives an optical reading of the amount of captured labelled gold spheres relative to the control line. Current results indicate that values of greater than 2.0 indicate the presence of a test line (negative result). The results show that onion downy mildew conidia were detected in samples using the lateral flow device on only a few sampling periods in the field. Presence of onion downy mildew conidia was tested using the lateral flow device in air samples collected on the 22, 24, 25, 26 May 2006 and the 5, 6, 7, 8 June 2004 (see Figure 21 section 5). Onion downy mildew was detected using the lateral flow device on the 5, 6 and 7 June 2006 corresponding to the presence of onion downy mildew conidial presence in high numbers.

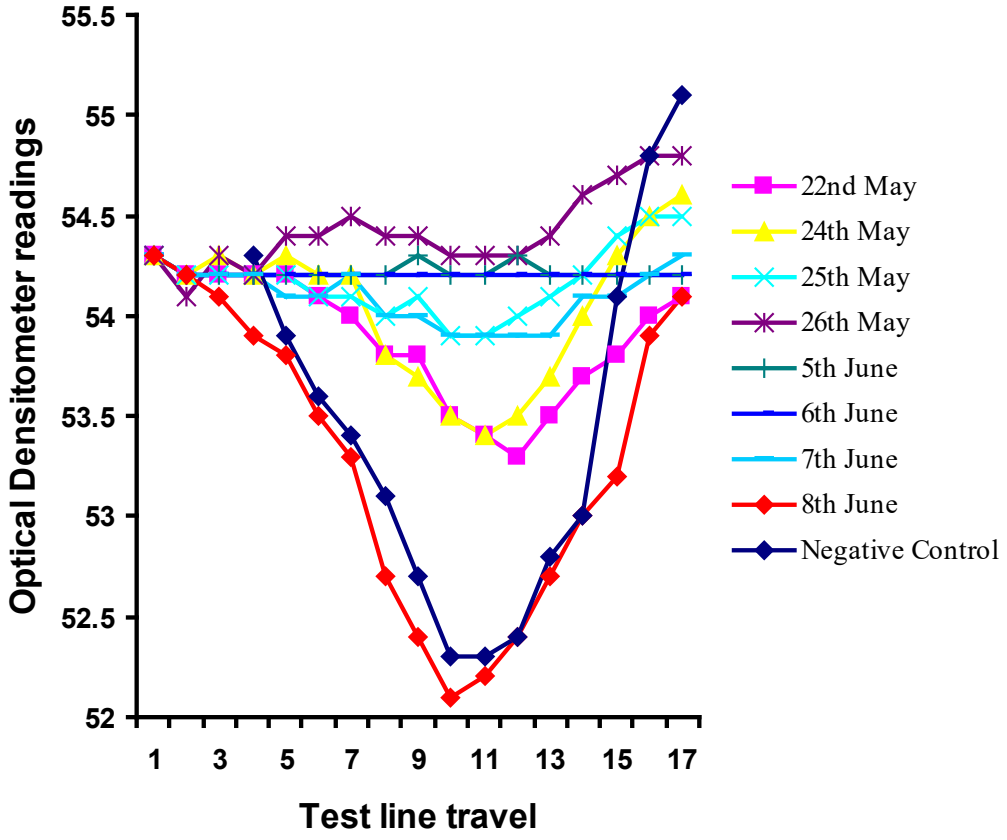


Figure 27 Quadscan readings of lateral flow test lines relative to control lines for onion downy mildew sampling periods

There was a relationship between the Quadscan reading and the number of onion downy mildew conidial number observed within each sample (Figure 26). Optical density values of approximately 54 were required for good line visualisation (negative result). However there

appeared to be an interaction between the presence of pollen spores in the sample and line formation (Table 8). This occurred on samples collected on the 24 and 25 May 2006 which had high levels of pollen in the sample and low levels of onion downy mildew. However these samples gave positive results with the prototype lateral flow device (no line formed). The numbers of test samples which could be used in these tests was limited due to the pattern of onion downy mildew disease in the seeding onion crops at Warwick HRI.

