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

HDC PROJECT FV 189a

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Grower Summary

FV 189a

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

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

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CONTENTS PAGE

Page

1. GROWER SUMMARY **1**
1.1 Headline 1
1.2 Background and expected deliverables 1
1.3 Summary of first year work on FV 189a 2
1.3.1 Development of specific antibodies for detecting and quantification of onion downy mildew conidia 2
1.3.2 Systems for detecting onion downy mildew in the field 2
1.4 Action points for growers 3
1.5 Anticipated practical and financial benefit 3

SCIENCE SECTION
2. INTRODUCTION **4**
2.1 Air borne disease problems affecting onion crops 4
2.2 Biology of *Peronospora destructor* on onion crops 4
2.3 Methods for determining the risk of air-borne diseases in onion crops 5
2.4 Using air-borne spore numbers within disease forecasting systems 6
2.5 Lateral Flow Device (Competitive Assay only) 6
2.6 Developing 'in field' tests for detecting the presence or absence of onion downy mildew inoculum 8

3. PRODUCTION OF ANTISERA **9**
3.1 Production of monoclonal antisera to *Peronospora destructor* 9
3.1.1 Introduction 9
3.1.2 Materials and Methods 9
3.1.2.1 Production of *P. destructor* immumogen for antibody production 9
3.1.2.2 Collection of *P. destructor* spores from leaf surfaces 9
3.1.2.3 Immunization of mice with *P. destructor* spores 10
3.1.3 Results 10
3.1.4 Conclusion 11
3.2 Production of polyclonal antisera to *Peronospora destructor* 12
3.2.1 Materials and Methods 12
3.2.1.1 Immunization of mice 12
3.2.2 Results 12
3.2.3 Conclusion 13
3.3 Cross reactivity tests with polyclonal antiserum (PAS 7346P) 14
3.3.1 Method 14
3.3.2 Results 15
3.3.3 Conclusion 16

4. DETERMINATION OF SPORE TRAPPING PERIODS FOR DOWNY MILDEW ON ONION **17**
4.1 Measuring the diurnal periodicity of downy mildew in air samples 17
4.1.1 Introduction 17
4.1.2 Materials and Methods 17
4.1.2.1 Infected bulb onion crop production 17
4.1.2.2 Spore trapping measurements 17
4.1.2.3 Micro-climate measurements 18

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4.1.2.4 Prediction of spore production in the field 18
4.1.3 Results 18
4.1.3.1 Environmental factors affecting periodicity of spore release by onion downy mildew 18
4.1.3.2 Predictions of spore production and spore release by onion downy mildew 20
4.1.4 Conclusions 21

5. EVALUATION OF TRAPPING FORMATS FOR ONION DOWNY MILDEW IN THE FIELD 23
5.1 Monitoring downy mildew conidia in air samples in a commercial crop of bulb onions 23
5.1.1 Materials and Methods 23
5.1.1.1 Trapping inoculum of *Peronospora destructor* in a commercial crop of bulb onions 23
5.1.1.2 Detection and quantification of collected spore samples 23
5.1.1.3 Micro-climate measurements 23
5.1.1.4 Prediction of spore production in the field 24
5.1.2. Results 24
5.1.2.1 Prediction of onion downy mildew conidial presence 24
5.1.2.2 Estimated numbers of downy mildew conidia using ELISA employing polyclonal antibody PAS 6346p 26
5.1.3 Conclusion 27
5.2 Monitoring airborne inoculum of the downy mildew pathogen in an inoculated overwintered seeding onion crop 28
5.2.1 Materials and Method 28
5.2.1.1 Monitoring downy mildew in air samples in relation to plant infection 28
5.2.1.3 Detection of downy mildew conidia in air samples using Immunofluorescence 28
5.2.1.4 Detection of downy mildew conidia in air samples by PTA ELISA 28
5.2.2 Results 29
5.2.2.1 Detection of downy mildew conidia in air samples using Immunofluorescence 29
5.2.2.2 Detection of downy mildew conidia in air samples using ELISA 29
5.2.3 Conclusion 30

6. DISCUSSION 31
6.1 Producing antibodies for use in lateral flow devices 31
6.2 Sample preparation from traps 31
6.3 Disease forecasting criteria based on inoculum detection 32

7. REFERENCES 34

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1. GROWER SUMMARY

1.1 Headline

Detection systems for spores of onion downy mildew are being developed. These will be used to detect onion downy mildew in the air before symptoms can appear on the crop. Specific antibodies have been produced which will be used in the development of these tests.

1.2 Background and expected deliverables

Downy mildew (*Peronospora destructor*) causes heavy yield losses in bulb and salad onion crops. Fungicidal control of downy mildew is often difficult. Fungicides are only effective, if they are applied before or immediately after disease first appears in the crop (Kennedy *et al*., 1998). Management systems such as DACOM are available which can predict the early development of downy mildew. A 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. This model is not yet available for use in commercial crops. Use of this system, which provides information on the timing of the first fungicide application, may result in better disease management. However inoculum can be imported into disease free crops from other localities/areas but assessments based on environmental risk alone do not take this factor into account. In order to avoid these problems new and rapid methods of detecting and quantifying pathogenic inoculum are required which can be used in conjunction with forecasting models. With this more precision approach there will be reductions in the amounts of fungicide required to control disease by eliminating unnecessary fungicide applications, which are based on weather information alone. In the current project "in field" tests for conidia of onion downy mildew are being developed. Producing these tests will require the development of specific antibodies, which can be used to visualise the presence of target inoculum (onion downy mildew conidia). The specificity of the antibodies will require testing in relation to a range of related and unrelated fungi, which are found in onion crops or in their vicinity. The ability of the trapping system to catch and retain the spores in sample tubes will also require testing and optimisation. Finally once the " in field" (lateral flow) tests have been derived it will be necessary to test the system under field conditions. Each stage in this developmental process will require validation and in this report the various stages in developing the components needed for these tests has been described.

The expected deliverables from this project are:

- Better detection of downy mildew in the field before disease is visible in the crop.

