Project title:	Brassicas: Development of a rapid field based immunomonitoring assay and its use in controlling disease establishment in transplanted crops.
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The results and conclusions in this report are based on an investigation conducted over one year. The conditions under which the experiment was carried out and the results obtained have been reported with detail and accuracy. However because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results especially if they are used as the basis for commercial product recommendations.

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1. GROWER SUMMARY

1.1 Headline

Prototype sampling kits for detecting Alternaria spores are being developed. These will be for use in association with disease forecasting models. Field trials of the first prototypes will begin in 2003/04.

1.2 The Problem

There are many air-borne fungal diseases, which affect vegetable brassicas in UK production. Ringspot (*Mycosphaerella brassicicola*) and dark leaf spot (*Alternaria brassicae*) are two of the most serious problems with the most widespread geographic distribution. In Brussels sprouts production 4 - 6 fungicide applications have been used to control these diseases and maintain the high quality of produce demanded by the market. These levels of fungicide usage often result from the need to prevent disease establishment within the crop. Many air-borne pathogens (ringspot) of vegetable brassicas have long latent / incubation periods (time between infection and appearance of disease). This means that success or failure of control is only apparent in some cases weeks after fungicide applications. Often this leads to diseases becoming well established in crops before the disease is really visible. Additionally many of these diseases are difficult to diagnose correctly, as they all first appear as small black spots. A major problem exists in differentiating ringspot from dark leaf spot in its early stages of development. Similarly older lesions of powdery mildew often resemble young fresh lesions of dark leaf spot. Different chemicals of first choice may be used to control dark leaf spot and powdery mildew.

1.2 New detection systems for inoculum

The research in this report describes the development of new detection systems which can be used to monitor the presence or absence of diseases in the air before it gets on to the plant. This is a new and revolutionary approach to crop protection, which will help growers and producers reduce their reliance on pesticides especially if they use the information within existing disease forecasting systems (such as Brassica*spot*). The system is being developed in the first instance for detecting ringspot (ascospores) and dark leaf spot (spores) in the air. The development of 'grower friendly' rapid field-based assays to monitor the air-spora and detect, differentiate and semi-quantify disease inoculum requires the development of a specific test format. This test format will be non-automated (meaning that growers or end users need to carry out the test in the field manually). The format that the test will take is described as a "lateral flow test" . This type of device is based on the recognition of target spores by antibodies,

which react with them specifically. The more specific the antibody the more accurate the results are likely to be.

1.3 Lateral Flow Device (Competitive Assay)

The principle of the competitive lateral flow assay relies upon the competition between antibodies for binding sites on sensitised latex particles. Antibodies (polyclonal or monoclonal) raised to a specific target spores, are bound by passive or covalent means to dyed latex particles. These sensitised particles are then applied on to a release pad, to produce a reservoir for release on to a test membrane, which will be the part observed by the end user. Two lines of reagents are immobilised on to the test membrane. The target reference or test line comprises of the target particle to be visualised (the spore or a component of it). The control line comprises of anti-species antibody, which will react to any unbound antibody. The release pad and membrane are assembled together with an absorbent pad into a plastic housing. Two to three drops of sample extract (obtained from an air sampler used to collect the spores) are added to the well, releasing the 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-antigen complex. Any sensitised 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 amounts of target spores induce complete inhibition of latex attachment to the test line, a result that is indicated by a single line of latex deposition (a positive result). Two lines of equal colour intensity indicate a negative result.

The competitive lateral flow device assay can be produced as a semi-quantifiable test. Use of reader technology allows the line intensity, and therefore level of latex accumulation to be calculated using reflectance photometry. 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. Internal control latex is labelled with particular species sera that, is independent of the antigen-sensitised particle interactions. Variations in line intensity can be distinguished using a reader, facilitating the differentiation between target concentrations.

1.4 Producing antibodies to ringspot and dark leaf spot spores

In the present study a range of antibodies have been raised against dark leaf spot and tested for their compatibility for use in lateral flow devices. Several antibodies have been raised which recognise dark leaf spot spores but do not recognise related organisms found on carrot and wallflower. These antibodies did not recognise spores

of several species of *Botrytis* either. This is a common fungal type found in many vegetable brassica crops and is responsible for storage losses in Dutch white cabbage. The work raising antibodies to ringspot ascospores is still ongiong at this time. The antibodies raised to dark leaf spot spores have been tested for their ability to quantify numbers of dark leaf spot conidia. The results showed that some antibody test lines reacted strongly with increasing number of dark leaf spot spores. Of the monoclonal cell lines tested Mab 220/1.D1.C2.E11 gave the best results with a linear relationship between the signal (absorbance) and the number of dark leaf spot conidia per microtitre well. It is unlikely that Mab 212.C6 will be used in further lateral flow development as it recognised only germinated dark leaf spot conidia. This antibody was unable to detect ungerminated dark leaf spot conidia.

1.5 Detecting and quantifying dark leaf spot spores in the presence of other fungal spores

Tests were carried out on the ability of dark leaf spot spores to be extracted from sample tubes which, had been used to collect them from air samples. In the experiments described in this report spores were trapped in eppindorf tubes within cyclone spore traps. In these types of traps all the sampled air is drawn through the 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 tube and deposited into the test format. Removal of dark leaf spot spores from the trap sample tube was not problematical and the system was best optimised by using a phosphate buffered saline extraction buffer. Tests were conducted where the ability of the antibodies to detect dark leaf spot spores in the presence of other spores was determined. Powdery mildew was chosen for these tests because it is a very common air-borne spore type.

The optimised system with the most specific monoclonal antibodies which, selectively detect conidia of dark leaf spot, were used in controlled environment tests where a source of dark leaf spot spores were present. Infected vegetable brassica trash, which contained both dark leaf spot pathogens (A. brassicae and A. brassicicola), was placed in a CE cabinet at 15 C and 80 % r.h.. A cyclone trap and a modified Burkard volumetric trap were placed within the cabinet with 4 trays each, containing 16 plants of Brussels sprouts cv. Cavalier. Tests were conducted in the presence of 20 plants of Brussels sprouts, cv. Golfer with sporulating lesions of powdery mildew. These tests showed that antibodies Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5 were highly specific to dark leaf spot spores (*A. brassicae* only). These antibodies therefore appear suitable for use within lateral flow tests for dark leaf spot. Both these antibodies did not appear to cross react with *A. brassicicola*. *Alternaria brassicae* and *A. brassicicola* were present in the spores samples (from air). However a good relationship between ELISA estimates of *A. brassicae* spore number and dark spot

lesion numbers on exposed Brussels sprout plant given, infection conditions after exposure, was observed. Despite the presence of *A. brassicicola* in the spore samples it appeared that only *A. brassicae* spores had given rise to infection on leaf material. This was confirmed when leaf lesions were plated on to agar. These results were also confirmed when no relationship was observed between ELISA value and leaf lesion number using antibody Mab 190.191.G8. This antibody reacts with both *A. brassicae* and *A. brassicicola* resulting in no correlation between ELISA value and leaf lesion number.

The antibodies raised and characterised in this study will be incorporated into lateral flow tests for detecting conidia of dark leaf spot in the field. Development of antibodies specific to acospores of the ringspot pathogen is still on going.

1.6 Action points for growers

- Growers should run forecast models to identify likely disease periods.
- When these disease risk periods appear it is hoped that growers can use a simple test to confirm if spores are in the air / crop.
- Prototype spore testing kits for Alternaria and ringspot are in the preliminary stages of development. It is hoped that these will be tested in the field in 2004.