Table 8. Visual assessment of test line for sampling periods

Field exposure period	Test line observation	No. of Onion downy mildew conidia/Pollen
22 May 2006	Yes	0/16
24 May 2006	Yes	0/0
25 May 2006	No	0/400
26 May 2006	No	0/592
5 June 2006	No	1264/0
6 June 2006	No	9808/0
7 June 2006	No	2560/0
8 June 2006	Yes	32/0

7.1.3 Conclusions

There were too few days on which these tests could be used when onion downy mildew conidia were present in the air. Nevertheless four days (5 – 8 June 2006) were identified as days when variable numbers of onion downy mildew were present in samples. The lateral flow device gave test line formation on the 8 June when very low levels of onion downy mildew were present in the test sample. However the prototype lateral flow device gave no test line formation on the 25 and 26 May 2006 when there was the presence of high levels of pollen in the sample. The limited results indicate that a prefiltration step will be needed to be applied to the test sample before it is placed in the lateral flow device. Further tests of the lateral flow device will be required to determine how this prefiltration step should be applied. Additional field tests will be required to check the sensitivity of the lateral flow device reactions with a concentration range of onion downy mildew conidia. The device can detect levels of onion downy mildew conidia in samples of between 250 – 500 conidia. However due to the very dry conditions which occurred during 2006 a full concentration range of onion downy mildew conidia in air-borne samples was not obtained. Further trials are required to obtain air-borne samples with differing onion downy mildew levels within them so that these tests can be carried out more fully.

7.2 Monitoring airborne inoculum of the onion downy mildew (*P. destructor*) in commercial bulb and salad onion crops

7.2.1 Introduction

Tests were conducted using the onion downy mildew lateral flow device to monitor the number of onion downy mildew conidia in samples and compare this with onion downy mildew disease development in commercial crops of bulb and salad onions. Trials were conducted during 2006 at two onion production sites in different areas of the UK. One site in Lincolnshire (Sleaford) was sited in a bulb onion crop. A second site in Warwickshire (Hampton Lucy) was sited in a salad onion crop.

7.2.2 Materials and Methods

7.2.2.1 Crop experimental design and crop disease observations

Trials were conducted in commercial crops at Sleaford (Allium and Brassica Centre, Wash Rd, Lincs.), Hampton Lucy (Bomfords, Luddington, Stratford on Avon) and run in conjunction with grower/consultants. At Sleaford a bulb onion crop was monitored and assessed for disease development. At Hampton Lucy the trial was located in a summer sown salad onion crop. The trial was located within an unsprayed plot (5 x 5 m) of a fungicide trial. Environmental conditions were recorded using a Skye DataHog II data logger. Observations were taken on disease levels on 5 – 10 marked plants which were tagged. Disease observations on tagged plants were taken weekly. The Warwick HRI downy mildew models were used to determine disease (infection and sporulation) risk on each day during the trial. The Skye dataHog II data logger was positioned adjacent to the trial site and provided information on temperature wetness duration, humidity and rainfall at 30 min intervals (with a 5 minute log interval).

7.2.2.2 Air sampling at each trial site

Air samples were taken continuously over a period of 6 weeks using a Burkard 7 day cyclone sampler at both trial sites. This sampler automatically changes the trapping vessel each day at a preset time period. The seven tubes within the sampler (one for each day) can be changed weekly by using fresh tubes. The Burkard 7 day cyclone sampler was operated for 24 H per day at a sampling volume of 16.5 litre of air min ^{2} at Hampton Lucy and a 72 H period at Sleaford. A Burkard 7 day volumetric air sampler (with the same characteristics as the 24 H glass slide sampler) was also used throughout the monitoring period at the Sleaford trial site. An additional cyclone sampler was used at the Hampton Lucy site where the trap vessel was replaced every 3 days by hand. During the three day periods the sampler was operated continuously at a sampling volume of 10 litre of air min ^{2}. The eppindorf sample collection vessels (from the cyclone samplers) were stored prior to assay development at -20ºC. Sample vessels from all traps were tested in the laboratory using the prototype dark leaf spot lateral flow device and the responses recorded.