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

1.3 Summary of first year work on FV189a

1.3.1 Development of specific antibodies for detecting and quantification of onion downy mildew conidia

Producing specific antibodies in test animals which recognise downy mildew conidia depends on using the correct immunogen. Using downy mildew spore washings triggered an immune response by the immunised animals to *Peronospora destructor.* Monoclonal cell lines which react to onion downy mildew have been raised in mice injected with spore washings. Tests are on going at present to determine the specificity and cross reactivity of antibodies produced by these cell lines. Initial results not included in this report indicate that several lines produce antibodies which react specifically with onion downy mildew conidia. In addition a polyclonal antibody was raised in rabbit against onion downy mildew conidial washings. However high background absorbances were observed when this polyclonal antibody was tested for its specificity. This may have resulted from the presence of other reactive antibody classes. By using purification techniques these problems were reduced. A level of cross reactivity was observed to the spores of a number of fungal species using this polyclonal antibody. However no reactivity was observed to *Hyaloperonospora parasitica w*hich causes downy mildew on vegetable brassicas. The polyclonal antibody was weakly cross reactive with *Botrytis cinerea* but did not react with *Botrytis allii*. Both species of *Botrytis* are commonly found in onion crops. Tests with *Botrytis squamosa* another pathogen present in onion crops have still to be carried out. The antibody reacted strongly with *Ascochyta rabiei* (blight of chickpea) and *Pyrenophora teres* (net blotch of barley).

1.3.2 Systems for detecting onion downy mildew in the field

Spore trapping studies were used to ascertain environmental factors required for spore dispersal and assess spore trapping formats for detecting onion downy mildew conidia. These experiments were conducted in a seeding crop of bulb onions which was extremely susceptible downy mildew infection and sporulation. No downy mildew symptoms were observed in the crop 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 and observed. The

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results of the trial show that the predictions of onion downy mildew sporulation were accurate particularly those from the MILIONCAST system in comparison to the DACOM onion downy mildew forecasting system. The environmental requirements for downy mildew spore dispersal were particularly difficult to ascertain. 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. Even if environmental criteria are present for spore dispersal no spores are dispersed if they have not been produced. 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. Further data sets will be required to ascertain all the critical factors necessary for downy mildew dispersal. Results indicated that conidia of onion downy mildew could be trapped within the vials in cyclone traps. Conidia could be extracted to give accurate estimates of conidial number when assessed by immunological methods (ELISA). The results (with a limited data set) were comparable to standard spore trapping techniques (Burkard seven day volumetric trap) as assessed using microscope counts.

1.4 Action points for growers

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

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

1.5 Anticipated practical and financial benefit

- The usage of the “in field “ test for onion downy mildew will improve the timing of the first application of fungicide for controlling this pathogen in onion crops.
- There will be less need for and reliance on metalaxyl based fungicides which should reduce the costs associated with onion downy mildew control in bulb and salad onion crops.

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

SCIENCE SECTION

2. INTRODUCTION

2.1 Air borne disease problems affecting onion crops

Downy mildew (*Peronospora destructor*) and onion leaf blight (*Botrytis squamosa*) can cause heavy yield losses in bulb and salad onion crops. Leaf blight of onion can be a problem in UK production however it is downy mildew which is the most serious air-borne disease in onion crops in the UK. In salad onions, *Botrytis* lesions themselves lead to a loss of quality. To control the disease, fungicide sprays such as chlorothalonil are often applied and these are also effective against downy mildew. Fungicidal control of downy mildew is often more difficult. Fungicides are only effective, if they are applied before or immediately after disease first appears in the crop (Kennedy *et al*., 1998). Fungicidal control is the only effective means of controlling the disease and avoiding crop loss. Plant density, poor orientation and irrigation may also be important in controlling the pathogen, particularly in salad onion crops.

2.2 Biology of *Peronospora destructor* on onion crops

The downy mildew pathogen sporulates during the night under high relative humidities of greater than 95% at temperatures of 6 - 22ºC provided there was no rainfall. Sporangial discharge is triggered when relative humidity falls below 59%. Conidia are thin walled and wind transported over considerable distances. Conidia have been detected at heights of 1500 ft. When conidia are deposited on leaf surfaces, they germinate between 1.5 and 7 h. Progress and spread of downy mildew is dependent on the survival of spore populations until conditions become favourable for germination (Sutton & Hildebrand, 1985). However viability is affected by the prevailing temperature and humidity conditions. At 10ºC, spore viability is unaffected by relative humidity. However, at temperatures of 30ºC viability declines rapidly at relative humidities above 55 %.

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

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

Management systems are available for both diseases (BOTCAST/DOWNCAST/ONION$_{SPOT}$) (Gilles & Kennedy, 2004) which can predict the early development of both downy mildew and *Botrytis* in onion crops. Predictions for downy mildew are based on the occurrence of environmental conditions, which affect downy mildew infection and sporulation. Temperature and relative humidity have the greatest effect on downy mildew sporulation. Downy mildew sporangia were produced most rapidly at 8 to 12°C after 5 h of high humidity during dark periods. The greatest number of sporangia was produced at 100% RH and sporulation decreased to almost nil when humidity decreased to 93% RH. A 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 this controlled environmental data. This model is not yet available within ONION$_{SPOT}$ but it is hoped that this model can be added to the system at some future date. The accuracy of MILIONCAST was compared to the accuracy of existing models based on DOWNCAST. MILIONCAST gave more correct predictions of sporulation than the DOWNCAST models and a random model. All models based on DOWNCAST were more accurate than the random model when compared on the basis of all predictions (including positive and negative predictions), but gave less correct predictions of sporulation than the random model (Gilles & Kennedy, 2004). Use of this system, which provides information on the timing of the first fungicide application, may result in better disease management. However inoculum can be imported into disease free crops from other localities/areas but assessments based on environmental risk alone do not take this factor into account. In order to avoid these problems new and rapid methods of detecting and quantifying pathogenic inoculum are required which can be used in conjunction with forecasting models. With this more precision approach there will be reductions in the amounts of fungicide required to control disease by eliminating unnecessary fungicide applications, which are based on weather information alone.

2.4 Using air-borne spore numbers within disease forecasting systems

Detection and quantification of airborne spore numbers can be used to predicted disease accurately before it is visible in the crop. Peaks of airborne spores are always detected prior to crops becoming infected. This, results from the requirement for a threshold of inoculum to initiate disease establishment in crops and this must coincide with favourable weather conditions.