1.7 Anticipated practical and financial benefit

With the development of test kits (lateral flow tests) it will possible for the grower to obtain this data on the risk to crops due to pathogenic inoculum in a rapid and inexpensive way. By using traps in conjunction with forecasts the grower will be able to assess the risks precisely from ringspot and dark leaf spot to his crops. If the tests are carried out on, samples obtained before rain events the likelihood of "rain outs" can be determined. Rainouts are events where spores are deposited out of the air by rainfall. These account for virtually all successful transmission events between and within crops. Using this information the grower will then be able to determine precisely which crops require fungicides and which do not. This offers the possibility of producing crops using very little or no fungicides by breaking the cycle of disease establishment and transmission. By using this approach the grower will be able to cope with reduced numbers of actives available in brassica production and any subsequent decline. By conducting tests before predicted rainfall events the grower will also be able to assess the need for protective applications of fungicide to his crops and optimise these very effectively.

2. INTRODUCTION

2.1 Air borne disease problems in vegetable brassica crops

Many air-borne fungal diseases affecting vegetable brassicas are common in UK production. Ringspot (*Mycosphaerella brassicicola*, and dark leaf spot (*Alternaria brassicae*) are the most serious problems with the most widespread geographic distribution. However a range of air-borne disease problems are prevalent including white blister (*Albugo candida*), light leaf spot (*Pyrenopeziza brassicae*), powdery mildew (*Erysiphe cruciferearum*) and downy mildew (*Hyaloperonospora parasitica*). Brussels sprout crops in many areas would normally receive 4 - 6 fungicide applications to control these diseases and maintain the high quality of produce demanded by the market. However these diseases are still problematical on cauliflower and broccoli crops and can lead to downgrading of produce or in severe cases complete crop loss. Fungicides are thus often used to prevent disease establishment within the crop. However many air-borne pathogens (white blister; light leaf spot and ringspot) of vegetable brassicas have long latent periods (time between infection and appearance of disease). This means that success or failure of control is only apparent in some cases weeks after fungicide applications. Often this leads to diseases becoming well established in crops before the disease is really visible. Additionally many of these diseases are difficult to diagnose correctly, as they all initially appear as small black spots (except white blister). A major problem exists in differentiating ringspot from dark leaf spot in its early stages of development.

2.2 New detection methods for air-borne spores of vegetable brassica pathogens

Within this project new and rapid methods of detecting and quantifying pathogenic inoculum in air samples are being developed. These methods can be used in conjunction with disease forecasters to accurately determine the type and actual amount of disease, which will occur. 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.

The principles involved in the development of rapid tests have been demonstrated within DEFRA project HH1759SFV. In this DEFRA project a new novel trapping system (MTIST spore trap) was developed and used to quantify spore type and number by processing particulates trapped from air samples using specific antibody based probes (Wakeham *et al*., 2002). By using a specific monoclonal antibody (which recognises the wall material of ringspot ascospores) a clear correlation between the number of ascospores trapped by the MTIST spore trapping device and

the absorbance values (generated by the immunoassay test - ELISA) was demonstrated. A relationship was also demonstrated between the amount of ringspot lesions (which appeared on exposed trap plants placed inside the controlled environment cabinet) and the immunoassay test (PTA ELISA) values. Mixed spore population tests in these studies suggested that there was no interaction between large and small propagules trapped. There was also a very good correlation between existing antibody based trapping systems (the B-7 Day immunofluorescence test) and the PTA ELISA indicating a useful bench mark for assessing the efficiency of other rapid systems such as lateral flow tests.

When used in the field the more accurate readings were obtained when system was optimised to trap inoculum of *M. brassicicola* ascospores only during periods when it was released. By using existing commercial or non commercial data loggers trapping could be activated when a light intensity of >0.003kwm^2 was observed together with a relative humidity (r.h.) of > 80 %. These two conditions are required for release of ascospores of *M. brassicicola*.

2.3 Significance of disease transmission in vegetable brassica crops

By using the modified version of the MTIST spore trap in the field it was demonstrated that epidemiologically significant levels of *M. brassicicola* inoculum in the air could be detected both reliably and rapidly. Despite weather conditions when the results were less accurate (eg. heavy rainfall) large numbers of ascospores could be detected at the ringspot source and up to 1.6 km away. The distance (and amounts) that ascospores will travel depended on the amount of disease at source. For example there were enough ascospores produced by an infected plot (of dimensions of 5x5m) to give high ascospore numbers in the air 1.6 km from source. The results have great epidemiological significance for the control of ringspot and other diseases in the field. Overwintered unsprayed cauliflower plots with heavy levels of infection are common in field vegetable production areas. These can on average be approximately 10 to 20 hectares in size and must represent considerable sources of inoculum for plantings carried out during the new season (May onwards). It is likely that these crops have the potential to spread significant amounts of disease over entire production areas. In addition it is also clear that summer-grown cauliflower and broccoli which have reduced spray numbers act as considerable sources of disease for over-wintered cauliflower and late season Brussels sprouts crops.

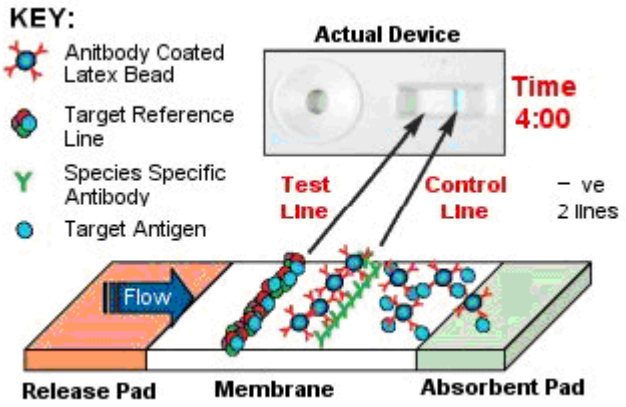
The tests developed within DEFRA project HH1759SFV can be optimised within lateral flow assays which means that the information on inoculum presence or absence

and amount can be ascertained by the end user in the field. These tests are relatively cheap to produce and use.

2.4 Lateral Flow Device (Competitive Assay)

The principle of the competitive lateral flow assay relies upon the competition for binding sites on sensitised latex particles. Antibodies (polyclonal or monoclonal) raised to a specific target spore, are bound by passive 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 (Figure 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.

Figure 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. 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. Internal control latex is labelled with particular species sera that is independent of the antigen-sensitised particle interactions. Variations in line intensity can be distinguished using a reader, facilitating the differentiation between target concentrations.

2.5 Accuracy of 'in field' tests for detecting the presence or absence of dark leaf spot and ringspot spores

Accurate 'in field' tests for inoculum, based on lateral flow devices, require specific antibodies that can differentiate between different types of pathogenic spores. However, it also depends on the trapping format for collecting the spores from the air. Detecting the presence or absence of inoculum will need to be ascertained at the same point as data loggers collecting the weather information. This is because the weather information will be required to activate the trap during periods of spore dispersal. The inoculum test information will also need to be used in conjunction with disease forecasts, which will be developed using the weather information collected by the data logger. This means that an important aspect of developing these 'in field ' tests will be the optimisation of the spore-trapping format. This will be required so that the lateral flow device will give accurate results. Inaccuracies could arise from either error associated with the lateral flow device or from the trapping format use to estimate the air-borne spore concentration.

2.6 Other pathosystems where air-borne spore numbers are used to forecast disease

Detecting airborne spores of fungal plant pathogens will be useful in crop protection if this could be done rapidly and accurately. For example it has been reported that one or two peaks in sporangial concentration in the air of the potato blight pathogen *Phytophthora infestans* preceded the first observed symptoms of the disease in the field (Bugiani *et al.*, 1998). These observations were validated in studies conducted by Philion (2003). In these studies the numbers of sprays applied to control potato blight could be successfully reduced without any impact on crop quality by monitoring the onset of thresholds of potato blight inoculum. Fungicide applications were initiated when the daytime airborne sporangial concentration reached 30 sporangia/m^3 (disease was not yet visible when this threshold was reached). By using this, criteria in combination with disease forecasts based on weather information the number of fungicide applications could be reduced with no impact on disease development.

Given that potato blight is a difficult pathogen to control great scope exists in applying this approach in the control of other less aggressive pathogens and in different localities. This means that disease can be predicted accurately before it is visible in the crop. 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. This work suggests that big reductions in fungicide applications could be made for control of a range of pathogens on a range of common vegetable crop types.

