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Field evaluation of a competitive lateral-flow assay for detection of *Alternaria brassicae* in vegetable brassica crops

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ABSTRACT

On-site detection of inoculum of polycyclic plant pathogens could potentially contribute to management of disease outbreaks. A 6-min, in-field competitive immunochromatographic lateral flow device (CLFD) assay was developed for detection of the *Alternaria brassicae* (the cause of dark leaf spot in brassica crops) in air sampled above the crop canopy. Visual recording of the test result by eye provides a detection threshold of approximately 50 dark leaf spot conidia. Assessment using a portable reader improved test sensitivity. In combination with a weather driven infection model, CLFD assays were evaluated as part of an in-field risk assessment to identify periods when brassica crops were at risk from *A. brassicae* infection. The weather-driven model over-predicted *A. brassicae* infection. An automated 7-day multivial cyclone air sampler combined with a daily in-field CLFD assay detected *A. brassicae* conidia air-samples from above the crops. Integration of information from an in-field detection system (CLFD) with weather driven mathematical models predicting pathogen infection have the potential for use within disease management systems.

Keywords: Immunoassay, *Alternaria brassicae*, lateral flow test, integrated disease management

INTRODUCTION

Fungal pathogens occurring on vegetable brassica crops can be difficult to control. The use of mathematical models to summarise the effect of environment on key life cycle stages of target pathogens has traditionally been used to determine the risk of pathogen infection in crops (Magarey et al. 2005). These systems however do not provide information on presence / absence of pathogenic inoculum. Approaches based on the direct measurement of fungal spores in the air have been reported previously (Carisse et al. 2005; Caulderon et al. 2001; Kennedy et al. 2000; Rogers et al. 2009; Wakeham and Kennedy 2010). These tests are a useful tool in crop protection if carried out rapidly and accurately. At present, commercial systems for estimating inoculum are laboratory based, often laborious and require specialist knowledge (Kennedy and Wakeham 2015). As a result, disease symptoms can be visible before laboratory analysis is complete.

Concerns over the use of pesticides and rising production costs provide a platform for the development of inexpensive, rapid and accurate diagnostic tools to improve management of crop diseases. Assays such as the immunochromatographic test strip (lateral-flow assay) have been used worldwide for home care diagnostic use since the 1980's (Yager et al. 2008) with current applications in human and veterinary, medicine, agriculture and environmental and forensic sciences (Wong and Tse 2009). Immunochromatographic tests provide a homogenous format (a system which is able to measure bound label without the need to separate bound and free label) with speed, simplicity of use, specificity, sensitivity and at low cost. Bangs Laboratories report a production cost of $0.35 per test and an average shelf life of 12 to 24 months (www.bangslabs.com; Technote 303). In Agriculture, where profit margins are often low, these type of tests have been used by growers to inform disease management decisions. Tests were originally developed in a flow through format (Miller et al. 1989). However a range of

immunochromatographic test strips are commercially available for the detection of viral, bacterial and fungal plant pathogens (Danks and Barker 2000). These types of tests have been developed to diagnose the presence or absence of individual pathogens on plants expressing disease symptoms.

There is the potential to apply this technology as an early warning system for the occurrence of inoculum in air samples. This development would require a system to collect and concentrate inoculum into a suitable sample vessel for further testing. Immunoassays have been developed to quantify airborne inoculum captured by passive deposition on to plant surfaces (Jamaux and Spire 1994) or impaction using rotor rods (Schmechel et al. 1996). Alternatively, an MTIST (microtitre immunospore air sampler, Burkard Manufacturing, Rickmansworth, UK) provides collection of air samples by impaction across 32 microtitre wells (Kennedy et al. 2000). Quantification of trapped particulates can then be subjected to enzyme-linked immunsorbent assay (ELISA) and with suitable antibodies multiplex assays can be devised (Wakeham et al. 2004). Most of these tests require laboratory processing facilities. A multi vial cyclone air sampler (Burkard Manufacturing Ltd) which collects air samples in successive into 8 x 1.5ml collection vessels at pre-determined time periods has been considered for use in PCR based methods (West et al 2008; West and Kimber 2015). In contrast harnessing this sampling technology with immunochromatographic test strips provides an opportunity to measure target inoculum in-situ at low cost by the end user e.g. a grower or consultant.

The combined use of these two technologies (air sampling and immunological processing) could prove a powerful tool across a range of disciplines. The predictive power of this approach could be improved further if used in conjunction with meteorological models (Jones and Harrison 2004; Zinc et al. 2012). For example, in horticulture reduced applications of fungicides has been

evaluated using a weather driven forecast model to predict risk of *Albugo candida* sporulation and infection periods (Minchinton et al. 2013). Combining this approach with concentration of inoculum in air sample could further improve the predictive outcome of the test and subsequent control of the disease.

This study reports on the development and evaluation of an immunochromatographic test device (CLFD) for risk assessment of the fungal plant pathogen *Alternaria brassicae* (dark leaf spot) in air samples. *A. brassicae,* causes dark leaf and pod spot on Brassica spp. and is of worldwide occurrence (Hong et al. 1996; Humpherson-Jones, 1992). A nectrotroph, which on host tissue produces asexual spores (condia) for wind dispersal (McCartney et al1998). Infection of crops by *A. brassicae* can result in severe yield losses (Parada et al. 2008). The relationship between important metereological parameters affecting infection and sporulation of *A. brassicae* have been incorporated into a disease forecast model (Kennedy and Graham, 1995). Integration of this information with an in-field detection system (CLFD) for *A. brassicae* in air is evaluated for improved control of dark leaf spot in *Brassica oleracea.*

MATERIALS AND METHODS

Monoclonal antibody production. Nine *Alternaria brassicae* isolates (Warwick HRI, University of Warwick, UK (Maude and Humpherson-Jones 1980)) were cultured on V8 juice agar (3g technical agar, 0.4g calcium carbonate, 20ml V8 juice (Campbell Soup Company, Camden, N.J.) for one week in darkness at 25º C. A 5cm square of mycelium from each inoculated plate was then removed, homogenised in 5 ml of sterile distilled water (SDW) and transferred in 500µl aliquots to 10 x 5ml sterile clarified V8 juice medium (Johnston and Booth 1983). The V8 mycelial