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/m3 (disease was not yet visible when this threshold was reached). By using this, criteria in combination with disease forecasts based on weather information the number of fungicide applications could be reduced with no impact on disease development. Given that potato blight is a difficult pathogen to control great scope exists in applying this approach in the control of other less aggressive pathogens and in different localities. This means that disease can be predicted accurately before it is visible in the crop. Often this comes well in advance of disease appearance because a threshold of inoculum is required to initiate disease establishment in crops and this must coincide with favourable weather conditions. Similar results were obtained using for *Botrytis* blight (*Botrytis squamosa*) on onion crops where thresholds of 15 - 20 conidia/m^3 could be used to reduce fungicide application by up to 100% (Carisse et al., 2003). Thresholds of inoculum required for disease establishment have also been reported for *M. brassicicola* which is the fungal pathogen responsible for ringspot on vegetable brassicas (Kennedy et al., 2000). In these studies (with the exception of ringspot) the information on spore number had to be collected manually using a microscope which was slow and time consuming. Tests which, can be conducted in the field are necessary if information on air-borne inoculum concentration is to be of more practical value. The use of air-borne spore numbers, as criteria, within forecasting systems is a new and exciting development in disease forecasting. One “in field test” which could be used in this respect is the lateral flow test for downy mildew inoculum.

2.5 Lateral Flow Device (Competitive Assay only)

Lateral flow assays rely upon the competition for binding sites by sensitised latex particles. Antibodies (polyclonal or monoclonal) raised to a specific target spore, are bound by passive

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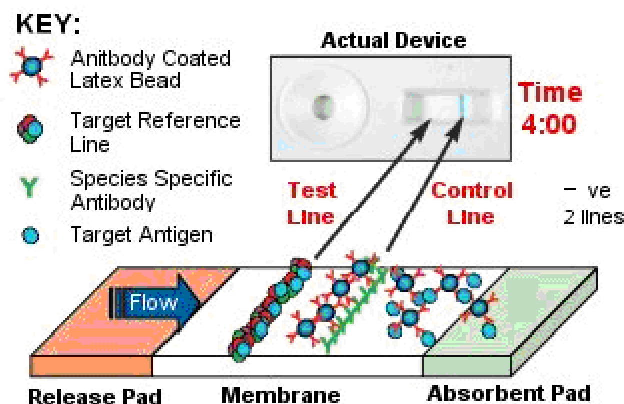
or covalent means to dyed latex particles. These sensitised particles are then applied using an immersion procedure on to a release pad, to produce a stable particle reservoir for release on to a nitro-cellulose-based membrane. Two lines of reagents are immobilised on to the membrane using a sophisticated reagent dispenser. The target reference or test line comprises an antigen or conjugate of the target to be identified 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 (Diagram 1). Two to three drops of sample extract obtained from an air sampler used to collect the spores) are added to the well, releasing the specific antibody bound latex particles, which then begin to flow across the membrane. If the target antigen is present in the sample extract, antibody binding will occur to produce a latex/antibody -antigen complex. Any antibody conjugated latex particles that fail to bind to an antigen will attach to the immobilised test line as they traverse the membrane; thus producing a visible line of deposited latex. The anti-species antibody then captures excess sensitised latex particles to produce an internal control line, providing a visible confirmation of latex flow. Sufficient target presence induces complete inhibition of latex attachment to the test line, a result that is indicated by a single line of latex deposition. Two lines of equal colour intensity indicate a negative result.

Diagram 1. The Lateral Flow Device



The competitive lateral flow device assay is a semi-quantifiable test. Use of reader technology allows the line intensity, and therefore level of latex accumulation to be calculated using reflectance photometry. However this is not yet available for use under field conditions. By introducing internal control latex 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, facilitating the differentiation between target concentrations.

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

If accurate 'in field' tests for inoculum are to be constructed they will require specific antibodies that can differentiate between different types of pathogenic spores recognising only downy mildew spores. If specific antibodies can be raised then the development of lateral flow tests which incorporate them can proceed. No antibodies are commercially available for downy mildew of onions. For this reason most of the first year work of the project must be devoted to the development and testing of onion downy mildew specific antibodies. This must be done by raising reactive cell lines in mice which produce specific antibodies. These are then tested for their specificity to downy mildew and their reactivity to a range of common air-borne fungi found in the field associated with onion crops. This would include other pathogens which are common in onion crops notably *Botrytis squamosa* and *Botrytis cinerea*. Other pathogens found in onion crops include *Cladiosporium allii cepae* Many of these pathogens survive on debris in the soil or are found on leaves on onion crops. Consequently tests which detect downy mildew on onions should not react with the conidia of other pathogens common in onion crops. For this reason extensive cross reactivity tests must be conducted using the specific antibody to check that it does not react with other fungal species. If the antibody does react with conidia of other fungi found in onion crops then it cannot be used for the development of “in field” tests for downy mildew conidia. The level of reactivity of the antibody is also important as this can be used to quantify the number of downy mildew conidia present in samples. In the first year of the work these aspects of the detection system for downy mildew are required.

3. PRODUCTION OF ANTISERA

3.1 Production of monoclonal antisera to *Peronospora destructor*

3.1.1 Introduction

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

3.1.2 Materials and Methods

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

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 2nd 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*.

3.1.3 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 1). 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).