3. PRODUCTION OF ANTISERA

3.1 Production of antisera to *Mycosphaerella brassicicola*

3.1.1 Materials and Methods

3.1.1.1 Production of ascosporic inoculum of *M. brassicicola*

The pathogen was isolated on a sprout leaf decoction agar (SLD: agar-amended filtrate from 100g fresh-weight senescent leaves homogenised in 140ml distilled water) from a single ringspot lesion on leaves of a diseased Broccoli crop at Freiston Shore, Lincolnshire. Leaves were surface sterilised by dipping in a 70 % ethanol solution followed immediately by placing in an aqueous sodium hypochlorite solution (4% w/v available chlorine) for 30 sec. Isolations were excised after 21-28 days growth, excess agar was removed and, a mycelial suspension produced in 10ml sterile distilled water (SDW) using an Ultra Turrax homogenizer (Janke Kunkel C., Stausen in Breisgau, Germany). Aliquots (0.5ml) of mycelial suspension were pipetted onto SLD agar Petri plates. The suspension was spread evenly over the entire surface of the agar, using a sterile plate spreader. The inoculated plates were partially air dried under sterile conditions and then stored at 17 ±2°C for 14 days in a growth room under warm white fluorescent / black light (Osram F7/AD/Phillips TLD 18 W/08 tubes). After which the cultures were examined under x 100 magnification at intervals of 4 – 5 days until pseudothecia were observed on the culture surface. Selected cultures were misted with sterile distilled water and, incubated as described above. Discharged ascopores of *M. brassicicola*, identified on the surface of the Petri dish lid, were then removed by agitation with a sterile plate spreader in 5ml of sterile distilled water.

3.1.1.2 Production of monoclonal antibodies to *M. brassicicola*

The collected ascosporic suspension was concentrated by first freeze-drying (Modulyo 4k, Edwards) and then rehydrating to a final volume of 5ml in phosphate buffered saline solution (PBS). Three female Balb C mice were immunised (by intraperitoneal injection) each with 50µl of the ascospore preparation mixed with an equal volume of Titermax adjuvant (Sigma T-2684). The mice were immunised twice more at 4 weekly intervals. Following tail bleeds and, employing a plate trapped antigen ELISA (PTA-ELISA Plate-Trapped Antigen Enzyme-Linked ImmunoSorbent Assay), a mouse was identified which exhibited a level of sensitivity to *M. brassicicola*. The selected mouse received a final pre-fusion boost (ascospore preparation mixed with adjuvant) and the spleen was removed four days later. A fusion was carried out according to standard CSL protocol and cell hybrids were fed on days 3,6, and 10. Cell culture supernatants were screened by PTA ELISA 14 days after cell fusion for the presence of antibodies, which recognised ascosporic epitopes of *M.brassicicola*.

3.1.2 Results

The initial screen was unsuccessful with false positives recorded. A plate-trapped antigen ELISA which had been optimised at HRI and, employing a streptavidin / biotin amplification system, was used to repeat the screen but no hybridoma cell lines were identified positive to *M. brassicicola*.

3.1.3 Conclusion

Previous work at HRI has established that polyclonal and monoclonal antisera can be raised to ascosporic inoculum of *M. brassicicola*. For that reason the remaining mice were re-boosted and, following tail bleeds, a further fusion will be carried out to identify positive cell lines. The optimised ELISA assay system will this time be used at the first screening stage. Tail bleeds are scheduled for May 2003.

3.2 Production of monoclonal antiserum to airborne stage of *Alternaria brassicae*

3.2.1 Materials and Methods

3.2.1.1 Condial production of *A. brassicae*

Isolates of *A. brassicae* (Table 1), taken from the HRI culture collection, were grown on a vegetable juice agar (V8) for one week. A 5cm cube of mycelium was then removed, homogenised in 5 ml of sterile distilled water and transferred in 500µl aliquots to 10 x 5ml sterile clarified V8 juice medium. This process was repeated for each isolate. To induce conidial production the V8 mycelial suspensions were agitated prior to incubation at 25º C. At a magnification of x 100 conidial production was noted seven days later. Conidia of *A. brassicae* were collected into SDW ($3.5x10^3$ conidia per ml), disrupted using a Fast Prep (Qbiogine, UK) and then dispatched to Central Science Laboratory, York for immunization (Immunogen Type A).

Table 1. Isolates of *A. brassicae* for use in immunization study

Code No.	Host	Area	Date isolated
AA3	Brussels sprout	Wellesbourne, HRI	1994
AA3-1	Brussels sprout	Wellesbourne, HRI	1994
AA3-2	Brussels sprout	Wellesbourne, HRI	1994
AA4	Brussels sprout	Wellesbourne, HRI	1992
AA5	Brussels sprout	Lincolnshire	1993
AA10-1	Kohl rabi	Lincolnshire	1994
AA10-2	Kohl rabi	Lincolnshire	1994
AA10-5	Kohl rabi	Lincolnshire	1994
AA11-4	Kohl rabi	Lincolnshire	1994

This procedure was repeated (after 6 months) however this time the collected conidia were suspended in a 0.1 % glucose solution. Following agitation for 1 hour on a wrist action shaker the conidial suspension was then sprayed directly on to disease free Brussels sprout seedlings (*Brassica oleracea* var. *gemmifera*) c.v. Golfer, each with three true leaves). To provide optimal disease conditions the inoculated plants were exposed to a relative humidity of 100% for 48 hours and thereafter retained in a glass house operating at a constant temperature of 18 ºC. Approximately two weeks after inoculation conidia of *A. brassicae* were identified and collected by agitating segments of *A. brassicae* sporulating leaf material in sterile distilled water for a period of 30 minutes. After which the plant debris was removed by filtering the collected suspension through a membrane of 97µm pore size. To remove bacteria, plant cell components and other leaf contaminants the conidial suspension received a final filtration through a membrane of 37µm pore size. The retained conidia of *A. brassicae* were resuspended in 5ml PBS ($1x10^5$ conidia per ml), and completely disrupted (broken open) using a Fast Prep (Qbiogine, UK) and then dispatched to Central Science Laboratory, York for immunization (Immunogen Type B)

3.2.1.2 Production of monoclonal antibodies to *A. brassicae*

Three female Balb C mice each received 50µl of immunogen preparation A (*in vitro* produced conidia of *A. brassicae*) mixed with an equal volume of Titermax adjuvant. The mice were immunised twice more at 14 day intervals using preparation A without the adjuvant. The mice were then tail bled and the sera screened using a plate trapped antigen ELISA (PTA-ELISA). A mouse was identified which exhibited a level of sensitivity to *A. brassicae* and, following a final pre-fusion boost, the spleen was removed four days later. The fusion was carried out according to standard CSL protocol. Cell hybrids were fed on days 3,6, and 10. Cell culture supernatants were screened by PTA ELISA 14 days after cell fusion for the presence of antibodies,

which recognised conidial epitopes of *A. brassicae*. To determine specificity selected *A. brassicae* positive cell lines were screened, by PTA-ELISA and immunofluorescence (IF), against a range of fungal species.

The immunization process was repeated but this time the mice were immunized with immunogen type B (which consisted of disrupted conidia of *A. brassicae* produced '*in planta*').

3.2.2 Results

Fusion 1 – Immunogen Type A: Four hybridoma cell lines were identified as positive to *Alternaria brassicae* (Figure 2). The cell lines were twice cloned, purified and isotyped as IgM. In cross-reactivity studies each however was found to be highly non-specific reacting with a range of fungi tested (Table 2).