suspensions were mixed and then incubated in darkness at 25º C. At a magnification of 100x, conidial production was observed seven days later. Using the method described by Lawrie (2002), conidia of *A. brassicae* were collected from liquid culture and suspended in phosphate buffered saline (PBS, pH 7.2) at a concentration of 10^5 conidia ml^{-1}. Using a Fast Prep device (Qbiogine FP120, Anachem Ltd, Luton, UK) the conidia were mechanically disrupted according to the manufacturer's guidelines (3 x 25 seconds at a speed setting of 5.5) and then aliquoted in to 50µl lots; this preparation was labelled 'antigen-A'. The fungal culturing procedure was repeated and *A. brassicae* conidia were suspended in a 0.1 % glucose SDW solution. Following agitation for 1 hour on a wrist action shaker, the conidial suspension was sprayed directly on to healthy Brussels sprout plants (*Brassica oleracea* var. *gemmifera*) c.v. Golfer. Inoculated plants were exposed to a relative humidity of 100% for 48 hours and thereafter retained in a greenhouse held at 18 ºC. Two weeks after inoculation *A. brassicae* conidia were observed on lesions. Leaf sections bearing sporulating lesions were detached and agitated in PBS for a period of 30 minutes. Leaf material was removed by filtering the suspension through a membrane of 97µm pore size. Conidia of *A. brassicae* were recovered on a 37µm pore size membrane and, resuspended in 5ml PBS (10^5 conidia ml^{-1}). Following mechanical disruption, as described above, the *A. brassicae* suspension was aliquoted in to 50µl lots; this preparation was labelled 'antigen-B'. Three female Balb C/Cj substrain mice were each immunised for induction of antibody secreting spleen cells (Kohler and Milstein, 1976) with 50µl of antigen-A mixed with an equal volume of Titermax adjuvant (Sigma-Aldrich T-2684). Three additional mice were injected with 50µl of antigen-B. The same mice were immunized on two further occasions at 14-day intervals without adjuvant.

Collected tail bleeds (Kohler and Milstein, 1975) were titrated against their respective homologous antigen preparation by plate trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA)

(Kemeny 1991). For each tail bleed, 10 paired wells of a 96 well Nunc Immunosorbent Polysorp flat-bottomed microtitre plate (model no. 475094, Life Technologies, Paisley, Scotland) were coated with 100µl^{-1} well of the homologous antigen type in PBS. Ten paired wells received 100µl^{-1} well of PBS alone for each tail bleed as a control. After overnight incubation at 18°C, unbound antigen was removed by inverting the individual microtitre plates and tapping them down on to absorbent towelling. The plates received four one-min washes of 200µl^{-1} well PBS. Wells were blocked with 200µl 3% casein buffer (3%[wt/vol] casein in PBS) and incubated in a Wellwarm shaker incubator (model no. W1031B, Denley Instruments Ltd., Sussex, UK) for 30 mins at 30°C. Residual blocking buffer was removed and wells washed four times for one min each with 200µl^{-1} well PBS 0.05% Tween 20 (PBST). Mice tail bleeds were diluted 1:100 in PBST and doubling dilutions made to 1:25600. A diluted tail bleed was applied (100µl^{-1} well) respectively to homologous antigen coated paired wells. The remaining homologous antigen paired well of each 10 paired well set received PBST alone. This process was repeated but with microtitre wells which had not been coated with *A. brassicae* antigen. After incubation in a Wellwarm 1 shaker incubator as previously described unbound material was removed and wells were washed as previously described four times for one min with PBST. Aliquots of 100µl of anti-mouse IgG (whole molecule) biotinylated antibody produced in goat (model no. B7264, Sigma-Aldrich Company Ltd, London, UK) diluted 1 in 500 PBST, were added to each well and incubated for 45 min. as above. After washing as previous, 100ul^{-1} well of Streptavidin peroxidase (model no. SS512, Sigma-Aldrich Company) diluted 1:10000 PBST was added to each well and incubated as above for 45 min. The microtitre wells were washed as previously described and each well received100µl of 3,3',5,5'-tetramethylbenzidence substrate (model no. T-3405 and P-4922, Sigma-Aldrich). The reaction was stopped by adding 25µl of 2M H_2SO_4. Absorbance was recorded at 450nm in an HT11 (Anthos Labtec Instruments, Salzburg, Austria) ELISA plate reader.

Mice with high titre tail bleeds (end point > 1:32000) to *A. brassicae* were identified. Following a final pre-fusion boost, the spleen was removed four days later. Spleen cell fusions were carried out according to a modified protocol (Kennett et al. 1978) with cell hybrids fed on days 3, 6, and 10. By PTA-ELISA, the cell culture supernatants were screened 14 days after cell fusion to the homologous antigen preparation as previously described.

Hybridoma cell lines identified as producing antibodies positive to *A. brassicae* antigen were screened for reactivity to other fungal species (Table 1) by PTA-ELISA. For this process, fungal plate washings were prepared on Supor 450 Membrane filter (Model no. HPWP09050; MERKMillipore, Darmstadt, Germany) covered inoculated agar plates (Wakeham et al. 1997). Spore suspensions of each fungal isolate were collected and adjusted to 10^5 spores ml^{-1} (Skottrup et al. 2007). The collected spores were mechanically disrupted using a Fast Prep device as described previously. By centrifugation for 5 minutes at 13,000g the particulate fraction was removed and the soluble spore fraction was retained for PTA-ELISA. Paired wells of a 96 well Nunc Immunosorbent Polysorp flat-bottomed microtitre plate (Life Technologies, Paisley, Scotland; model. no. 475094 A) were coated with $100\mu l^{-1}$ well of each fungal washing (Dewey et al. 1989). Unbound antigen was removed after overnight incubation at 18°C (Wakeham et al. 1997) and the PTA ELISA process carried out as previously described with an *A. brassicae* positive cell culture supernatant. The process was repeated twice for each of the *A. brassicae* positive cell culture supernatants.

The immunoglobulin class produced by each *Alternaria* positive cell line was determined using an Isostrip mouse monoclonal isotyping kit (model no. 11-493-027 001, Roche Diagnostics, Burgess

Hill, West Sussex, UK). One of the cell lines (coded EMA 212) was selected and the tissue culture supernatant purified using a High Trap™ IgM purification HP column according to the manufactures instructions (model no. 17-5110-01, GE Healthcare Little, Chalfont). The effects of antigen modification with protease and periodate on antibody binding (EMA 212) was determined by the method of Bossi and Dewey (1992). To determine antigen site expression, *A.brassicae* conidia (Maude and Humpherson-Jones, 1980) were collected using the method described by Lawrie (2002). Conidia were germinated in 0.01% glucose solution on multiwell glass slides (model no. MIC3412, Scientific laboratory supplies, Nottingham, UK) and by immunofluorescence probed with antibodies produced by EMA 212 cell line (Kennedy et al. 1999b).

Competitive immunochromatographic test device. Immunochromatographic test strips consisted of a carrier material containing dry reagents that are activated by applying a liquid sample. Movement of this liquid allows passage across various zones (test framework) where molecules are attached and exert specific interactions with target analytes. Results are generated within 5 – 10 minutes by the formation of a control and test lines as appropriate to the sample and the test type**.** The development of a control line provides confirmation that the test is valid. In a competitive format (CLFD) the test line result is counter intuitive i.e. as the target analyte in a sample increases test line colour intensity decreases. At a high concentration of target analyte no test line is visible and a positive result is recorded. The test can be made semi-quantitative with the use of a reader (Wong and Tse 2009).