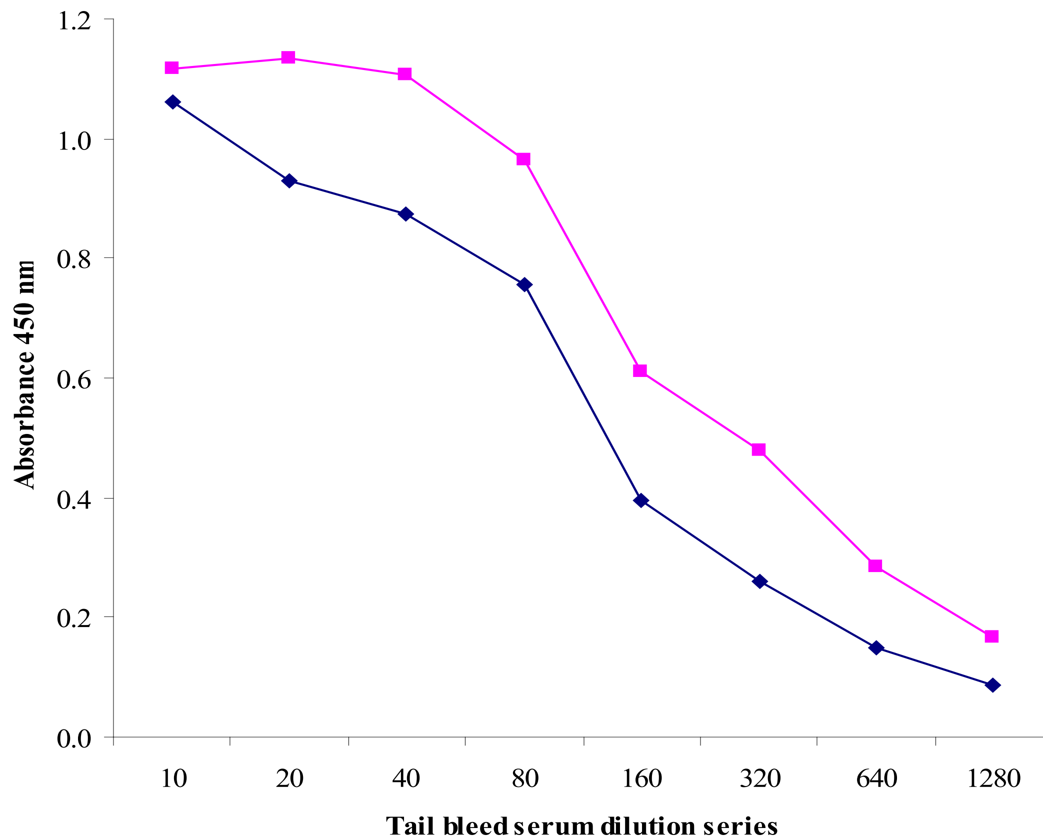


Figure 1. **Titration of mice tail bleeds (PAS 7996 and 7997) to freshly collected sporulating spores of *P. destructor* in Phosphate buffered saline (PBS) by PTA-ELISA**

3.1.4 Conclusion

Using A soluble concentrate (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

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lateral flow assay where antigen solubility is critical for movement during the aqeous 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 mice

A female White New Zealand rabbit (coded PAS 7346) received an initial immunization of a 500µl *P. destructor* spore suspension (2.6 x 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 3.1.1.2) 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 (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.

3.2.2 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 2).

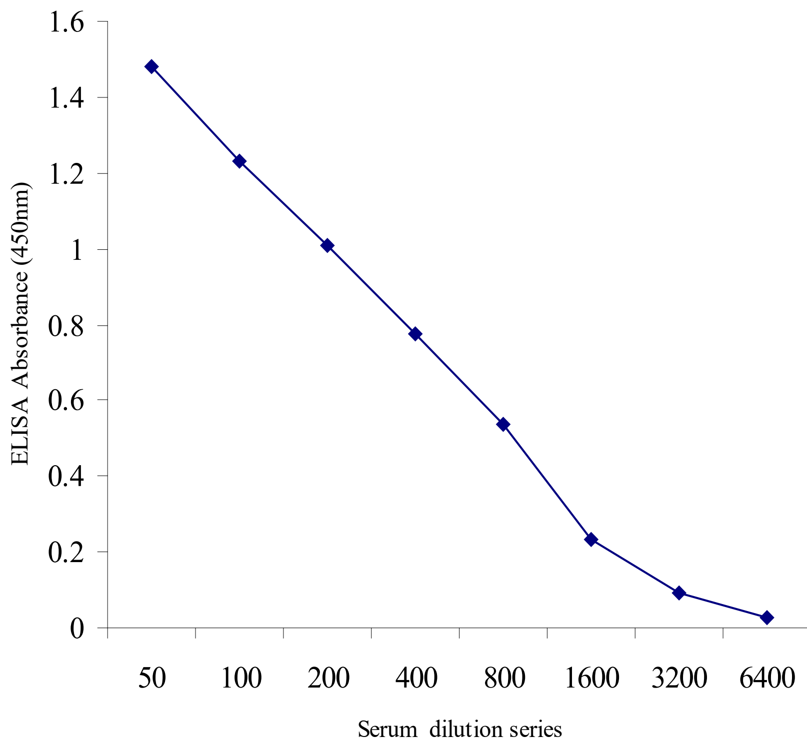

Figure 2. **Titration of rabbit IgG purified polyclonal antiserum (coded PAS 7346p) to freshly collected downy mildew spores (*P. destructor*) in Phosphate buffered saline (PBS) by PTA-Protein A Horseradish peroxidase ELISA.**

3.2.3 Conclusion

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 the mice (Section 3.113) 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 microtiter wells were

blocked with 200 µl of 1 % casein buffer (1 % (w/v) casein PBS) and incubated at 37 º C for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200 µl PBS Tinc 0.05 % Tween 20 (PBSTincTw). Following this procedure wells received 100 µl per well of 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 microtiter 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 3). 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).