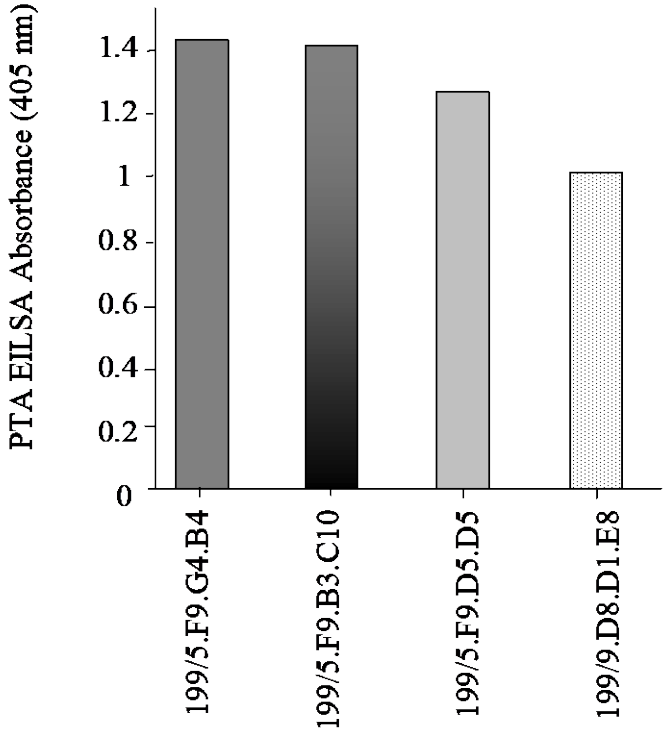


Figure 2. Recognition by PTA ELISA of hybridoma cell lines to *Alternaria brassicae* coated microtitre wells

Table 2. Reactivity of Fusion 1. *A. brassicae* positive cell lines

	Cell line reactivity			
Fungi Tested	**199/5.F9.G 4.B4**	**199/9.D8.D 1.D10**	**199/5.F9.B3. C10**	**199/5.F9.D 5.D5**
Alternaria brassicae	□	□	□	□
Alternaria brassicicola	□	□	□	□
Alternaria cheiranthi	□	□	□	□
Alternaria dauci	□	□	□	□
Alternaria raphani	□	□	□	□
Eurotium sp.	□	□	□	□
Fusarium culmorum	□	□	□	□
Stemyphillium sp.	□	□	□	□

Fusion 2 – Immunogen Type B: Five hybridoma cell lines were identified as positive to *A. brassicae.* These were twice cloned and isotyped as IgM. In preliminary reactivity studies the selected clones exhibited a low level of recognition to fungal species outside the *Alternaria* genus. However with the exception of clone 190/191.C8 all clones exhibited a level of recognition to *Alternaria dauci* (Figure 3). It should be noted however that a later test revealed no reactivity with *A. dauci* when conidia remained ungerminated and intact (i.e. non-disrupted) when tested with monoclonals (MAbs) 220/1.D1.C2.E11 and 220/2.G2.C9.G11. The selected clones will be further tested against a greater range of air-borne fungi to determine specificity.

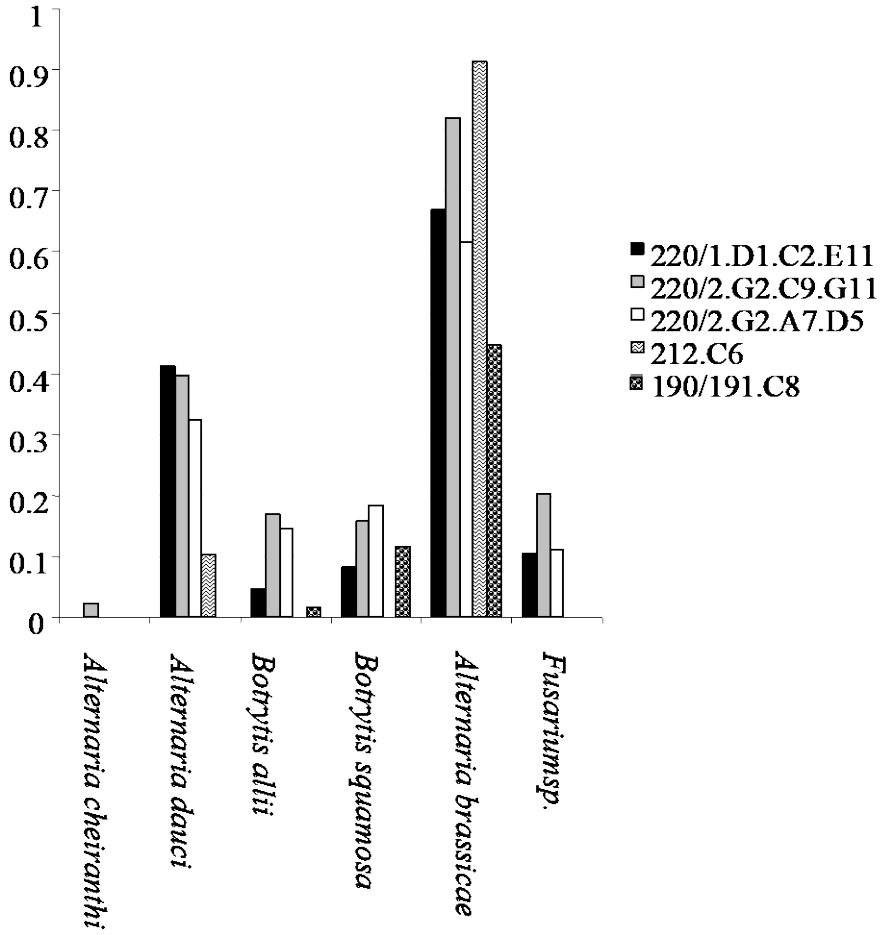


Figure 3. Reactivity screening of cloned cell lines (Fusion 2) by PTA ELISA

3.2.3 Conclusion

Five cell lines have been selected which will prove useful in the development of a lateral flow device for the rapid detection and semi-quantification of trapped airborne inoculum of *A. brassicae*. Further tests will be carried out to determine the level of specificity of each clone against an increased range of fungal targets. Employing a DAS-ELISA (double antibody sandwich ELISA) the use of these antibodies in combination will be examined to determine whether increased sensitivity and specificity can be achieved. If successful this format will be taken forward to lateral flow development. A final immune boost of an A. *brassicae* conidial suspension has been given to the remaining mouse and a hybridoma screen will take place during May 2003 for the selection of further clones positive to *A. brassicae*.

4. DEVELOPMENT AND OPTIMISATION OF IMMUNOMONITORING ASSAY FORMATS

4.1 Materials and Methods

4.1.1 Collection of *Alternaria* spores

Brassica leaf material exhibiting dark leaf spot symptoms was collected from a commercial site in Lincolnshire. To promote spore production the leaf material was incubated in an enclosed chamber at a relative humidity (r.h.) of 97% for 48 hours. After which any fungal spores, which had been produced and released in to the airborne environment of the chamber, were monitored using a Burkard eppindorf cyclone sampler (Plate 1). Over a 76 hr period 9 sample collections were made. After each exposure period the eppindorf collection vessel was removed, sealed and stored at -20ºC.

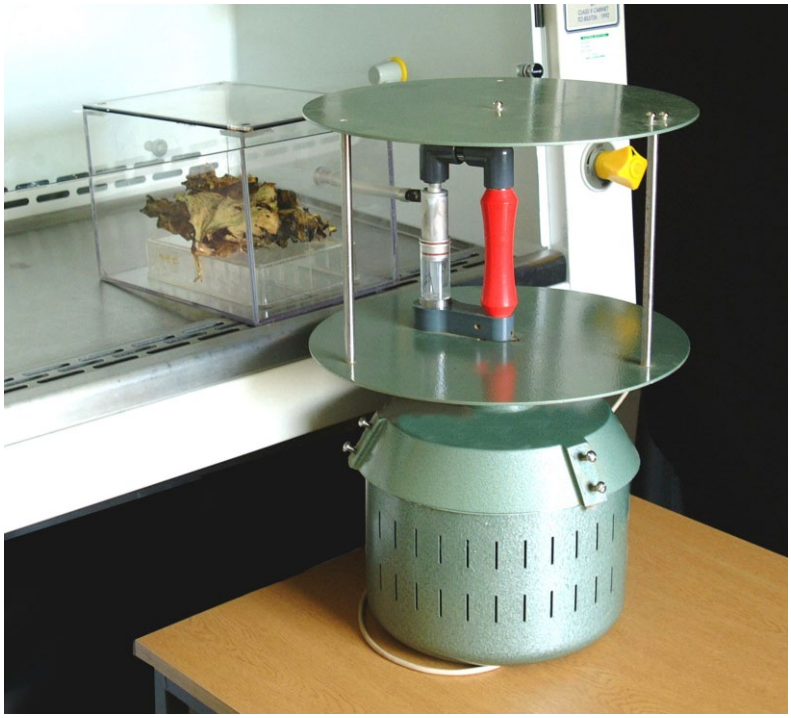


Plate 1. Collection of airborne spora in to an eppindorf vessel employing a Burkard cyclone sampler.