The test framework was constructed using a Millipore 135 HiFlow™ cellulose ester membrane direct cast on to a Mylar backing (model no. SHF2400225, Millipore Corp., Bedford, MA.)

attached at either end to an absorbent pad (model no. GBOO4, Schleicher and Schuell, Dassel, Germany) and a sample pad (model no. T5NM, Millipore Corp., Bedford, MA.). A control line of a non-immune anti-mouse serum and a test line of *A. brassicae* (antigen-A), each adjusted to 0.5mg ml $^{-1}$ in PBS were independently applied to the cellulose ester membrane surface using a flat-bed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK) operating at a line travel speed of 50m s^{-1}. The sprayed membranes were air dried overnight at room temperature (18 to 20ºC) and cut in to 5 mm strips. A 500µl volume of EMA 212 (1mg ml^{-1}) was mixed with 375µl of a goat anti-mouse IgM 40nm gold conjugate (Code BA GAMM 40, British Biocell, International, Cardiff, UK) made to 2 ml in PBS and incubated on a roller incubator for 3 hours. EMA 212 antibody bound gold particles were then collected by centrifugation (4000 xg) and resuspended to a final volume of 1.625ml in application buffer (20mM sodium phosphate buffer, 100mM sodium chloride, 0.25% trehalose, 0.1% sucrose, pH 7.2). Sixty µl of the EMA 212 antibody gold conjugate solution was then pipetted on to individual CLFD sample pads and air dried at 37ºC for 30 min. Each pad was attached to the CLFD test strip. The CLFDs were mounted within a plastic housing device (model no. SH 003, European Veterinary Laboratory, Woerden, Netherlands).

Alternaria brassicae conidia (Warwick HRI, University of Warwick; AA3, AA4 isolated from Brussels sprouts and AA10-1M isolated from Khol rabi) were produced in planta as described above (antigen-B) and collected in extraction buffer (0.05M Tris HCL, 0.15M NaCl, 0.4% Triton X100, 0.2% Tween 20, 0.2% BSA, 0.12% Geropan). For each isolate, a doubling dilution series was made to provide 100µl aliquots of *A. brassicae* ranging from 6x10^4 conidia per aliquot. Aliquots of each conidial suspension were applied drop wise to the sample pads of individual CLFD. A negative control of three CLFD received extraction buffer alone. After an assay time of

6 min the development of test and control lines were recorded visually by eye and then by optical densitometry using a laboratory-based Biodot Quadscan device (BioDot Ltd, Chichester, Sussex, UK).

The fungal species tested previously by PTA-ELISA were grown in sterile culture as described previously but were collected in CLFD extraction buffer. A culture of *M. brassicicola* (single-spore isolate CH195001) was maintained on sprout decoction agar with illuminated low intensity sub-stage fluorescent lighting for production of pseudothecia (Kennedy et al. 1999b). Ascospores were identified on the underside of each Petri dish lid (bright field microscopy 100x) and removed in CLFD buffer by gentle agitation with a glass spreader (Kennedy et al. 1999b). Similarly, *Pyrenopeziza brassicae* mating types MAT-1 and MAT-2 (Foster et al., 2002) were cultivated on compost agar for apothecial development and the production of ascospores (Gilles et al., 2001). For each fungal species spore concentrations were adjusted to $< 10^5$ ml^{-1} with CLFD buffer. A 100µl aliquot of each fungal sample was applied drop wise to the sample pad of individual *Alternaria* CLFD. After an assay time of 6 min data the CLFD was recorded visually for test line development and using a Quadscan reader.

Preliminary field trial study. For a 3-week period an over-wintered, heavily infected (dark leaf spot, ringspot and white blister) field plot (20 m x 10 m) of Brussels sprouts (c.v. Golfer) was monitored continuously, for the presence of *Alternaria brassicae* spores in the air, using a Burkard cyclone sampler (model no. MEI0073, Burkard Manufacturing, Rickmansworth, Hertfordshire, U.K.) at a site in Warwickshire, U.K. (OS grid reference SP278552). After each sampling period (1 or 3 days) the 1.5 ml sample collection vessel from the cyclone spore trap was removed and taken to the laboratory where 110µl volume of extraction buffer was then added. The contents of the

collection vessel were gently mixed .using a disposable micro pipette (model no. 50504NU, Alpha Lab Ltd, Eastleigh, UK) and a 100 µl aliquot transferred to a sample pad of an *Alternaria* CLFD with assay time and data collection as described above. The remaining 10µl of extraction buffer was removed from the sample collection vessel and examined using a microscope for the presence of *A. brassicae* conidia (expressed m^{3-1} of air sampled). The risk of *A. brassicae* infection for each of the field sampling periods was assessed by placing six healthy, greenhouse-grown *B. oleracea* trap plants (c.v. Golfer, 10 true leaves) in the field plot adjacent to the spore trap. After each air sampling period trap plants were removed and placed in an isolated environment of 100% humidity for 48 hrs at 16° C to fulfil the environmental requirements for infection by *A. brassica*e (Kennedy et al. 1999a). Additionally, six healthy, greenhouse-grown *B. oleracea* plants (which were not exposed to the field) were included as controls. The plants were then air-dried and retained in a glasshouse at a temperature of 12 to 14ºC for 21 days. Plants were visually examined for the expression of dark leaf spot on leaves. Confirmatory isolations from these lesions were recorded on sprout leaf decoction agar to confirm the presence of *A. brassicae* (Kennedy et al. 1999b).

Field risk assessment studies. In conjunction with a weather driven infection model (Kennedy and Graham, 1995; Wakeham and Kennedy, 2010) *A. brassicae* CLFD assays were evaluated in commercial fields of Brussel sprouts (OS grid reference TF509615, Lincolnshire, UK) and cauliflower (OS grid reference SD430235, Lancashire, UK). Within the fields, three trial plots (15 x 15 m) were marked. No fungicide application or treatments were made to the crop in these areas. During a six week period, daily air samples were collected using a Burkard multi-vial cyclone sampler adapted for field use (model no. ME10029; ME10031; ME10034, Burkard Manufacturing, Rickmansworth, Hertfordshire, U.K.). The samplers were pre-set for an automatic change of the trapping vessel (1.5 ml microfuge tube) each day at a pre-set time. The tubes within each sampler

(one tube for each day) were replaced weekly. The samplers were operated for 12 hours in each day between 5 am and 5pm to match the diurnal periodicity of *A. brassicae* conidial dispersal (Kennedy et al. 1999a). At collection, a 110 µl volume of extraction buffer was added to each of the tubes and the contents mixed as previously described. A 100 µl aliquot was transferred from each tube to a sample pad of an *A. brassicae* CLFD. After 6 min, data on the CLFD were observed visually and with a field portable one-step digital immunochromatographic test strip reader (European Veterinary Laboratory, Woerden, Netherlands). The remaining 10 µl suspension of each sample was examined in the laboratory under bright field microscopy (x 400) for the presence of *A. brassicae* conidia. Throughout the sampling period, Brassica leaf spot infection periods were monitored (Kennedy & Graham 1995; Wakeham & Kennedy 2010). For this purpose, canopy positioned wetness sensors and temperature and humidity environmental conditions were monitored with a SKYE Datahog II weather station (Skye Instruments Ltd, Powys, UK) at intervals of 30 min. Throughout the trial period, visual dark leaf spot disease assessments were taken in the unsprayed plots of each crop. Leaves of 10 plants in each plot were tagged and numbered. Recordings were made weekly and isolations were taken from identified dark leaf spot lesions as previously described.