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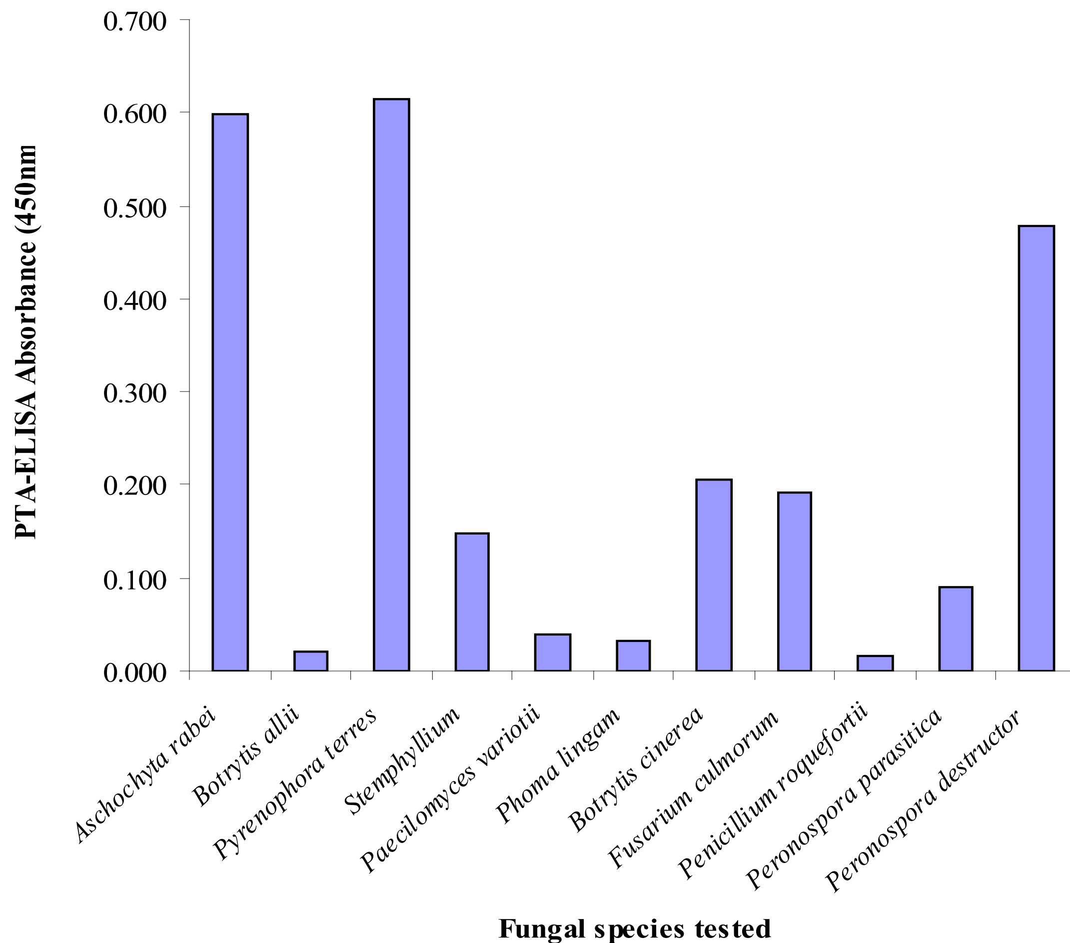

Figure 3. Cross-reactivity of polyclonal antibody PAS 7346p to a range of fungal organisms found in the field

3.3.3 Conclusion

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 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.

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4. DETERMINATION OF SPORE TRAPPING PERIODS FOR DOWNY MILDEW ON ONION

4.1 Measuring the diurnal periodicity of downy mildew in air samples

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.

4.1.2 Materials and Methods

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 was introduced into the centre of the plot. Downy mildew lesions were observed on plants close to the points of inoculation. Downy mildew infection of the plot was not extensive due to hot and dry conditions which occurred during July and August 2003. The crop was over-wintered to produce a seeding crop in spring 2004.

4.1.2.2 Spore trapping measurements

A 24 H volumetric spore traps (Burkard Scientific Ltd., Rickmansworth, Hertfordshire, England) were operated continuously (Hirst, 1953) 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.

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 logger. Measurements were collected by GSM portable phone Link (Skye Instruments Ltd, Llandrindod Wells, Powys). 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.

4.1.2.4 Prediction of spore production in the field

Disease forecasting models were used to predict when downy mildew conidia were produced under prevailing environmental conditions. The 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.

