Field Evaluation of a Competitive Lateral-Flow Assay for Detection of *Alternaria brassicae* in Vegetable Brassica Crops

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Abstract

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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 *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 overpredicted *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.

Fungal pathogens occurring on vegetable brassica crops can be difficult to control. The use of mathematical models to summarize 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). However, these systems do not provide information on the presence or absence of pathogenic inoculum. Approaches based on the direct measurement of fungal spores in the air have been reported previously (Carisse et al. 2005; Calderon et al. 2002; 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 1980s (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, and 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 types 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 onto plant surfaces (Jamaux and Spire 1994) or impaction using rotor rods (Schmechel et al. 1996). Alternatively, a microtiter immunospore air sampler (MTIST; Burkard Manufacturing Ltd., Rickmansworth, Hertfordshire, UK) provides collection of air samples by impaction across 32 microtiter wells (Kennedy et al. 2000). Quantification of trapped particulates can then be subjected to enzyme-linked immunosorbent assay (ELISA) and, with suitable antibodies, multiplex assays can be devised (Wakeham et al. 2004). Most of these tests require laboratory processing facilities. A multivial cyclone air sampler (Burkard Manufacturing Ltd.) which collects air samples into successive collection vessels (8 by 1.5 ml) at predetermined time periods has been considered for use in polymerase chain reaction (PCR)-based methods (West and Kimber 2015; West et al. 2008). 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; Zink et al. 2012). For example, in horticulture, reduced application 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 an 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 (competitive immunochromatographic lateral flow 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 and Fitt 1996; Humpherson-Jones 1992). A necrotroph on host tissue produces asexual spores (conidia) for wind dispersal (McCartney and Fitt 1998). Infection of crops by *A. brassicae* can result in severe yield losses (Parada et al. 2008). The relationship between important meteorological parameters

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affecting infection and sporulation of *A. brassicae* has 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 *A. brassicae* isolates (Warwick HRI, University of Warwick, UK) (Maude and Humpherson-Jones 1980) were cultured on V8 juice agar (3 g of technical agar, 0.4 g of calcium carbonate, and 20 ml of V8 juice [Campbell Soup Company, Camden, NJ]) for 1 week in darkness at 25°C. A 5-cm square of mycelium from each inoculated plate was then removed, homogenized in 5 ml of sterile distilled water (SDW), and transferred in 500- μ l aliquots to 10 $\times$ 5 ml of sterile clarified V8 juice medium (Johnston and Booth 1983). The V8 mycelial suspensions were mixed and then incubated in darkness at 25°C. At a magnification of $\times$ 100, conidial production was observed 7 days later. Using the method described by Lawrie et al. (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 (Qbiogene FPI20; Anachem Ltd., Luton, UK), the conidia were mechanically disrupted according to the manufacturer's guidelines (3 $\times$ 25 s at a speed setting of 5.5) and then aliquoted into 50- μ l lots; this preparation was labeled "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 h on a wrist-action shaker, the conidial suspension was sprayed directly onto healthy Brussels sprout plants (*B. oleracea* var. *gemmifera* 'Golfer'). Inoculated plants were exposed to a relative humidity of 100% for 48 h and thereafter retained in a greenhouse held at 18°C. 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 min. Leaf material was removed by filtering the suspension through a membrane of 97- μ m pore size. Conidia of *A. brassicae* were recovered on a membrane of 37- μ m pore size and resuspended in 5 ml of PBS (10^5 conidia ml⁻¹). Following mechanical disruption, as described above, the *A. brassicae* suspension was aliquoted into 50- μ l lots; this preparation was labeled "antigen-B". Three female Balb C/Cj substrain mice were each immunized for induction of antibody-secreting spleen cells (Köhler and Milstein 1976) with 50 μ l of antigen-A mixed with an equal volume of Titermax adjuvant (Sigma-Aldrich T-2684). Three additional mice were injected with

50 μ l of antigen-B. The same mice were immunized on two further occasions at 14-day intervals without adjuvant.