RESULTS

MAb selection. Five hybridoma cell lines were identified by PTA-ELISA as producing antibodies with recognition sites to *Alternaria brassicae* antigen. Three of the cell lines emanated from mice immunised with antigen preparation-A. Two of these produced antibodies isotyped as IgG1 subclass. The other cell line produced IgM class. The remaining two cell lines were derived from mice immunised with antigen preparation-B and isotyped as producing IgM and IgG2a subclass. Only one of these hybridoma cell lines (coded EMA 212, isotype IgM and yielded from antigen

preparation A) provided an antibody type which could be used to detect conidial preparations of *A. brassicae* in a CLFD format (data not presented). When this hybridoma cell line was assessed by PTA-ELISA some reactivity to other *Alternaria* species was observed (Fig 1). Although, with a 10 fold difference observed between *A. brassicae* and the other the *Alternaria* species tested this was at a low level. Of those species tested outside of the genus little or no reactivity with EMA 212 was observed.

MAb characterisation. Effects of heat treatment, chemical and enzymatic effect of periodate oxidation and protease digestion on antigen binding by the antibody type produced by hybridoma cell line EMA212 are shown in Tables 2-4. Antibody binding was not affected by heat treatment of the *A. brassicae* conidial antigen (Table 2). Both periodate and protease reduced antibody binding at each of the temperatures and time periods tested (Table 3,4). This would suggest that the antibody recognised both carbohydrate and protein moieties of a glycoprotein. As heat treatment of the protein did not diminish the reactivity by the monoclonal antibody this would suggest linear epitopes in the protein molecule. Differential sensitivity to chemical and enzymatic modification illustrates the need to study a range of incubation periods and temperatures. The antibody produced by EMA 212 cell line was observed by immunofluorescence to bind to an epitope associated with the germ tube of the conidium which dissipated with mycelial growth (Fig. 2).

Competitive immunochromatographic test (CLFD). Using a monoclonal antiserum (produced by hybridoma cell line EMA 212), an immunochromatographic assay was developed to provide a visual indicator of *A. brassicae* presence within an air sample. This is a competitive assay and is counter intuitive i.e. test line depletion is dependent on increasing *A. brassicae* concentration (Fig. 3). The test is recorded positive for *A. brassicae* when no test line is observed by visual eye

observation. With an assay time of 6 min. aprox. 50 *A. brassicae* spores 100 μl^{-1} sample volume are required for depletion of the test line. Test sensitivity was increased aprox. five-fold with a laboratory based optical density (OD) reader (Fig. 4) and provided potential for quantitative measurement of *A. brassicae* in air samples. When other fungal species were tested with the *A. brassicae* CLFD a visible test line was evident and OD test line values for each recorded > 5.0 i.e. test negative for *A. brassicae*.

Preliminary field trial study. During the 3 week monitoring period an over-wintered crop of Brussels sprouts inoculated with *A. brassicae*, five 12 hour day periods of air sampling were identified by the CLFD-assay as positive for conidia of *A. brassicae*. For each of these periods, no visible CLFD test line was recorded and the OD test line values recorded by the Quadscan reader gave a value of < 2 OD. On each of these dates the total numbers of dark leaf spot lesions on the 6 trap plants exceeded 13. For all other sampling periods, all trap plants were negative for dark leaf spot lesions (*A. brassicae*).

Field risk assessment studies. Daily *A. brassicae* infection score ratings over the 6-week observation period resulted in ten days designated 'moderate risk' (infection score 100 to 150) and 15 days as 'high risk' (infection score >151). Infection scores < 100 were interpreted as 'low risk'. With the weather-driven model for each week of the six week observation period, at least at least one daily *A. brassicae* infection risk period was identified (Figure 5). In contrast, CLFD assay performed daily on collected air samples detected *A. brassicae* on only three days (13^{th},14^{th} and 23^{rd} Aug) by both visual and field-portable strip reader. Microscopic examination of each daily collected field air sample showed a correlation (r^2= 0.8081) with the number of *A. brassicae* conidia trapped and the corresponding field portable EVL immunochromatographic test strip reading (Fig. 6).

For the three dates where the CLFD test determined *A. brassicae* to be at an air sample threshold risk in the crop, the environmental model recorded a moderate or high risk of *A. brassicae* infection. Evaluation of the Brussels sprout tagged plants prior to the start of the experiment (3rd August) revealed a level of dark leaf spot incidence already within the crop (10 lesions plant^{-1}). Thereafter two distinct phases of dark leaf spot development occurred on the tagged Brussels sprout plants over the six week period (Fig. 7). The second phase of dark leaf spot symptom expression was recorded on the tagged plants after the 26th August.

At the second commercial site (cauliflower crop) the weather driven model identified 16 days when the crop was at risk of *A. brassicae* infection. However, visual assessment of the CLFD assay indicated that *A. brassicae* inoculum was either absent or at a low level. Using the portable in-field reader, a single CLFD assay showed a digital reading below 2 (EVL test reading at 1.6 for the 9-10th September). For this period, *A. brassicae* conidia were identified by bright field microscopy within the collected air sample (5 conidia m$^{3-1}$ air sampled). Ten days later dark leaf spot establishment (at a low level), was observed within non-fungicide treated areas of the cauliflower crop (Fig. 8).

DISCUSSION

In this study two preparation types of *Alternaria brassicae* conidia were used for the production of antibody producing hybridoma cell lines from female Balb C mice. IgM and IgG subclass were produced by *A. brassicae* positive cells lines irrespective of the growth medium used for conidial antigen (V8 juice agar culture (antigen A) or Brussels sprout plants (antigen B)). When tested by

ELISA or lateral flow, none of the *A. brassicae* positive cell lines produced antibodies which could be used in a double antibody sandwich (DAS) (data not supplied). A DAS assay requires target analyte (antigen) to bind simultaneously to both the reporter and an immobilized 'capture' antibody. This cannot be accomplished with small analyte molecules that may have a single antigenic determinant. Additionally, steric hindrance may prevent simultaneous binding. When the target analyte consists of a small molecule, CLFDs are often preferred as they do not require an analyte to provide the linkage between the capture and reporter antibody (Qian and Bau, 2004). Similarly, this is the case with PTA-ELISA and the processes by which each of the cell lines were initially screened for antibodies reactive to *A. brassicae* antigen.