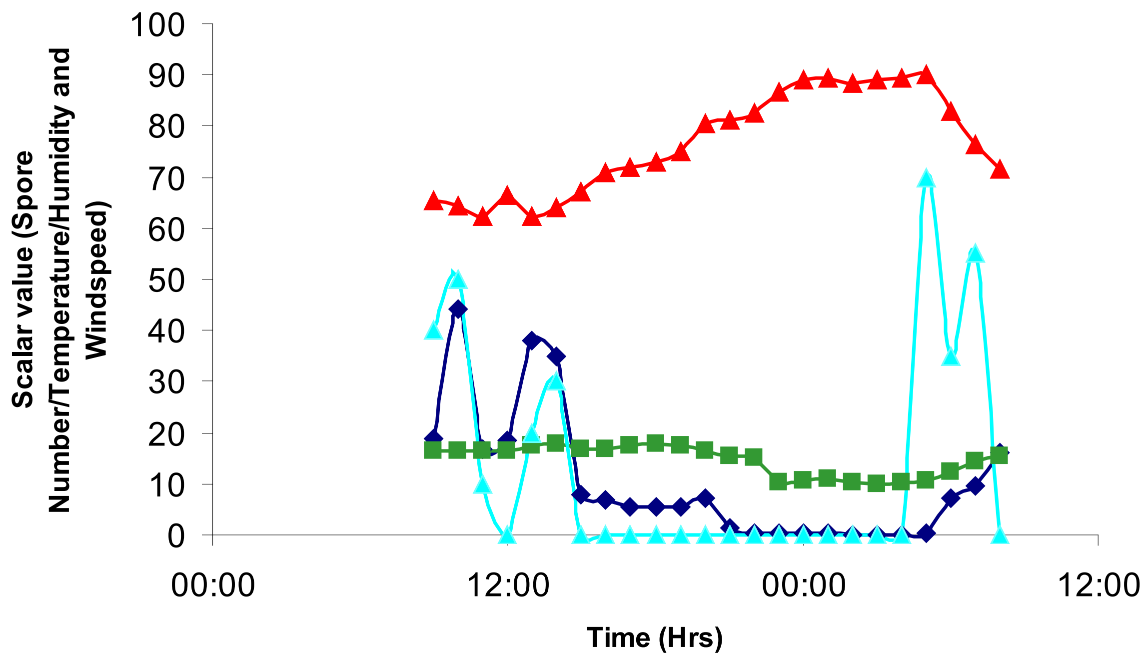
4.1.3 Results

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

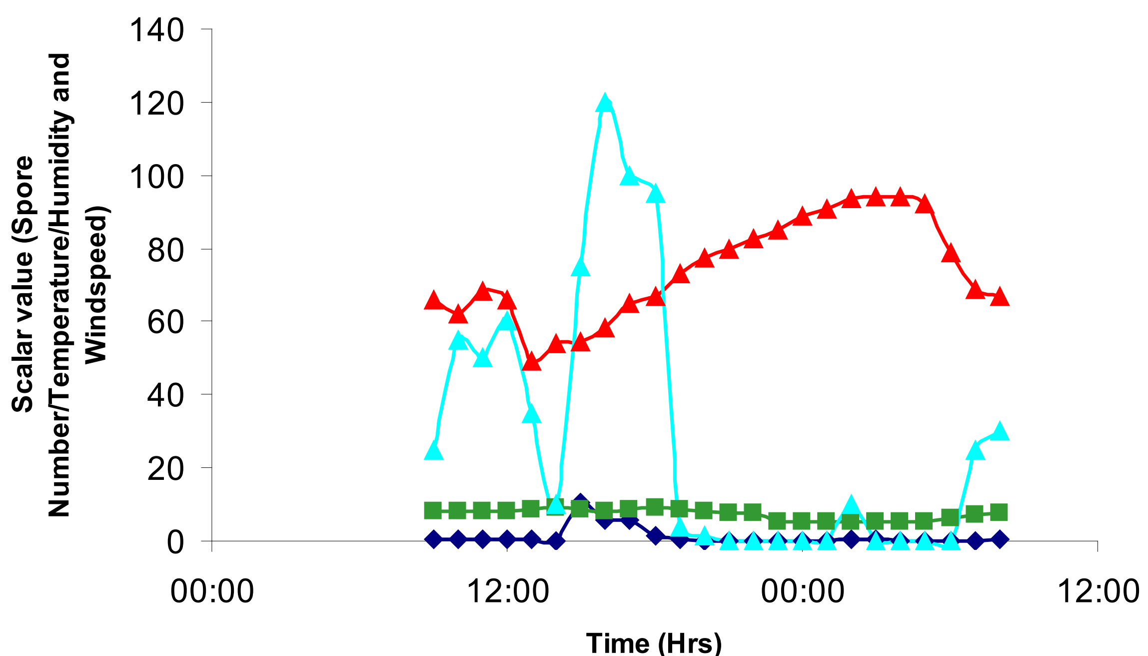
The effect of major environmental parameters on onion downy mildew release is shown in Figures 4 a, b and c. These figure show three days where spore production by downy mildew was predicted. The numbers of trapped downy mildew conidia are shown in relation to the diural variation in weather conditions 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 4, 5, and 6

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 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)

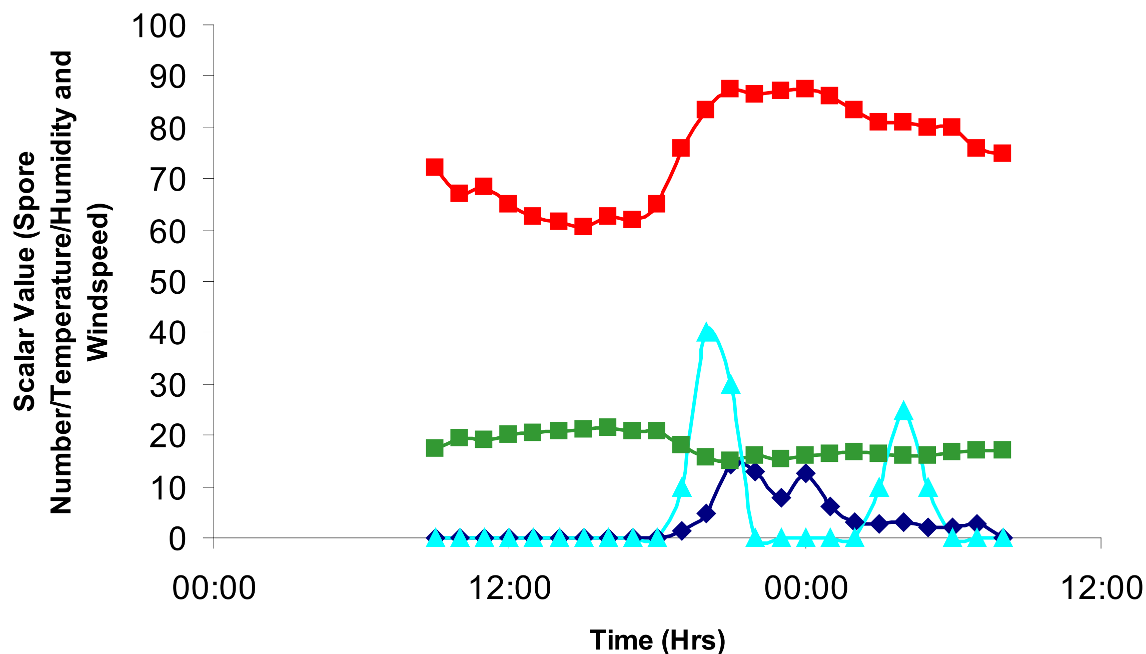


Figure 4. **Numbers of airborne conidia of downy mildew trapped x 10 $^{-2}$ (♦) over a) 3 June 2004, b) 4 June 2004 and c) 5 June 2004 and corresponding temperature (■), humidity (■) and x10 windspeed at 5 km h for 5 sec (▲) recorded at the monitoring site.**

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.

4.1.3.2 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 Table 2. The two models used use 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.

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4.1.4 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 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).

Table 2. Predictions of onion downy mildew conidial production at Warwick HRI

Date (2004)	Predicted Sporulation	
	MILIONCAST	DACOM
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	-	-

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5.0 EVALUATION OF TRAPPING FORMATS FOR ONION DOWNY MILDEW IN THE FIELD

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.

5.1.1 Materials and Methods

5.1.1.1 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. Description of the 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 microtiter 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.

5.1.1.2 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 3.3.2. Absorbance values (clour changes) could then be compared with the numbers counted by light microscopy.

5.1.1.3 Micro-climate measurements

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

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5.1.1.4 Prediction of spore production in the field

Disease forecasting models were used to predict when downy mildew conidia were produced under prevailing environmental conditions. The 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 (see section 4.1.1.4) .

5.1.2 Results

5.1.2.1 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 3. 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	-	+

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

The estimated numbers of downy mildew conidia trapped in air samples for each 3 – 4 day trapping period is shown in Figure 5. The absorbance value represents a colour change. 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. 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 at the time of this report. Direct microscope observations would be required to determine if downy mildew were present in the sample in each trapping period.