7.2.2.3 Detection and quantification of onion downy mildew using lateral flow devices

Approximately 80µl of extraction buffer was added to each of the collected eppindorf vessels (cyclone spore sampler) and, using a Gallenkamp Spinmix, agitated for a period of 3 minutes at high speed. A 80µl aliquot of each spore suspension was then applied to a sample pad of an individual competitive lateral flow device. Determination of test line development was made

by visual assessment and, using a Quadscan, (Biodot, Chichester). Each device was scanned on two occasions using the Quadscan reader. Due to the number of prototypes available it was not possible to carry out lateral flow tests on all sampling periods. However by utilising disease forecasts at the site a number of risk days could be compared with non risk days for the presence or absence of onion downy mildew inoculum at the required concentration for disease development to occur.

7.2.2.4 Visual microscopic counts of dark leaf spot from air samples

Samples used in tests with lateral flows were checked visually for the presence or absence of onion downy mildew conidia. Approximately 10µl of extraction buffer was removed from each sample vessel prior to testing with the lateral flow device and placed on a microscope slide. Estimates of the numbers of onion downy mildew conidia were taken by counting the number of onion downy mildew conidia in each 10µl sample before multiplying by 8.

7.2.2.5 Prediction of Onion downy mildew sporulation in the field

(see section 3.4.1.2.4)

7.3 Results

7.3.1 Detection of onion downy mildew conidia in air samples using lateral flow devices and disease development at Sleaford 2006 .

The results of using dark leaf spot lateral flow device on air samples collected at Sleaford are shown in Table 9. The Quadscan reader device gives an optical reading of the amount of captured immunogold on the test and control line. When more immunogold is captured on either line the visualisation of the line is more discrete. However use of the reader enables the device to used semi-quantitatively to determine the amount of onion downy mildew present in the sample. It was not possible to test all days where air samples were taken due to the numbers of tests required. The following days were designated according to the model to be high risk periods for onion downy mildew development. These were the 27 June 2006, 4, 5, 6, 20, 23, 24, 28 and 29 July 2006 and the 3, 5 , 6 and 7 August 2006. The trap developed a fault during the final week of July and samples could not be collected. The trap was removed during early August 2006. Using the onion downy mildew lateral flow prototype gave positive results during all periods tested (the absence of the test line) (Table 9). However all samples were contaminated with soil which probably accounted for the positive result. The 3 day sampling period enhanced this contamination problem. The lack of a control line formation in these tests with samples from Sleaford suggests that the positive results were an error obtained from the malfunction of the device with the test samples used in it.

Table 9. Onion downy mildew conidial concentration per m^3 at Sleaford in 2006

Field exposure period	Onion downy mildew conidia m^3	Immunogold Line Reading	
		Test	Control
3 -7 July	0	0.15*	0.15
17 – 21 July	0	0.15*	0.15