Interestingly, of the *A. brassicae* positive hybridoma cell lines selected by this method only one produced an antibody type (Antigen Type A, IgM producing cell line) which could be used within a CLFD assay format. Antibody performance in a lateral flow assay can be very different to that in ELISA where long incubation times and lower surface concentration exist. For lateral flow (LFD) an antibody with high affinity is required (O'Farrell, 2013). Typically, IgM antibodies are considered to be of lower affinity to that of IgG preparations (Makela, 1997). For this reason it was surprising that the IgG cell lines did not prove suitable for use. However, high affinity may not be the sole driver in this type of assay as affinity needs to be driven by a fast on-rate (k_{on} or association rate constant). Unlike the ELISA, the test line zone of the LFD provides limited time for interaction between the antibody and analyte. A time of 1 and 6s for binding has been reported. Although a little more contact time exists between antibody and analyte from the conjugate pad with the "effective" binding reaction starting with resolubilization of the conjugate and ends after the conjugate passes the test line. This time is typically on the order of 10-20 s (Brown 2009). Malmborg (1992) reported by surface plasmon resonance that IgM anti-Tn alpha antibodies

showed one order of magnitude higher association rate constants, as compared with the IgG antibodies. Also, IgM multi-valency (10 binding sites compared to 2 of IgG) leads to a large effect on the dissociation rate resulting in high binding avidity (King, 1998). The IgM antibody used in this study (EMA 212 hybridoma cell line) provided clear test line development in the CLFD format within 6 min. By eye a detection threshold of approximately 50 *A. brassicae* conidia was achieved. Assessment using a portable reader (optical densitometry) improved test sensitivity.

Initial reactivity screening of the hybridoma cell lines to other spore types was carried out by PTA-ELISA prior to lateral flow development. The ELISA format lends itself to high throughput screening of multiple antigens and antibodies and provides a quick primary screen. As the fungal structure is complex variation in antigen concentration and type is likely to occur between species. Plant pathology papers often report fungal plate washings and adjustment by dilution or weight for immunoreactivity studies rather than protein concentration (Dewey et al 1990, Bermingham et al. 1995, Kennedy et al. 2000, Meyer et al. 2000). For air sampling it is standard to relate pollen and fungal spore number by enumeration (British Aerobiology Foundation, 1995). By using spore concentration as a normalisation factor provides a better measure when relating to the field situation (spores cubic metre^{-3-1} air sampled). Skottrup (2007), reported in the development of monoclonal antibodies for the detection of *Puccinia striformis* urediniospores the adjustment of fungal spores for ELISA reactivity testing to 10^5 ml^{-1} rather than by protein concentration.

Using this methodology, the IgM antibody produced by cell line EMA 212 showed some reactivity to other *Alternaria* species when tested by PTA-ELISA. Although, interpreting ELISA data to establish useful thresholds for antibody specificity and sensitivity is arbitrary and dependent on the test requirement. Frequently, positive thresholds used are multiples from two to four of the control

mean (Sutulu et al. 1986). With negative controls replicating the solution containing the antigen (Dewey et al. 1997). Using these parameters, the PTA- ELISA test provided a good measure of discrimination between *A. brassicae* and the other *Alternaria* species tested with a 10 fold difference by PTA-ELISA. No significant reactivity was observed to the species tested outside of the *Alternaria* genus. This was also observed when EMA 212 was incorporated within a CLFD format and additional vegetable brassica pathogen tests included (*Mycosphaerella brassicicola* (ringspot) and *Pyrenopeziza brassicae* (light leaf spot). It is however important to note the low number of fungal species tested in this study. High throughput sequencing methods suggest that as many as 5.1 million fungal species exist (Blackwell, 2011). A weakness of antibody reactivity testing is the number of isolates that can be accessed and screened easily. Unlike PCR based technologies there is no bioinformatics tool available to quickly and remotely screen hybridoma culture supernatants for specificity to target organisms (Ye et al. 2012).

In this study, both IgG and IgM- producing hybridoma cell lines were selected and with reactivity to the two types of *A. brassicae* antigen preparations used. Both isotypes are frequently reported on in the development of monoclonal antibodies for fungal diagnostic assays (Werres and Steffens, 1994; Skottrup et al 2007) and found equally cross-reactive to the fungal species tested (Dewey et al. 1989). The structurally complex nature of fungi, often with shared immunodominant antigens, makes the development of species specific antibodies difficult (Drouhet, 1986; Notermans and Soentoro, 1986; Priestley and Dewey, 1993). No value of improved specificity or sensitivity could be drawn from the use of different *A. brassicae* conidial antigens (spores produced in culture or in planta) for monoclonal antibody production.

Nevertheless, in combination with prediction of infection, the *A. brassicae* CLFD assay demonstrated potential for 'in-field' risk assessment to identify periods when brassica crops were at risk from *A. brassicae* infection. Although for widespread commercial application, the test may require refinement to reflect potential reactivity of the diagnostic probe with other spore types. The developmentof disruptive technologies over recent years provides application of DNA aptamers, molecular beacons and quantum dot LFD assays which, if improved specificity is required, could prove useful (Bruno 2014; Sajid et al. 2015; Wang et al. 2014).

Developing accurate risk assessments for plant (and animal) diseases is an important area within epidemiological research. Improving risk assessments for plant diseases in agricultural cropping systems has been useful in the reduction of pesticide applications in the environment (Bugiani et al. 1995; Fry and Fohner 1985; Kennedy and Graham 1995; Wakeham and Kennedy 2010). However, the use of direct detection and quantification of pathogenic inoculum in risk assessments for plant diseases in agricultural production and biosecurity is not routine. One reason for the low usage of direct detection systems has been the cost and speed at which results can be processed and obtained by end users. Detection systems often rely on laboratory facilities in conjunction with specialist knowledge. For these reasons infection risk (based on environmental conditions) is often used as a risk criteria for controlling plant pathogens in agricultural production systems (Gilles et al. 2004; Magarey et al. 2005). However, although infection is an important part of the plant pathogens life cycle it does not adequately estimate the real risk of the development of crop diseases. For example, in the present study infection of *A. brassicae* based on temperature and wetness duration (two major criteria) gave an infection risk on most days within a susceptible crop. By incorporating information on inoculum availability (CLFD test) reduced the number of days when the crop was identified as at

risk of infection. The study demonstrates the potential to forecast disease risk in the field at a relatively low cost.

Development of the disease in the field will depend on a number of factors. The plant growth stage that infection occurs, cultivar, environmental conditions and inoculum concentration are limiting factors of *A. brassicae* disease development on oilseed rape (Hong and Fitt 1995). In this study, *A. brassicae* conidial concentrations were identified as being above a disease risk threshold on three occasions in a Brussels sprout crop using on-site CLFD assays. After the first risk period had been identified an increase in dark leaf spot symptoms occurred between 14 and 21 days later in the crop. Thereafter increasing lesion development was observed in the crop. In a cauliflower crop, the disease was first observed on plants 10 days after a CLFD assay predicted a risk of *A. brassicae*. Under optimal conditions in a controlled glasshouse very small lesions on cauliflower were first observed 6 day after inoculation (Duhan and Suhag 1990).

Risk assessment which include direct measurement of numbers of plant pathogenic spores in the air should prove useful in predicting the occurrence of pathogens in crops. This is particularly important in systems where pathogen infection causes cosmetic damage and downgrades value by its occurrence on for example Brussels sprout buttons. In vegetable crops the use of F_1 hybrids means that pathogen occurrence is usually uniform within the production area. The occurrence of pathogenic inoculum results from interactions with other crops in the area produced under a different production schedule. For example, oilseed rape which *A. brassicae* infects, is often unsprayed and grown in proximity to vegetable brassica production. Freshly transplanted summer vegetable brassica crops are often produced adjacent to overwintered and unsprayed vegetable brassica crops. Detecting inoculum will be useful as many of the approved fungicides which control *A. brassicae*

infection have systemic activity allowing good control even when applied after infection has occurred.