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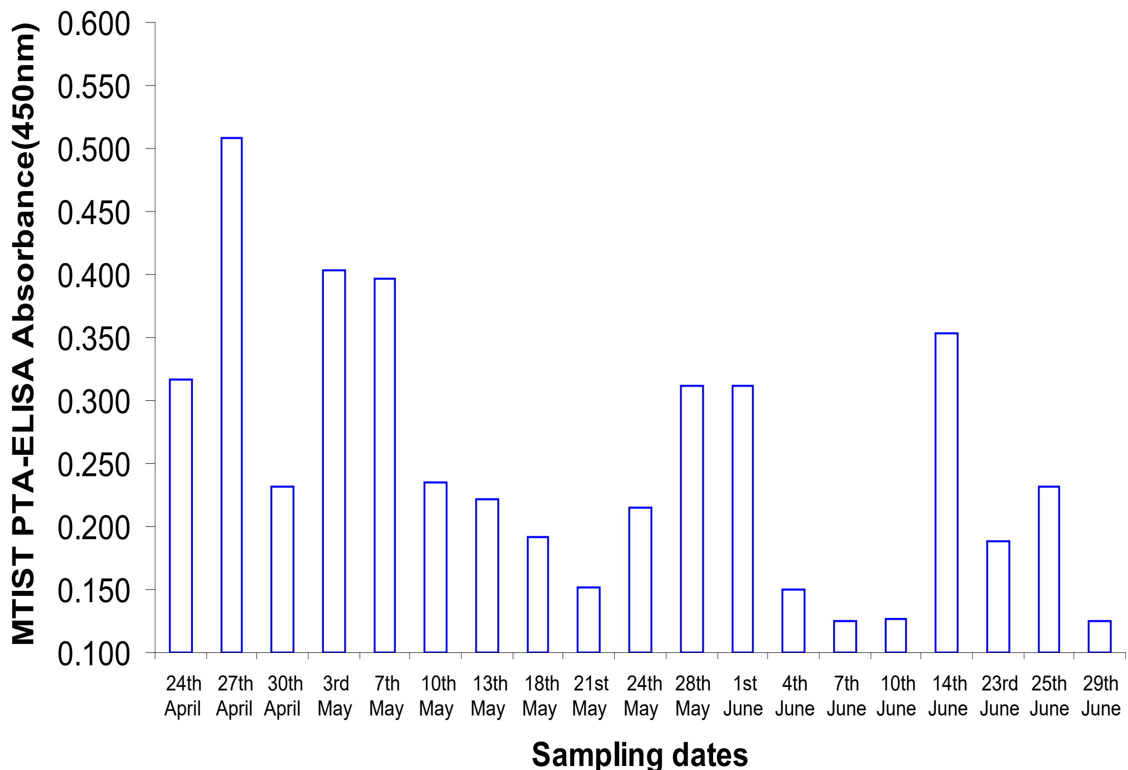

Figure 5. **ELISA values obtained for each trapping period at Sleaford 2004 employing polyclonal antibody PAS 6346p.**

5.1.3 Conclusion

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 in Figure 5 which leaves 3 strips available for further evaluation with other antibodies.

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

5.2.1 Materials and Methods

5.2.1.1 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.

5.2.1.3 Detection of 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. Afterwhich 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 (Sugna E-2377) and diluted in PBS TC. The slide was incubated as described above but this time in darkness. The slide was washed as desribed 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.

5.2.1.4 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 weel 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

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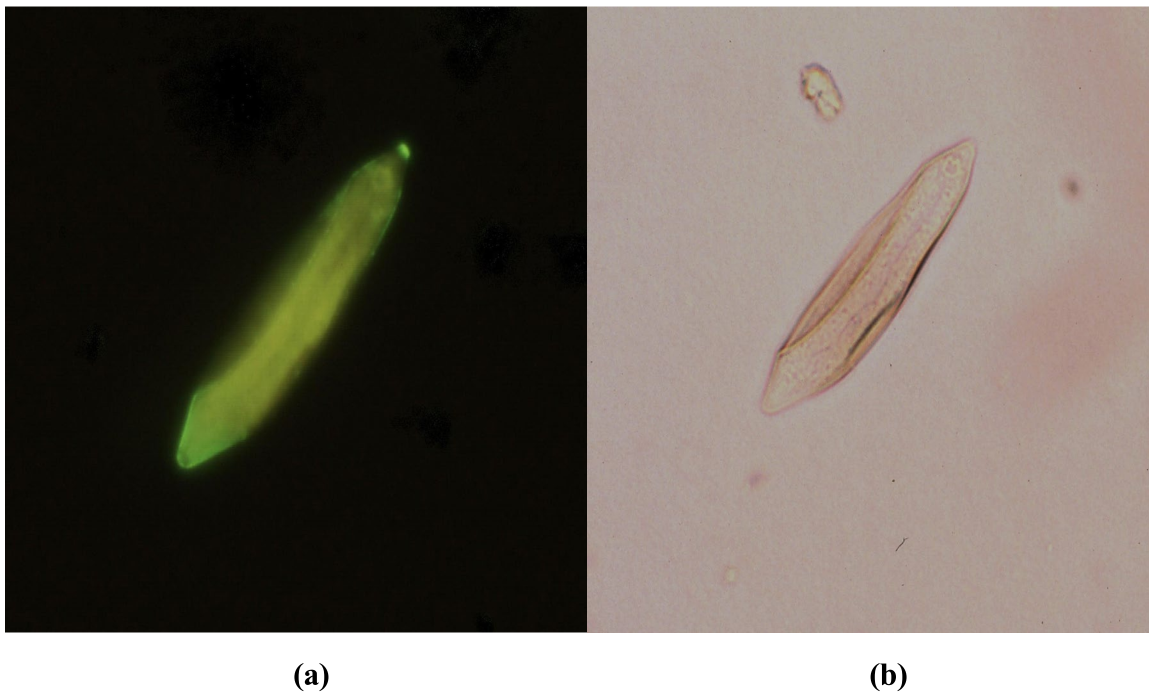
against absorbent towelling. To block unbound sites each well received 200ul 1% Casein in PBS. The remainder of the ELISA was as described above in Section 3.3.1

5.2.2 Results

5.2.2.1 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 1). Reactivity of the antiserum was noted however to a number of other trapped air spora but was at a low level.



(a) **(b)**

Plate 1 Onion downy mildew conidia as visualised by (a) immunofluorescence using polyclonal antibody PAS 7346p and (b) using bright field microscopy

5.2.2.2 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 parasitica*) 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.