4.1.2 Preparation of spore samples for immunoassay

To each eppindorf sample 500µl of distilled water was added the sample was agitated and, the collected number of *A. brassicae* spores determined (Table 3). To determine the optimal buffer for use in an immunoassay detection system 100µl of each sample

was mixed with 1ml of sterile distilled water (pH 7), 1ml carbonate buffer (pH 9.6) and 1ml of Phosphate buffered saline solution (pH 7). To an ELISA microtitre well 80µl of each sample type was aliquoted and, for each sample type, three replicate wells were included. From a range of wells the conidial numbers of *A. brassicae* present, was determined using a Nikon TMS inverted microscope.

Table 3. Number of *Alternaria brassicae* conidia trapped into an eppindorf collection vessel during each of the sampling periods

Sample	Total number of *A. brassicae* conidia trapped
1	225
2	5000
3	1620
4	330
5	7500
6	375
7	250
8	145
9	13.5

4.1.3 Immunoassay process

The samples were incubated overnight in an enclosed chamber at 18ºC. After which unbound material was removed and the microtitre wells were washed once 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 MAb cell line 220/1.D1.C2.E11 (diluted 1:2 PBST TincTw). Following incubation as above, wells were washed three times for one min each with 200 µl PBSTincTw. 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 antibodies of cell line 220/1.D1.C2.E11. Wells were washed as described above and to each well 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). Conidial counts of *A. brassicae* in a selection of the microtitre wells was carried out before the ELISA procedure, following the Casein block stage, each of the antibody stages and post ELISA.

The ELISA process was repeated with each of the selected *Alternaria* MAb cell lines (220/2.G2.C9.G11 and 212.C6, 190/191.C8) and as a negative control PBS alone was included at the primary antibody stage.

4.2 Results

A high level of *Alternaria* sporulation was observed on the infected leaf material following humidity treatment. The cyclone spore trap when used in conjunction with the eppindorf capture vessel proved useful in the collection of airborne spores of *Alternaria brassicae*. The use of water to remove spores from the eppindorf collection vessel proved satisfactory as a method for determining *Alternaria* spore catch. However when used as a coating buffer water proved inhibitory reducing antigen binding. Only using MAb cell lines 220/2.G2.C9.G11 and 220/2.G2.A7.D5 could a level of correlation be observed between the numbers of *Alternaria* spores present and the absorbance values derived by ELISA (Figure 4a). Assay sensitivity was improved when a carbonate buffer was employed. However using a phosphate buffered saline coating solution proved optimal in the development of a highly sensitive and rapid quantification assay for airborne inoculum of *Alternaria brassicae*. The same result was observed when MAb cell lines 220/2.G2.C9.G11, 220/2.G2.A7.D5 or 190.191.C8 were used within the assay format (Figure 4b). No correlation could be derived using MAb cell line 212.C6 regardless of the coating buffer system used.

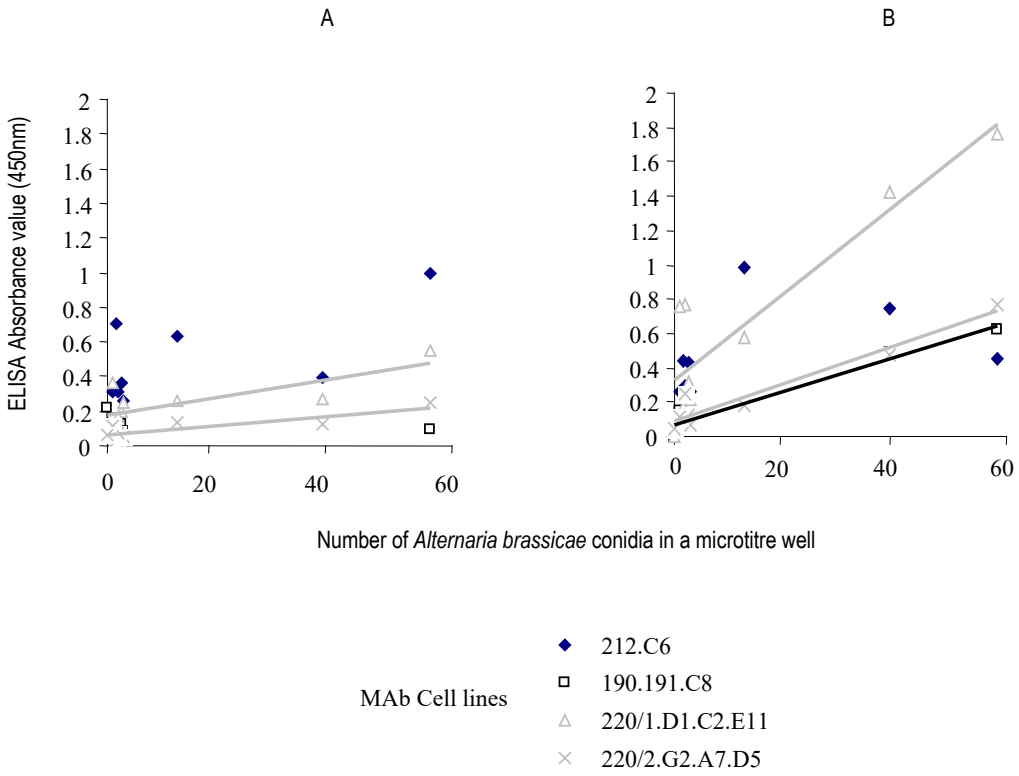


Figure 4. Relationship between the number of *Alternaria brassicae* spores pipetted in to a microtitre well using (A) Water as a coating buffer and (B) PBS as the coating buffer and, the resulting ELISA absorbances.

4.3 Conclusion

Conidia in air samples are trapped in collection vessels from which estimates of numbers will be needed. However, there is no information on the optimal procedure for removing and resuspending conidia in solution so that their numbers can be estimated. In tests reported here removal of trapped air spora from the eppindorf tubes did not prove problematical and they could be readily suspended in a liquid solution. Employing, a Phosphate buffered saline solution (PBS) was optimal for use within an immunoassay system employing MAbs 220/1.D1.C2.E11, 220/2.G2.C9.G11 and 190/191.C8. Future development of the lateral flow system will incorporate, as the buffer component; a Phosphate buffered saline solution. It is unlikely that MAb 212.C6 will be used within the developed lateral flow system, recognising only germinating conidia of *A. brassicae* and, as such, unable to detect and quantify ungerminated spores of *A. brassicae*.

5. DETERMINATION OF BIOLOGICAL INTERFERENCE WITH THE DEVELOPED ASSAY TEST

5.1 Materials and Methods

5.1.1 Production of Alternaria brassicae (dark leaf spot) and Erysiphe *crucifearum* (powdery mildew)

Brassica leaf material heavily infected with *Alternaria brassicae* was collected from the field in Lincolnshire and Cornwall. Leaf material was placed in a misting hood for 48hr at 97% humidity in the glasshouse. Material was dried off after exposure and incubated under room temperature for a further 12-hr period. Leaves were examined for the presence of conidia of *A. brassicae* using a binocular microscope. Powdery mildew was maintained in the glasshouse on plant material of Brussels sprouts Cv. Golfer. Plants were produced in Hassey trays before infection with powdery mildew at the 4 true leaf stage. After infection (which was achieved by rubbing infected leaf material on the surface of uninfected leaf material) plants were maintained under dry conditions in a glasshouse without overhead watering.