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). Similarly the occurrence of detected inoculum was shown before increased disease in the field in the two cropping systems reported on in this study. Recently it has been shown that spore sampling network devices maybe a suitable approach for early detection of incoming inoculum (Skelsey et al. 2009). When combined with decision support systems this approach represents a potential aid for targeting the optimal timing of disease control products against *Phytophthora infestans* (Fall et al. 2015). In horticultural crops, there is considerable interaction between transplanting date and spatial location. Humpherson-Jones (1982) reported wind transport of *A. brassicae* conidia of up to 1.8km. The epidemiology of *A. brassicae* on local horticultural brassica crops may relate to harvesting of *B. napus* (Skjoth et al. 2012). Directly measuring airborne inoculum could improve the estimation of risk resulting from the interaction of arable and horticultural brassica crops. Although, the number of air samplers and siting of these within a locality requires further research (West and Kimber 2015).

Immunochromatographic tests provide a suitable test format to detect and quantify inoculum '*in-situ*' (Kennedy and Wakeham, 2008; Thornton et al. 2004). The presence of relatively high spore concentrations as a prerequisite for infection means that detecting very low numbers of spores in many cases may not be necessary. Studies for the ascosporic fungi *Pyrenopeziza brassicae* and *Mycosphaerella brassicicola* (Brassica light and dark leaf spot) have demonstrated that spore inoculum occurs at high concentrations in the air before crop-to-crop transport is possible (Gilles et

al. 2004; Wakeham and Kennedy, 2010). Non-molecular methods are therefore adequate and can provide a low-cost approach when compared to the use of molecular methods (Shan 2011). Although, it should be noted that considerable advances have been made towards the deployment of DNA based systems to the field and in reducing analysis cost (Thiessen et al. 2015). However, as immunochromatographic devices do not require a pre-extraction or a DNA amplification stage they have considerable advantages both in cost and simplicity of use. Also, the cross-contamination of spores from collected field air samples are not likely to prove problematic as has been reported with the deployment of molecular methodologies in the field (Reiger 2013).

The improvement of immunochromatographic readers since the study was carried out also provides the potential of smartphones for use as a CLFD reading system (Sangdae et al. 2013). The synergistic use of these two technologies demonstrate the potential to help the agri-food industry to assess and predict disease potential in a cost effective way.

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Fig. 1. Reactivity of monoclonal antiserum (hybridoma cell line coded EMA 212) to fungal species : *Alternaria dauci, Alternaria alternata, Alternaria cheranthi, Alternaria brassicae, Penicillium waksmanni, Phoma lingam, Stemphyllium lycopersici, Pyrenophora dictyoides, Botrytis squamosa, Aschochyta fabae, Aureobasidium pululans*, *Fusarium solani* by plate-trapped enzyme-linked immunosorbent assay.

Fig. 2. Visualization of *Alternaria brassicae* on a glass microscope slide with monoclonal antiserum (hybridoma cell line coded EMA 212) labelled with a fluorescein conjugate as viewed by (a) bright field microscopy (b) UV episcopic fluorescence (filter wavelengths at 450–560 nm).

Fig. 3. Visual assessment (by eye) of competitive immunochromatographic assay strips (CLFDs) for risk of *Alternaria brassicae* inoculum in air samples: A, test line development indicates low or no risk; B, no test line development – risk of *Alternaria brassicae* inoculum.

Fig. 4. Assessment by optical densitometry of competitive immunochromatographic assay strips (CLFDs) for measurement of *Alternaria brassicae* (serial doubling dilution series of *A. brassicae* conidial spore concentration (6000 to aprox. 6 conidia)).

Fig. 5. Daily infection score ratings generated with a weather-based model that identifies infection periods of the dark leaf spot brassica pathogen (*Alternaria brassicae*), Lincolnshire, UK (OS grid reference TF509615).

Fig. 6. Relationship between the number of daily *Alternaria brassicae* conidia sampled by a multivial cyclone sampler and the corresponding value generated by immunochromatographic strip-type assay for presence of *A. brassicae* condia.

Fig. 7. Dark leaf spot disease development in two UK brassica commercial crops: Brussels sprouts (□) Lincolnshire (OS grid reference TF509615) and cauliflower (♦) Lancashire (OS grid reference SD430235) during 2005.

Table 1.

Species	Growth Media	Morphological Classification	Host isolated
Alternaria brassicae	V8 agar	Ellis, 1971	*B.oleracea*
Alternaria dauci	V8 agar	Ellis, 1971	*D. carota*
Alternaria cheranthi	V8 agar	Ellis, 1971	*C. cheiri*
Alternaria alternata	V8 agar	Ellis, 1971	*F. vespa*
Penicillium waksmanni	PDA	Pitt, 1988	Soil
Phoma lingam	PDA	Punithalingam and Holliday, 1975	*B. napus*
Stemphyllium lycopersici	PDA	Ellis, 1971	*S. lycopersicum*

Pyrenophora dictyoides	PDA	Ellis, 1971 (Drechslera state)	*L. perenne*
Botrytis squamosa	PDA	Ellis, 1971	*A. cepa*
Aschochyta fabae	PDA	Punithalingam and Holliday, 1975	*V. faba*
Aureobasidium pululans	PDA	Ellis, 1971	Air
Fusarium solani	PDA	Booth,1971 Nelson et al., 1983	*A. cepa*

PDA, Potato Dextrose Agar; V8 Juice Agar

Table 2

Absorbance values from PTA-ELISA tests with heat-treated *Alternaria brassicae* antigens by using MAb EMA 212

		PTA-ELISA Absorbance (450nm)[a]	
Time (min)	Temperature (°C)	PTA ELISA	Reduction (%)
0	n/a	0.936±0.045	
1	100	0.961±0.006	0
3	100	0.986±0.025	0
5	100	0.999±0.044	0

[a]Each value represents the mean of three replicate values ± standard errors.

Table 3

Absorbance values from PTA-ELISA tests with periodate-treated *Alternaria brassicae* antigens by using MAb EMA 212

		PTA-ELISA Absorbance (450nm)[a]		
Time (h)	Temperature (°C)	Periodate	Control	Reduction (%)
5	4	0.223 ±0.012	0.371±0.005	40
5	37	0.150±0.006	0.324±0.00	54
24	4	0.133±0.014	0.409±0.034	67
24	37	0.192±0.038	0.263±0.028	27

[a]Each value represents the mean of three replicate values ± standard errors.

Table 4

Absorbance values from PTA-ELISA tests with protease-treated *Alternaria brassicae* antigens by using MAb EMA 212

		PTA-ELISA Absorbance (450nm)[a]		
Time (h)	Temperature (°C)	Protease	Control	Reduction (%)
5	4	0.119 ±0.009	0.332±0.005	64
5	37	0.135±0.016	0.341±0.004	60
24	4	0.104±0.003	0.453±0.034	77

24	37	0.090±0.023	0.398±0.028	100	

[a]Each value represents the mean of three replicate values ± standard errors.