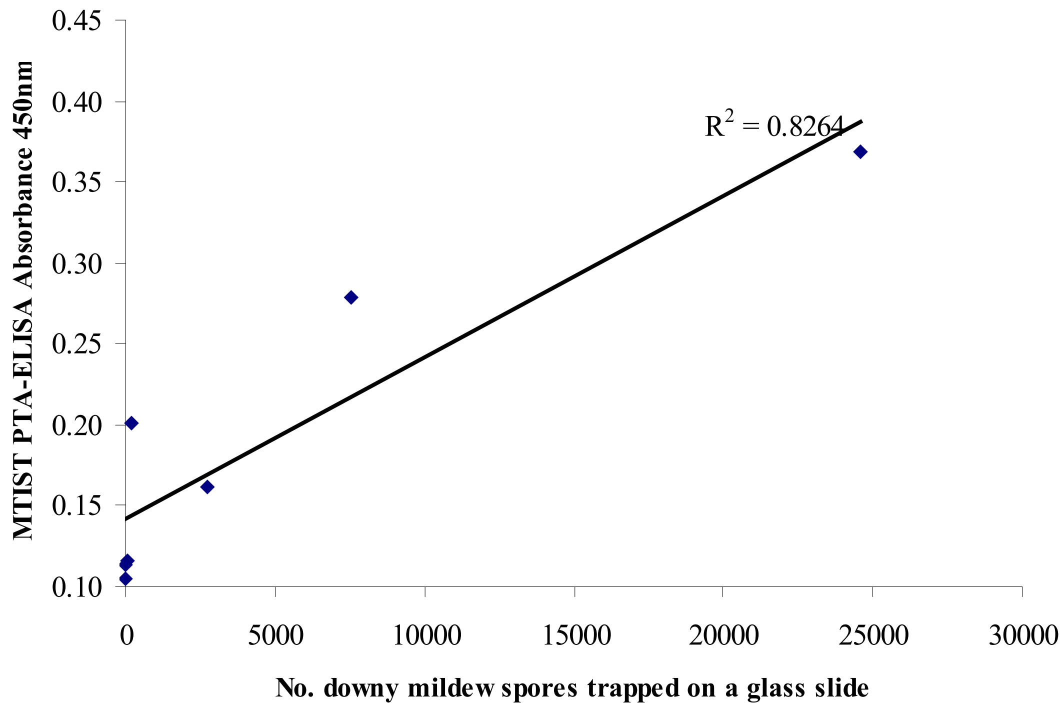


Figure 6. The relationship between the number of onion downy mildew conidia and absorbance from PTA ELISA.

5.2.3 Conclusion

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 parasitica* (downy mildew) conidia, should remove this problem and enable the development of a rapid, reliable quantitative assay.

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6.0 DISCUSSION

In the current project "in field" tests for conidia of onion downy mildew are being developed. Producing these tests will require the development of specific antibodies, which can be used to visualise the presence of target inoculum (onion downy mildew conidia). The specificity of the antibodies will require testing in relation to a range of related and unrelated fungi, which are found in onion crops or in their vicinity. The ability of the trapping system to catch and retain the spores in sample tubes will also require testing and optimisation. Finally once the lateral flow tests have been derived it will be necessary to test the system under field conditions. At each stage in this developmental process validation will be required and in this report the various stages in developing the components needed for these tests has been described.

6.1 Producing antibodies for use in lateral flow devices

Within this project monoclonal antibodies have been raised which selectively recognise components of the spore of *Hyaloperonospora destructor* (onion downy mildew). The antibodies raised to onion downy mildew conidia have been tested against a few other fungal species found in onion crops and a number of other common fungi found in the field. Raising monoclonal antibodies requires the use of spore washings as an immunogen. Earlier attempts to produce monoclonal antibodies using whole spores as the immunogen were unsuccessful. A polyclonal antibody was also raised to conidia of onion downy mildew. The polyclonal antibody was weakly cross reactive with *Botrytis cinerea* but did not react with *Botrytis allii*. The antibody reacted strongly with *Ascochyta rabiei* (blight of chickpea) and *Pyrenophora teres* (net blotch of barley). Some of the antibodies raised appear specific enough be used in lateral flow test kits to detect for *P. destructor* conidia. Work is still ongoing in raising additional monoclonal antibodies to *P. destructor* conidia.

6.2 Sample preparation from 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. Knowing the specific criteria which enables onion downy mildew to become airborne would be important. In the experiments described in this report spores were trapped in eppindorf tubes (the collection vessel) within cyclone spore traps. In these types of traps all the sampled air is drawn into an eppindorf tube on to which the spores are impacted. In order for the accuracy of the system to be realised all spore material must be removed from the eppindorf and deposited into the test format. For field use, the test format will be the lateral

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flow device. However as these are not yet available the tests were carried out by counting the numbers of spores after samples had been removed from the eppindorf tube and placed in microtitre wells.

In other experiments in the field the numbers of sampled conidia were detected (if present) using ELISA. This immunoassay format is complimentary to that of a lateral flow device. Removal of onion downy mildew spores from the trap sample tube was not problematical and the system was best optimised by using phosphate buffered saline. Important criteria would be to use the disease forecasting models to determine when to sample for onion downy mildew in the air. Two disease forecasting systems (DACOM and MILIONCAST) were used to determine when onion downy mildew conidia were produced. In these tests (on a limited data set) the MILIONCAST system proved to be more accurate in determining if onion downy mildew spores are produced. If these criteria were fulfilled then sampling time must be determined when favourable environmental conditions for conidial dispersal occur. From results obtained in this report the occurrence of wind speed's between 3 – 5 km sec^{-1} during the period after spore production would be important sampling criteria for onion downy mildew. Downy mildew conidia are relatively large and it is therefore not surprising that wind speeds at these levels would be required to disperse conidia. Under these conditions the onion downy mildew conidia are probably dispersed quite widely.

6.3 Disease forecasting criteria based on inoculum detection

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. Using these techniques the critical date for applying fungicide applications to the crop can be identified. However it is unclear how this information can be applied to bigger cropping areas. 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. This type of action is called a "rain out" and it means that by checking, for pathogenic inoculum in the air at locations before predicted rainfall, the likelihood of risk to the crop can be ascertained. 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.

Other workers have demonstrated that this approach can be used to control the potato blight pathogen *Phytophthora infestans*. 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. By using this, criteria in combination with disease forecasts based on weather information the number of fungicide applications could be reduced with no impact on disease development. This means that damaging disease outbreaks can be predicted accurately before it is visible in the crop giving time for the grower to take action to control potential damage. These forecasts will not be based on future weather events, which by themselves are too unreliable to use in disease forecasting systems. Similar results were obtained using 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). In all of these studies, 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. 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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