21 - 24 July	0	0.15*	0.15
24 – 25 July	0	0.15*	0.15
3 – 6 August	0	0.15*	0.15

* Soil contamination in the sample

7.3.2 Development of onion downy mildew at Sleaford 2006.

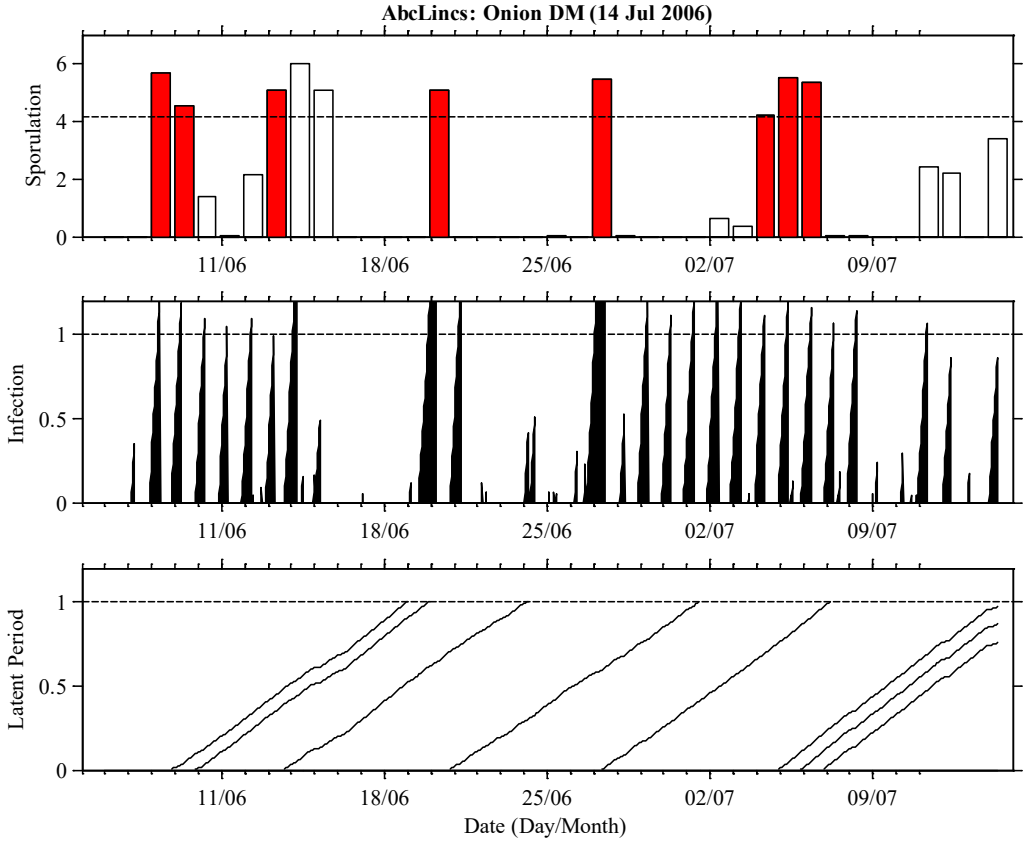
The tagged plants and other crop plants at Sleaford did not show any signs of infection by onion downy mildew. No onion downy mildew was recorded in unsprayed plots in an associated fungicide trial situated at the same site.

7.3.3 Prediction of onion downy mildew at Sleaford in 2006

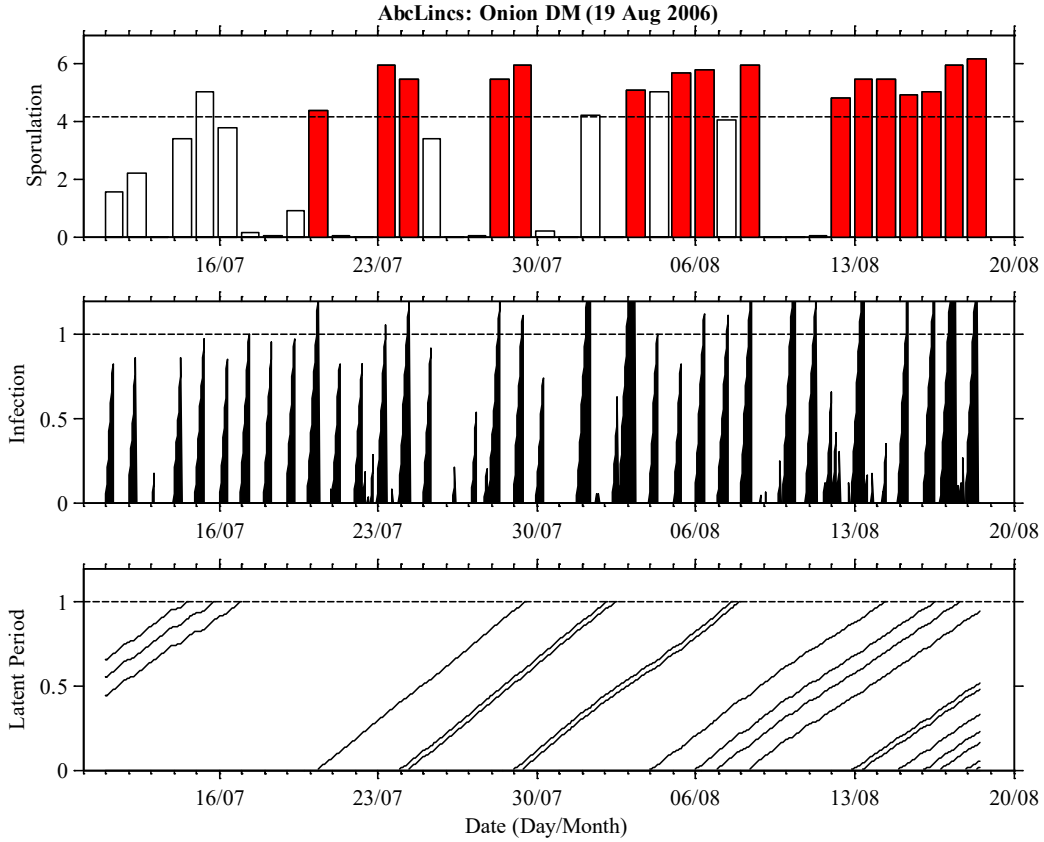
The results of using the Warwick HRI onion downy mildew disease forecasting system at the Sleaford site in 2006 are shown in Figure 28a and 28b. When infection and sporulation are predicted as having occurred at the same time a red coloured bar is shown on the bar chart of Figure 28a and 28b. The presence of this criteria can be used as a means of determining when it is more accurate to trap onion downy mildew conidia in the air. There few periods when environmental conditions were suitable for onion downy mildew development at Sleaford. Weather conditions during much of June and July 2006 at Sleaford were very dry and hot. Consequently there were few periods when onion downy mildew was predicted in the field at Sleaford. The following days were designated according to the model to be high risk periods for onion downy mildew development. These were the 27 June 2006, 4, 5, 6, 20, 23, 24, 28 and 29 July 2006 and the 3, 5 , 6 and 7 August 2006. On these days sporulation by the pathogen was predicted at the site. Completion of latent period is another criteria which could be used to determine the onset and duration of onion downy mildew trapping. These periods are also shown on Figure 28a and 28b. The latency period during June and July 2006 was approximately 10 days. Latent periods were completed over the 14, 15, and 16 July 2006 and the 30 July – 2 August 2006. Latent periods were also completed during the 8 – 10 August 2006.