Collected tail bleeds (Köhler and Milstein 1975) were titrated against their respective homologous antigen preparation by plate-trapped antigen (PTA)-ELISA (Kemeny 1991). For each tail bleed, 10 paired wells of a 96-well Nunc Immunosorbent Polysorp flat-bottomed microtiter plate (model number 475094; Life Technologies, Paisley, Scotland) were coated with the homologous antigen type in PBS at 100 μ l well⁻¹. Ten paired wells received PBS alone at 100 μ l well⁻¹ for each tail bleed as a control. After overnight incubation at 18°C, unbound antigen was removed by inverting the individual microtiter plates and tapping them down onto absorbent toweling. The plates received four 1-min washes of PBS at 200 μ l well⁻¹. Wells were blocked with 200 μ l of 3% casein buffer (3% [wt/vol] casein in PBS) and incubated in a Wellwarm shaker incubator (model number W1031B; Denley Instruments Ltd., Sussex, UK) for 30 min at 30°C. Residual blocking buffer was removed and wells were washed four times for 1 min each with PBS plus 0.05% Tween 20 (PBST) at 200 μ l well⁻¹. Mice tail bleeds were diluted 1:100 in PBST and doubling dilutions were made to 1:25600. A diluted tail bleed was applied (100 μ l well⁻¹) 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 microtiter 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 1 min each with PBST. Aliquots of 100 μ l of antimouse immunoglobulin G (IgG; whole molecule) biotinylated antibody produced in goat (model number B7264; Sigma-Aldrich Company Ltd., London) diluted 1:500 in PBST were added to each well and incubated for 45 min, as above. After washing as previous, streptavidin peroxidase at 100 μ l well⁻¹ (model number SS512; Sigma-Aldrich Company) diluted 1:10000 in PBST was added to each well and incubated as above for 45 min. The microtiter wells were washed as previously described and each well received 100 μ l of 3,3',5,5'-tetramethylbenzidine substrate (model number T-3405 and P-4922; Sigma-Aldrich). The reaction was stopped by adding 25 μ l of 2M H₂SO₄. Absorbance was recorded at 450 nm in an HT11 (Anthos Labtec Instruments, Salzburg, Austria) ELISA plate reader.

Mice with high titer tail bleeds (end point > 1:32000) to *A. brassicae* were identified. Following a final prefusion boost, the spleen was removed 4 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-covered (model number HPWP09050; MERCKMillipore, Darmstadt, Germany) 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 min at 13,000 $\times$ g, 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 microtiter plate (model number 475094 A; Life Technologies) were coated with each fungal washing at 100 μ l well⁻¹ (Dewey et al. 1989). Unbound antigen was removed after overnight incubation at 18°C (Wakeham et al. 1997) and the PTA-ELISA process was 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* sp.-positive cell line was determined using an Isostrip mouse monoclonal isotyping kit (model number 11-493-027 001; Roche Diagnostics, Burgess Hill, West Sussex, UK). One of the cell lines

Table 1. Fungal species cultured for immunoassay reactivity studies

Species	Growth media ^a	Morphological classification	Host isolated
<i>Alternaria brassicae</i>	V8 agar	Ellis 1971	<i>Brassica oleracea</i>
<i>A. dauci</i>	V8 agar	Ellis 1971	<i>D. carota</i>
<i>A. chervanthi</i>	V8 agar	Ellis 1971	<i>C. cheiri</i>
<i>A. alternata</i>	V8 agar	Ellis 1971	<i>F. vespa</i>
<i>Penicillium waksmani</i>	PDA	Pitt 1988	Soil
<i>Phoma lingam</i>	PDA	Punithalingam and Holliday 1975	<i>B. napus</i>
<i>Stemphyllium lycopersici</i>	PDA	Ellis 1971	<i>S. lycopersicum</i>
<i>Pyrenophora dictyoides</i>	PDA	Ellis 1971 (Drechslera state)	<i>L. perenne</i>
<i>Botrytis squamosa</i>	PDA	Ellis 1971	<i>A. cepa</i>
<i>Aschochyta fabae</i>	PDA	Punithalingam and Holliday 1975	<i>V. faba</i>
<i>Aureobasidium pululans</i>	PDA	Ellis 1971	Air
<i>Fusarium solani</i>	PDA	Booth 1971; Nelson et al. 1983	<i>A. cepa</i>

^a V8 agar = V8 juice agar and PDA = potato dextrose agar.