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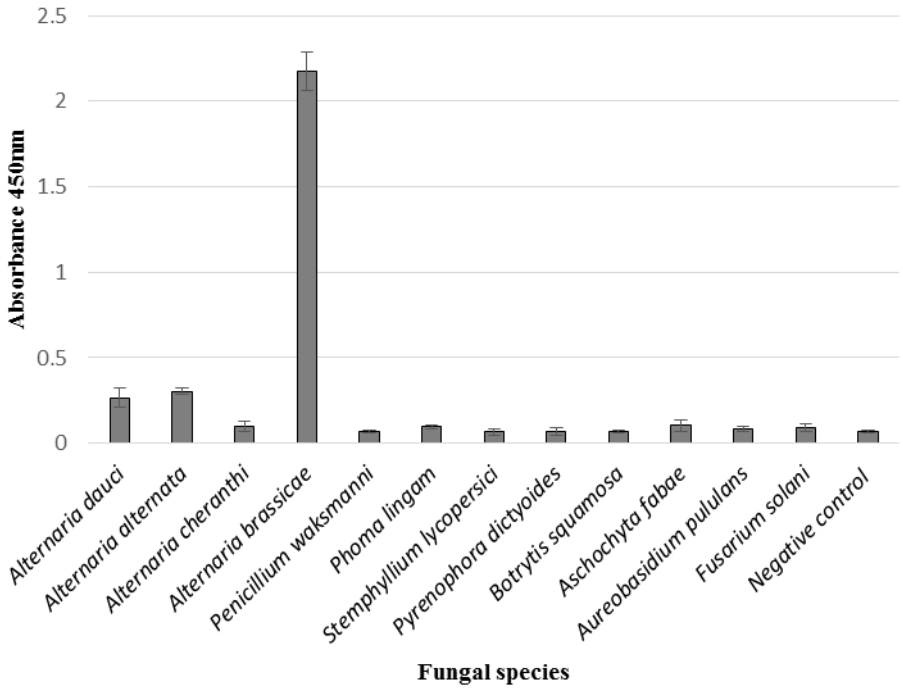

Fig. 1. Reactivity of monoclonal antiserum (hybridoma cell line coded EMA 212) to fungal species : *Alternaria dauci, Alternaria alternata, Alternaria cheranthi, Alternaria brassicae, Penicillium waksmanni, Phoma lingam, Stemphyllium lycopersici, Pyrenophora dictyoides, Botrytis squamosa, Aschochyta fabae, Aureobasidium pululans, Fusarium solani* by plate-trapped enzyme-linked immunosorbent assay.

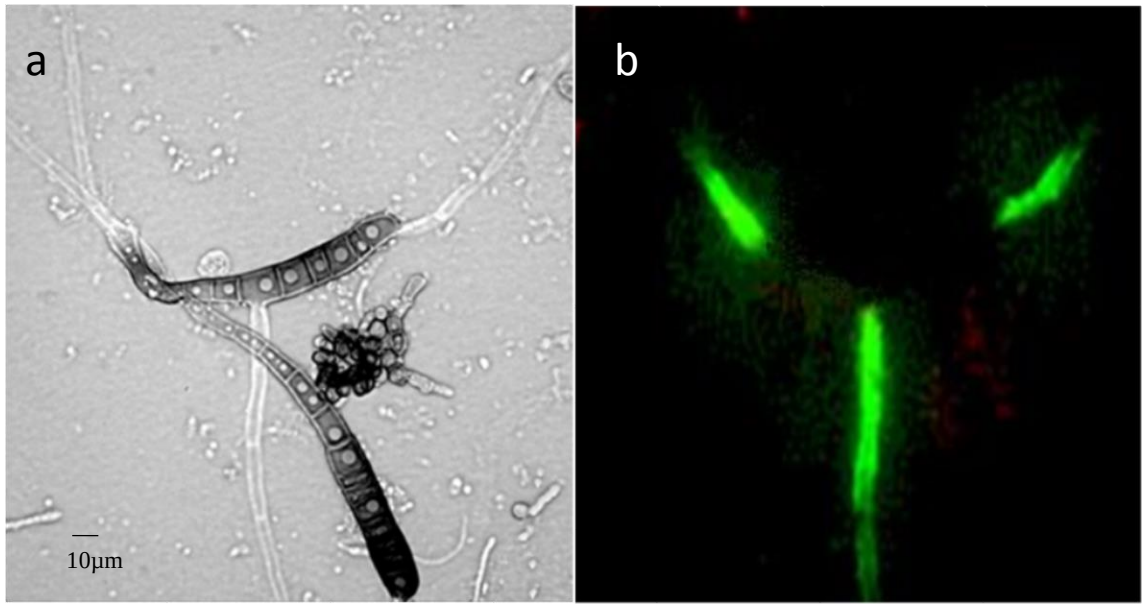

Fig. 2. Visualization of *Alternaria brassicae* on a glass microscope slide with monoclonal antiserum (hybridoma cell line coded EMA 212) labelled with a fluorescein conjugate as viewed by (a) bright field microscopy and (b) UV episcopic fluorescence (filter wavelengths at 450–560 nm).

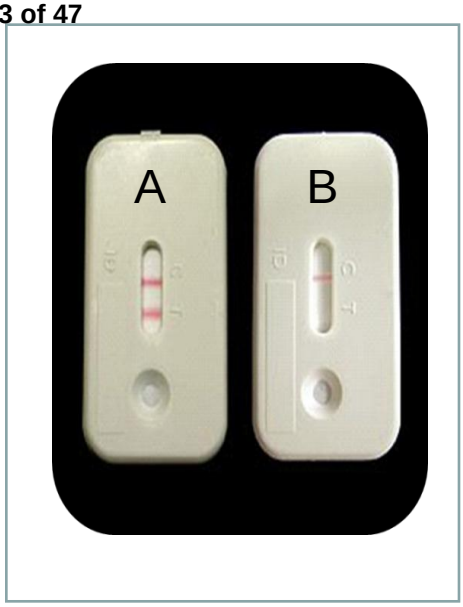

Fig. 3. Visual assessment (by eye) of competitive immunochromatographic assay strips (CLFDs) for risk of *Alternaria brassicae* inoculum in air samples: A, test line development indicates low or no risk; B, no test line development – risk of *Alternaria brassicae* inoculum.