Figure 28. Prediction of onion downy mildew at Sleaford 2005 (a) 8 June 2006 – 14 July 2006 (b) 14 July 2006 - 18 August 2006

(a)



(b)



7.3.4 Detection of onion downy mildew using lateral flow devices at Hampton 2006

Results of testing air samples collected at Hampton are shown in Table 11. Samples at the Hampton site were collected daily (24 H sampling periods). Risk periods were designated as having occurred on the 5, 6, 10, 11, 12, 20, 21,23, 24 July 2006 and the 1, 6 and 8 August 2006. Approximately 80µl of buffer was added to each sample tube and mixed. The sample was then transferred to the lateral flow device. Use of the Quadscan reader enables the device to used semi-quantitatively to determine the amount of onion downy mildew present in samples at Hampton. An additional cyclone trap where the tubes were changed at three day intervals was also located at the site. However sample tubes in this trap were not used because of insect contamination problems. The results showed that all sampling periods tested gave positive results in that there was no test line formation (Table 10).

Table 10. Onion downy mildew conidial concentration per m^3 at Hampton in 2006

Field exposure period	Onion downy mildew conidia m^3	Immunogold Line Reading	
		Test	Control
5 July 2006	0	0.15	0.15
6 July 2006	0	0.15	0.15
10 July 2006	0	0.15	0.15
11 July 2006	0	0.15	0.15
12 July 2006	0	0.15	0.15
20 July 2006	0	0.15	0.15
23 July 2006	0	0.15	0.15
24 July 2006	0	0.15	0.15
1 August 2006	0	0.15	0.15
6 August 2006	0	0.15	0.15
8 August 2006	0	0.15	0.15

The results show that over the trial period no onion downy mildew conidia was detected at the site during any of the 24 H sampling periods. However no control line was observed on any lateral flow test suggesting that the samples had caused clogging of the device and suggesting that the positive results obtained were an error. This may have resulted from the sampling times used to collect air samples which in most cases were too long.

7.3.4.1 Development of onion downy mildew at Hampton 2006.

The tagged plants and other crop plants at Hampton did not show any signs of infection by onion downy mildew. No onion downy mildew was recorded in the field where the trial was located.

7.3.5 Prediction of onion downy mildew at Hampton 2006

The results of using the Warwick HRI onion downy mildew disease forecasting system at the Hampton site in 2006 are shown in Figure 29. The interpretation of the output is the same as previously described for Sleaford. There few periods when environmental conditions were suitable for onion downy mildew development at Hampton. Weather conditions during much the trial period at Hampton were like Sleaford hot and dry. There were few periods when onion downy mildew was predicted in the field at Hampton. High risk periods for onion downy mildew development occurred on the 5,6,10,11,12,20,21,23,24 July and the 1,6 and 8 August 2006. On these days sporulation by the pathogen was predicted at the site. There were too few latency periods to designate this as a criteria for testing sampling tubes at the Hampton site.