5.1.2 Sampling of *Alternaria brassicae* (dark leaf spot) and *Erysiphe cruciferarum* (powdery mildew)

After spore production was observed on leaves of infected leaf material (dark leaf spot) and infected plant material (powdery mildew), each type of material was placed in a controlled environment cabinet (Sanyo Gallenkamp, Loughborough, Leicestershire, U.K; Cat No. SGC970/C/RO HFL) operating at 80 % r.h. with continuous light. Over a 9 hr sampling period discharged air spora were collected by impaction into the eppindorf sample vessel of a Burkard cyclone sampler. After which the eppindorf was removed, sealed and stored at -20ºC. This process was repeated for a further eight sampling periods. For each sampling period 1ml of PBS Tinc (Phosphate buffered saline solution, 0.05 %Tincture of Merthiolate added as an anti-bacterial agent) was added to the eppindorf collection vessels and the collected spores resuspended in the solution. The total number of *Alternaria* (dark leaf spot) and *Erysiphe cruciferarum* (powdery mildew) was determined by bright field microscopy at a magnification of x 160. The remaining sample was aliquoted into ten microtitre wells (80µl per well) of a Polysorp microtitre strips (catologue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, Scotland) before incubation overnight at 18ºC. After which an ELISA was carried out, as described above, employing MAbs 220/1.D1.C2.E11, 220/2.G2.C9.G11 190/191.C8 and 212.C6. A negative control employing PBS alone was also included.

The sampling efficiency of the cyclone spore trap was determined by positioning a Burkard 24-hr glass slide sampler adjacent to the cyclone spore trap. The Burkard 24-hr spore trap (similar characteristics to the 7-day sampler) has been used routinely for monitoring fungal air-spora and, in a variety of air sampling studies. Detection and quantification of impacted air spora on the glass slide was by bright field microscopy and at a maginification of x160.

For each sampling period 20 disease-free *B. oleracea* (Brussels sprouts) seedlings (cv. Golfer, 3 true leaves) were positioned in the controlled environment cabinet. Following each of these sampling periods the plants were removed, and placed into an environment of 100 % humidity for 48 hrs. The plants then were removed and retained in a glasshouse, at a temperature of 15 º C for 5 days. Plants were visually examined for expression of dark leaf spot lesions (*Alternaria*). To confirm dark leaf spot symptoms infected leaf tissue was removed; surface sterilised for 1 minute in aqueous sodium hypochlorite (4 % w/v available chlorine) and isolations made on to V8 juice agar. Comparisons between counts of conidial numbers (and those of other fungi e.g. powdery mildew), ELISA absorbance, and lesion numbers were carried out to compare the sensitivity of the system using the antibodies raised as described in section 2.

5.2 Results

5.2.1 Comparison of spore counts in cyclone traps with Burkard 24 H volumetric traps.

Comparisons of the numbers of conidia of *Alternaria* spp. (*A. brassicae* and *A. brassicicola*) trapped in a cyclone trap (the type of trap to be used in conjunction with the lateral flow test) with of the numbers trapped on a 24 h volumetric spore trap is shown in Figure 5. There was a close relationship between the two estimates of dark leaf spot spore number from the two traps ($r^2 = 0.9782$). However the numbers of conidia trapped in the cyclone trap was lower by a factor of 10 in comparison to the 24 h volumetric trap. Similar results were obtained for comparisons of conidial counts of *A. brassicae* alone (Figure 6) where the relationship between the numbers in the two traps was very close ($r^2 = 0.966$). There was again a ten-fold order of magnitude difference between the two traps.

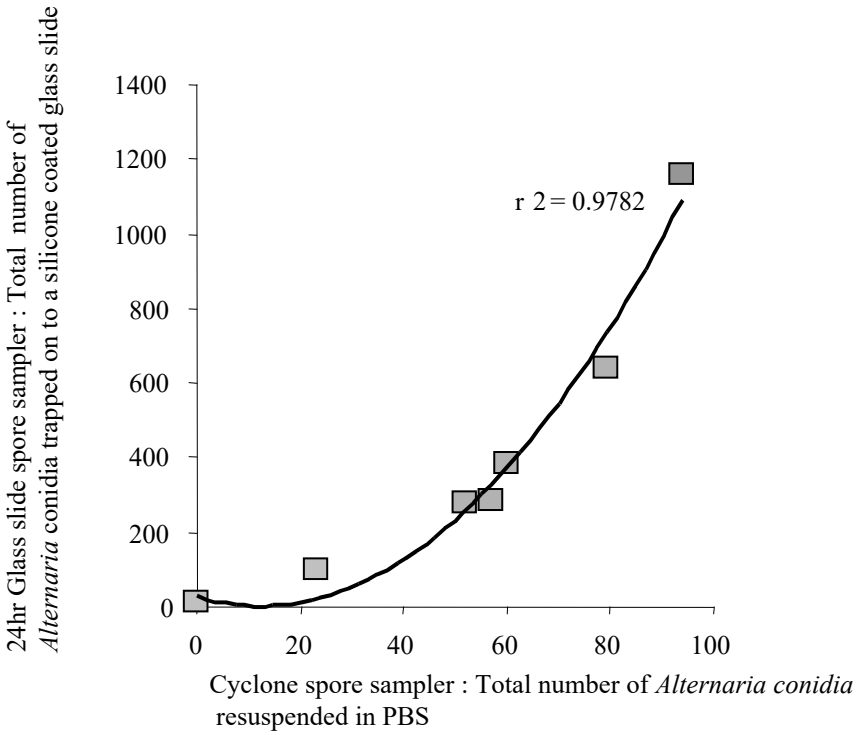


Figure 5. Collection efficiency of the Burkard cyclone sampler and 24hr glass slide sampler for airborne conidia of *Alternaria spp*.

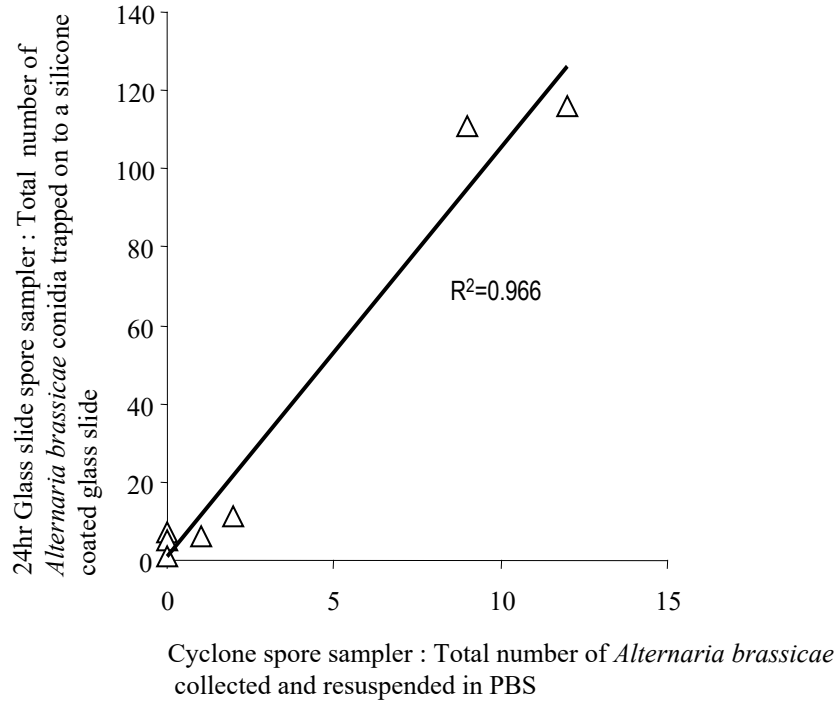


Figure 6. Collection efficiency of the Burkard cyclone sampler and 24hr glass slide sampler for airborne conidia of *Alternaria brassicae*

5.2.2 Comparison of dark leaf spot conidial numbers (*Alternaria* sp.) estimated by light microscopy and ELISA in the presence of other spores.