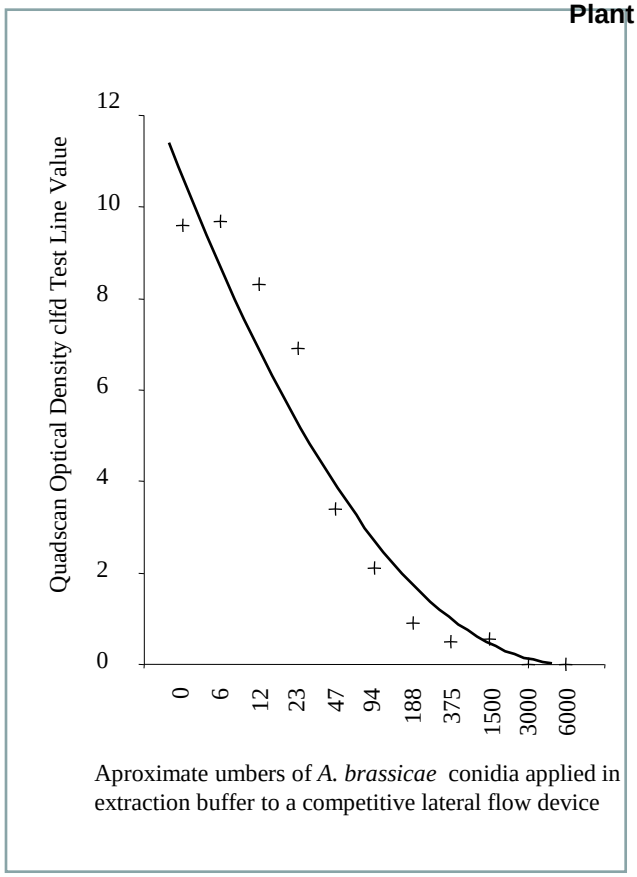

Fig. 4. Assessment by optical densitometry of competitive immunochromatographic assay strips (CLFDs) for measurement of *Alternaria brassicae* (serial doubling dilution series of *A. brassicae* conidial spore concentration (6000 to aprox. 6 conidia).

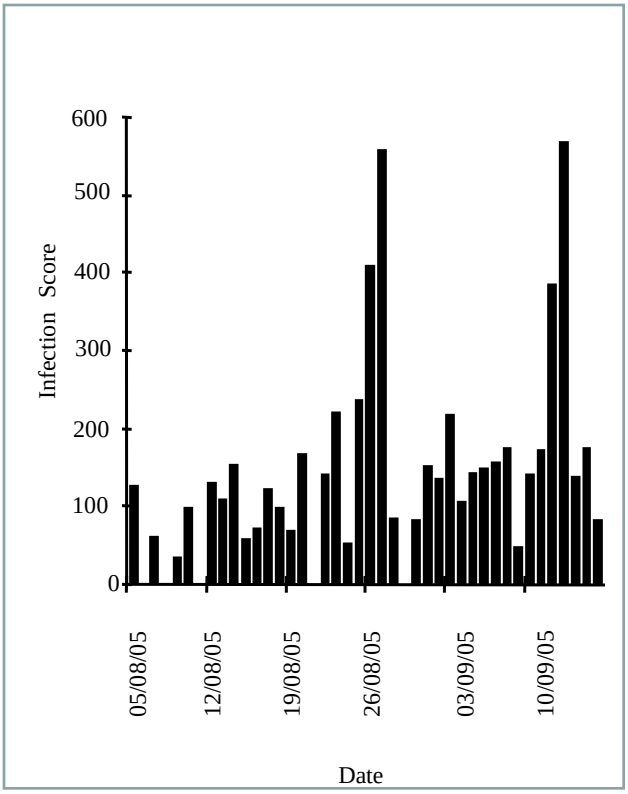

Fig. 5. Daily infection score ratings generated with a weather-based model that identifies infection periods of the dark leaf spot brassica pathogen (*Alternaria brassicae*), Lincolnshire, UK (OS grid reference TF509615).

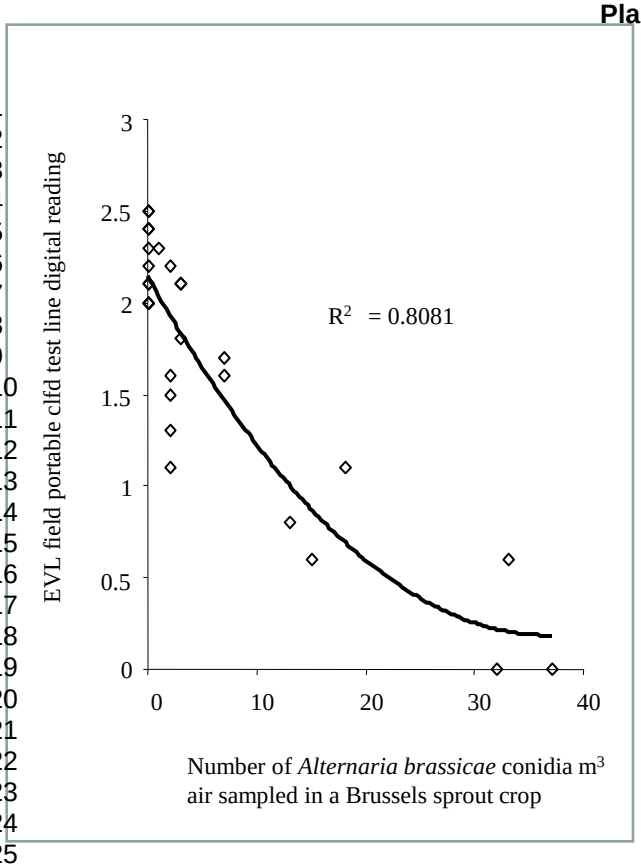

Fig 6. Relationship between the number of daily *Alternaria brassicae* conidia sampled by a multivial cyclone sampler and the corresponding value generated by immunochromatographic strip-type assay for presence of *A. brassicae* condia.

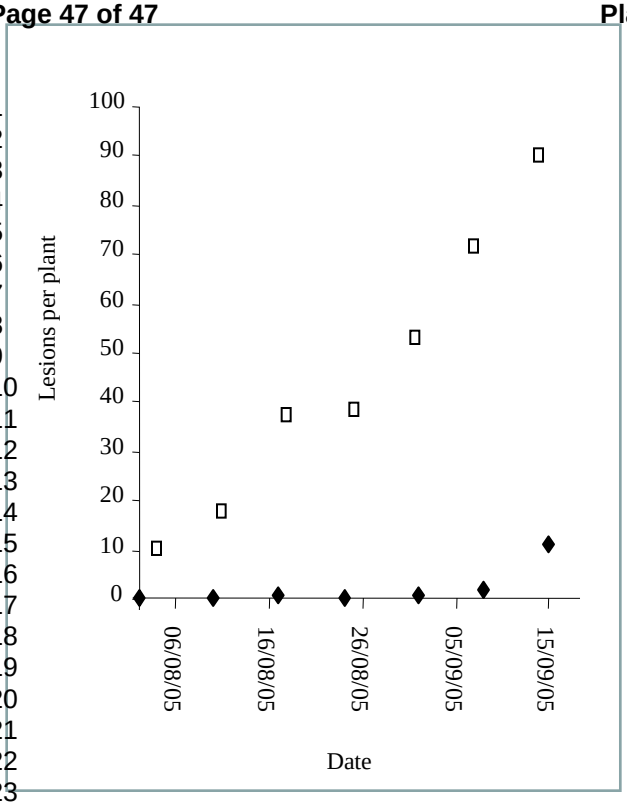

Fig. 7. Dark leaf spot disease development in two UK brassica commercial crops: Brussels sprouts (□) Lincolnshire (OS grid reference TF509615) and cauliflower (♦) Lancashire (OS grid reference SD430235) during 2005.