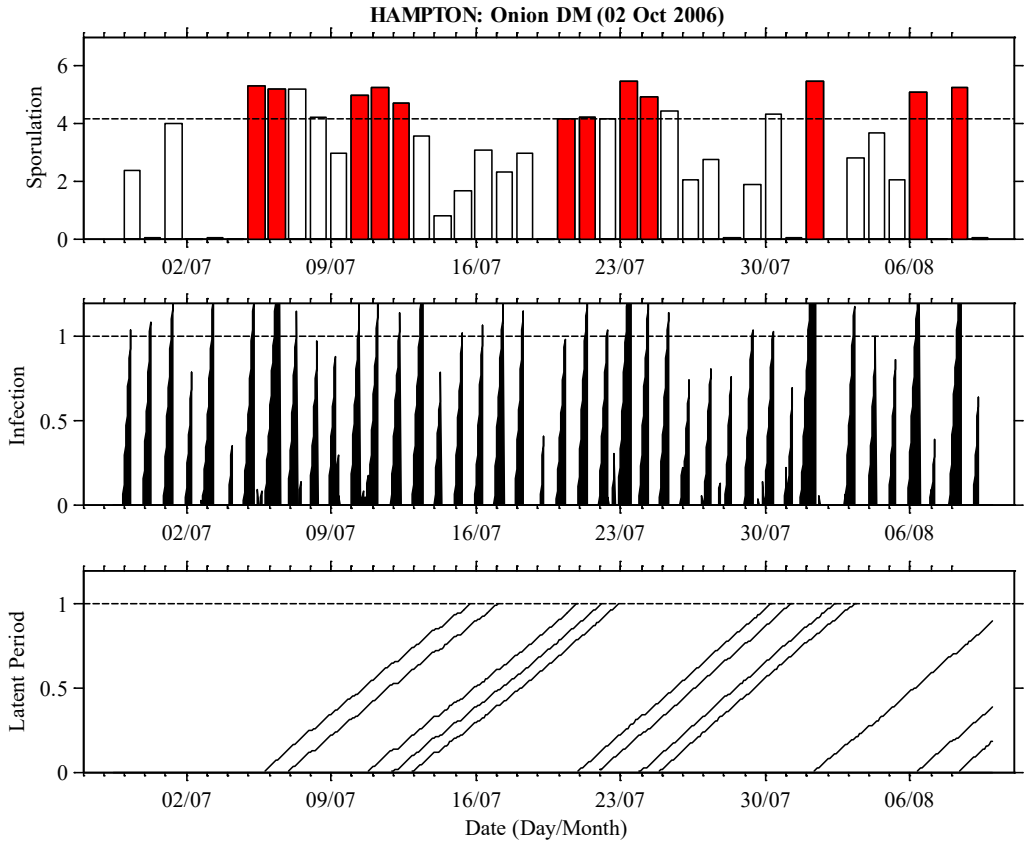


Figure 29. Prediction of onion downy mildew at Hampton in 2005

7.3.6 Conclusions

Tests carried out with the onion downy mildew prototype lateral flow device during 2006 were not satisfactory. Due to extremely hot conditions which occurred in June and July 2006 no downy mildew was observed in the crop or detected in the samples visually. An additional problem was the sampling regime used within commercial crops. Three day periods or even 24 H periods were too long to obtain samples which could be used with the current onion downy mildew prototype lateral flow device. Extended sampling times were used to increase the possibility of detecting very low levels of onion downy mildew. Sampling times of 12 H used in trials at Warwick HRI were more successful in giving accurate positive results. These trials had the added benefit of having onion downy mildew development at the sampling site however this resulted in only four positive sample days which was still too few to properly test the device. Tests with the device at Warwick HRI suggested that the device may be affected by the presence of grass pollen (although this is unclear). During 2006 the highest levels of grass pollen on record were observed.

8.0 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 and further investigated in Year 3 of the project.

8.1 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.

8.2 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.

8.3 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.

8.4 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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