The numbers of *Alternaria* sp. spores as estimated by ELISA and by microscope counts from the cyclone trap sample is shown in Figure 7. The results show a clear relationship between spore counts using a microscope and ELISA absorbance values (r^2 = 0.8544) employing monoclonal antibody (Mab) 190.191.G8. This antibody recognised spores of both *A. brassicae* and *A. brassicicola* and did not cross react in the presence of high numbers of *Erysiphe cruciferarum* (powdery mildew) and other spores (*Botrytis* sp., *Cladiosporium* and *Phoma* sp.). Although the numbers of dark leaf spot spores were generally low (<100) the samples were consistent with those previously obtained from the cyclone trap. Conidia may have been retained within the eppindorf collection vessel. There was no relationship between the ELISA absorbance values and the numbers of other spores present in the microtitre wells.

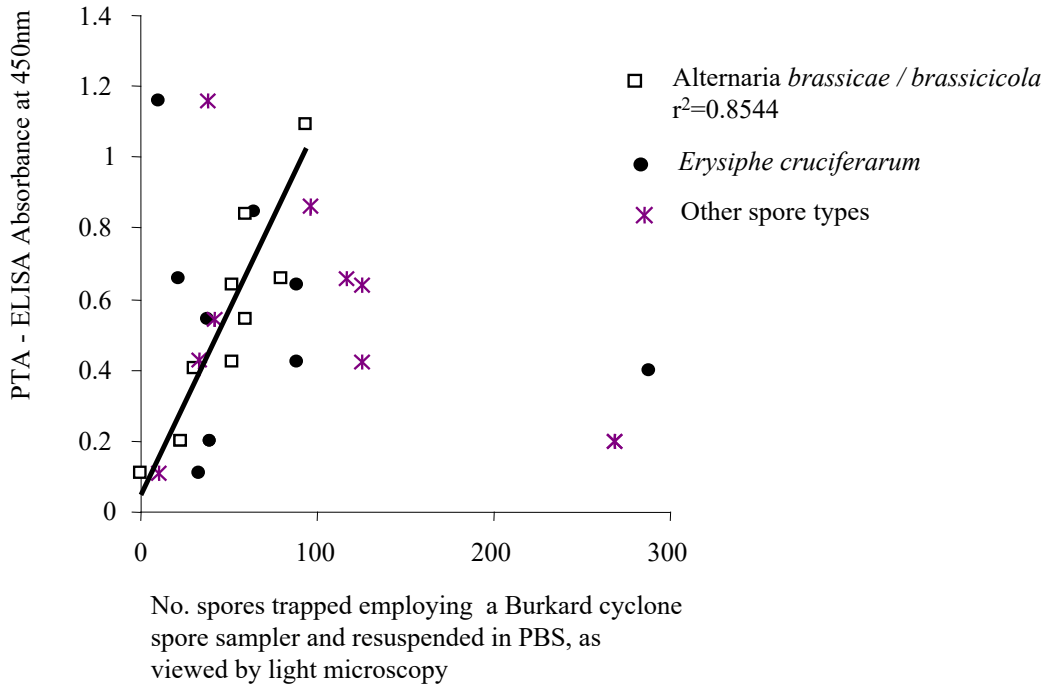


Figure 7. Selective detection and quantification of *A brassicae* and *A. brassicicola* by PTA -ELISA employing Mab 190.191.G8 in a mixed spore sample

5.2.3 Comparison of antibodies for detection of *Alternaria brassicae* by ELISA in mixed spore population

The results of using antibodies Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5, for the detection of dark leaf spot conidia (*A. brassicae* only), is shown in Figure 8. The sample also contained the presence of a range of other spore types found on brassica tissues. There was a very close relationship between the numbers of dark leaf spot spores in samples obtained from a cyclone trap and the ELISA value. The sample in the cyclone trap was derived by sampling the air in a CE cabinet where infected leaf material (containing conidia of *A. brassicae*) had been introduced. Although only low spore numbers of *A. brassicae* were present there was a relationship between spore number and ELISA values of $r^2 = 0.8131$ and $r^2 = 0.8049$ using Mab antibody clones 220/1.D1.C2.E11 and 220/2.G2.A7.D5 respectively. Both antibodies did not recognise *A. brassicicola* which, was also present in these samples.

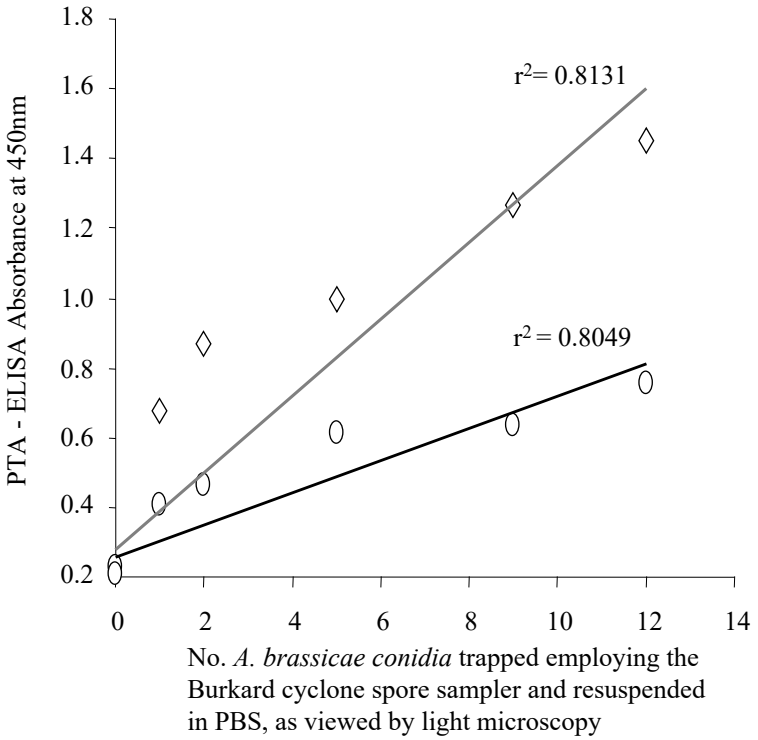


Figure 8. Selective detection and quantification of trapped airborne conidia of *A. brassica* by PTA-ELISA employing Mabs 220/1.D1.C2.E11(◊)and 220/2.G2.A7.D5 (0) in a mixed spore population.

5.2.4 Comparison of ELISA estimates of dark leaf spore number and dark leaf spot lesion numbers on plants

There was a very close relationship between the numbers of dark leaf spot lesions on plants exposed to dark leaf spot spores and the ELISA values obtained when using antibodies Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5 (Figure 9). After exposure to conidia plants were maintained under leaf wetting conditions for 48 H at temperatures which would result in infection by *A. brassicae*. Lesion numbers were counted 10 days after infection conditions had ceased. Isolations on to agar from leaf lesions confirmed that the dark leaf spot lesions resulted from infection by *A. brassicae* only despite the presence of *A. brassicicola* in the spore samples. A relationship between lesion number and ELISA value of $r^2 = 0.6744$ and $r^2 = 0.6747$ using antibodies Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5 respectively was observed. There was no relationship between lesion number and ELISA value using antibody Mab 190.191.G8.

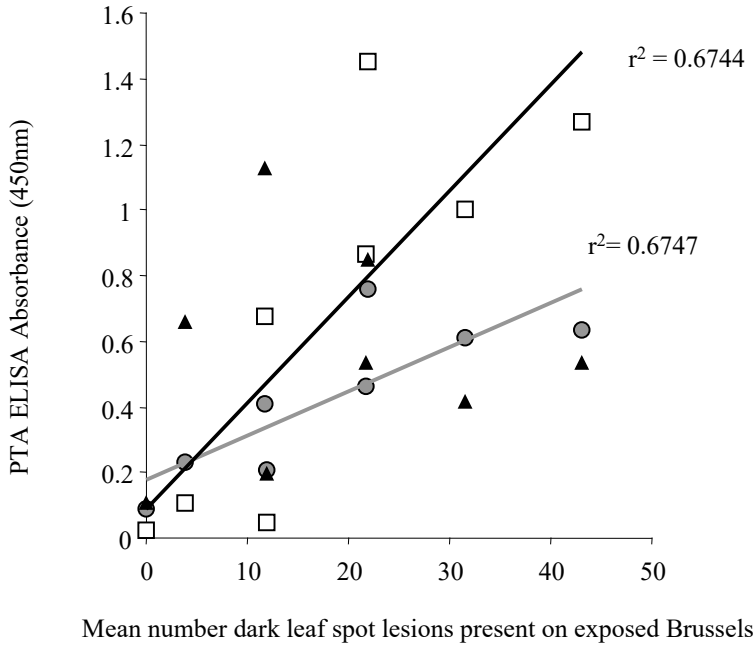


Figure 9. Relationship between predicted *A. brassicae* presence (PTA ELISA) and dark leaf spot disease development employing Mabs 190.191.G8 (▲) Mabs 220/1.D1.C2.E11(□)and 220/2.G2.A7.D5 (●) in a mixed spore population.

5.3 Conclusions

The results show that there was a very close relationship between the numbers of dark leaf spot spores trapped in the cyclone spore trap and the numbers trapped by a 24 h volumetric trap. The volumetric trap represents the total number of conidia collected. However the numbers from the cyclone trap had to be estimated by removing them from the capture vessel (the eppindorf tube). This may explain the difference in magnitude between the two counts. The results show that antibody Mab 190.191.G8 recognises conidia of both *Alternaria brassicae* and *Alternaria brassicicola* which can both cause dark leaf spot. However *A. brassicae* is the most important cause of dark leaf spot in vegetable brassica crops. This probably results from differences in environmental requirements for both organisms. *Alternaria brassicae* can infect and sporulate on vegetable brassica tissues at much lower temperatures in comparison to *A. brassicicola*. This explains why *A. brassicicola* is only found on vegetable brassica crops occassionally and is more important on oilseed rape crops.

Antibodies Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5 were highly specific to dark leaf spot spores (*A. brassicae* only). It is highly likely that these antibodies can be successfully used within lateral flow tests for dark leaf spot which, are now under development. This was further confirmed when ELISA values dervived from air samples using these two antibodies were compared with dark spot lesion numbers on exposed plants. Despite the presence of *A. brassicicola* in the spore samples it appeared that only *A. brassicae* spores had resulted in infection on leaf material. This was confirmed when leaf lesions were plated on to agar. Conversely no relationship was observed between ELISA value and leaf lesion number when antibody Mab 190.191.G8 was used. Results from study indicate that this antibody **(190.191.G8)** reacts with both *A. brassicae* and *A. brassicicola*.

6. DISCUSSION

Producing 'in field' tests based on lateral flow tests will require the development of specific antibodies, which can be used to visualise the presence of target inoculum (dark leaf spot and ringspot spores). The specificity of the antibodies will require testing in relation to a range of related and unrelated fungi, which are found in vegetable brassica 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. Additionally information on the requirements necessary for the transfer of trapped spores in the sample capture tube to the lateral flow test will be needed. It will also be necessary to test the system under CE conditions to relate numbers of trapped spores to the signal potentially generated by the test and also the numbers of spots on vegetable brassica plants. Finally once the lateral flow tests have been derived it will be necessary to test the system under field conditions. Each stage in this developmental process requires validation and in this report the various stages in developing and testing lateral flow tests for inoculum detection has been described.

6.1 Producing antibodies for use in lateral flow devices

Within this project monoclonal antibodies have been successfully raised which selectively recognise components of the spore of *Alternaria brassicae* (dark leaf spot). Antibodies are still being raised to selectively recognise components of the ascospore of *Mycosphaerella brassicicola*. The antibodies raised to dark leaf spot spores have been tested against a few *Alternaria* species and a number of other common fungi found in the field. The first set of antibodies produced (fusion 1), were highly reactive to *A. dauci* (*Alternaria* found on carrots), *A. cheiranthi* (*Alternaria* found on wallflower) and *Stemphyphillium* which causes symptoms on a range of symptoms on horticultural crops similar to *Alternaria*. However the second set of antibodies raised contained clones highly selective to *A. brassicae* (the dark leaf spot pathogen of vegetable brassicas). Although there was some reactivity to *A. dauci* this could be explained by the fact that the tests with *A. dauci* were carried out with very high conidial concentrations which would have biased the results. It is likely that these clones are not reactive to *A. dauci*, in comparison to *A. brassicae* (at the same concentrations of conidia).

Some of the antibodies raised appear specific enough be used in lateral flow test kits to detect *A. brassicae* conidia. Work is still ongoing in raising monoclonal antibodies to *M. brassicicola* ascospores.

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. 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 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. Additionally the numbers of sampled conidia were tested using ELISA. This immunoassay format is complimentary to that of a lateral flow device. Removal of dark leaf spot spores from the trap sample tube was not problematical and the system was best optimised by using phosphate buffered saline. Of the monoclonal cell lines tested Mab 220/1.D1.C2.E11 gave the best results with a linear relationship between the ELISA signal (absorbance) and the number of dark leaf spot conidia per microtitre well. It is unlikely that Mab 212.C6 will be used in further lateral flow development as it recognised only germinated dark leaf spot conidia. This antibody was unable to detect ungerminated dark leaf spot conidia.

6.3 Controlled environment tests detecting dark leaf spot in the air from an infected source

The optimised system with the most specific monoclonal antibodies which, selectively detect conidia of dark leaf spot, were used in controlled environment tests where a source of dark leaf spot spores were present. Infected vegetable brassica trash, which contained both dark leaf spot pathogens (A. brassicae and A. brassicicola), was placed in a CE cabinet at 15 C and 80 % r.h.. A cyclone trap and a modified Burkard volumetric trap were placed within the cabinet with 4 trays each, containing 16 plants of Brussels sprouts cv. Cavalier. Tests were conducted in the presence of 20 plants of Brussels sprouts, cv. Golfer with sporulating lesions of powdery mildew. These tests showed that antibodies Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5 were highly specific to dark leaf spot spores (*A. brassicae* only). These antibodies therefore appear suitable for use within lateral flow tests for dark leaf spot. Both these antibodies did not appear to cross react with *A. brassicicola*. *A. brassicae* and *A. brassicicola* were present in the spores samples (from air). However a good relationship between ELISA estimates of *A. brassicae* spore number and dark spot lesion numbers on exposed Brussels sprout plant given infection conditions after exposure was observed. Despite the presence of *A. brassicicola* in the spore samples it appeared that only *A. brassicae* spores had resulted in infection on leaf material. This was confirmed when

leaf lesions were plated on to agar. These results were also confirmed when no relationship was observed between ELISA value and leaf lesion number using antibody Mab 190.191.G8. This antibody reacts with both *A. brassicae* and *A. brassicicola* resulting in no correlation between ELISA value and leaf lesion number.

6.4 Disease forecasting criteria based on inoculum detection

The information could be used within crop protection programmes to eliminate over spraying of fungicides. This would be particularly useful early in the season as a method of preventing disease transfer between over wintered crops and freshly transplanted 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. By assessing air samples for ringspot or dark leaf spot, the need for fungicide applications can be determined. Protective applications of fungicide can therefore be precisely timed at different locations. This will help the brassica industry meet the current difficulties with declining numbers of active fungicides available for use on brassica crops by optimising fungicide application further.

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). This approach has also been used successfully

to control light leaf spot (*Pyrenopeziza brassicae*) on Brussels sprouts in Scotland (Gilles *et al*., 2001). 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 brassica industry. However to develop a full system for vegetable brassicas will require further work to include measures of inoculum for all diseases which, affect this crop.

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