(coded EMA 212) was selected and the tissue culture supernatant purified using a High Trap IgM purification HP column according to the manufacturers instructions (model number 17-5110-01; GE Healthcare, Little Chalfont, UK). 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 et al. (2002). Conidia were germinated in 0.01% glucose solution on multiwell glass slides (model number MIC3412; Scientific Laboratory Supplies, Nottingham, UK) and by immunofluorescence probed with antibodies produced by the 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 to 10 min 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 counterintuitive (i.e., as the target analyte in a sample increases, test line color 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 semiquantitative 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 onto a Mylar backing (model number SHF2400225; Millipore Corp., Bedford, MA) attached at either end to an absorbent pad (model number GBOO4, Schleicher and Schuell, Dassel, Germany) and a sample pad (model number T5NM; Millipore Corp.). A control line of a nonimmune antimouse serum and a test line of *A. brassicae* (antigen-A), each adjusted to 0.5 mg 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, Chichester, West Sussex, UK) operating at a line travel speed of 50 m s⁻¹. The sprayed membranes were air dried overnight at room temperature (18 to 20°C) and cut into 5-mm strips. A 500-μl volume of EMA 212 (1 mg ml⁻¹) was mixed with 375 μl of a goat antimouse IgM 40-nm 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 h. EMA 212 antibody-bound gold particles were then collected by centrifugation (4,000 × g) and resuspended to a final volume of 1.625 ml in application buffer (20 mM sodium phosphate buffer, 100 mM sodium chloride, 0.25% trehalose, and 0.1% sucrose, pH 7.2). The EMA 212 antibody gold conjugate solution (60 μl) was then pipetted onto individual CLFD sample pads and air dried at 37°C for 30 min. Each pad was attached to the CLFD test strip. The CLFD strips were mounted within a plastic housing device (model number SH 003; European Veterinary Laboratory, Woerden, The Netherlands).

A. brassicae conidia (Warwick HRI; AA3 and AA4 isolated from Brussels sprouts and AA10-1M isolated from kohlrabi) were produced in planta as described above (antigen-B) and collected in extraction buffer (0.05 M Tris HCL, 0.15 M NaCl, 0.4% Triton X100, 0.2% Tween 20, 0.2% bovine serum albumen, and 0.12% Geron). For each isolate, a doubling dilution series was made to provide 100-μl aliquots of *A. brassicae* of 6 × 10⁴ conidia/aliquot. Aliquots of each conidial suspension were applied drop wise to the sample pads of individual CLFD strips. A negative control of three CLFD strips received extraction buffer alone. After an assay time of 6 min, the development of test and control lines was recorded visually by eye and then by optical densitometry using a laboratory-based BioDot Quadscan device (BioDot Ltd.).

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 *Mycosphaerella brassicicola* (single-spore isolate CH195001) was maintained on sprout decoction agar with illuminated low-intensity substage fluorescent lighting for

production of pseudothecia (Kennedy et al. 1999b). Ascospores were identified on the underside of each petri dish lid (×100 bright-field microscopy) 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 <10⁵ ml⁻¹ with CLFD buffer. A 100-μl aliquot of each fungal sample was applied drop wise to the sample pad of individual *Alternaria* CLFD strips. After an assay time of 6 min, CLFD data were recorded visually for test line development and using a Quadscan reader.

Preliminary field trial study. For a 3-week period, an overwintered, heavily infected (dark leaf spot, ringspot, and white blister) field plot (20 by 10 m) of Brussels sprouts (Golfer) was monitored continuously for the presence of *A. brassicae* spores in the air using a Burkard cyclone sampler (model number MEI0073; Burkard Manufacturing Ltd.) at a site in Warwickshire, UK. (Ordnance Survey National Grid [OS] 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 a 110-μl volume of extraction buffer was then added. The contents of the collection vessel were gently mixed using a disposable micropipette (model number 50504NU; Alpha Lab Ltd., Eastleigh, UK) and a 100-μl aliquot was transferred to a sample pad of an *Alternaria* CLFD with assay time and data collection, as described above. The remaining 10 μl of extraction buffer was removed from the sample collection vessel and examined using a microscope for the presence of *A. brassicae* conidia (expressed per cubic meter 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 (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 h at 16°C to fulfill the environmental requirements for infection by *A. brassicae* (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 14°C 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 reference TF509615; Lincolnshire, UK) and cauliflower (OS reference SD430235; Lancashire, UK). Within the fields, three trial plots (15 by 15 m) were marked. No fungicide application or treatments were made to the crop in these areas. During a 6-week period, daily air samples were collected using a Burkard multivital cyclone sampler adapted for field use (model numbers MEI0029, MEI0031, and MEI0034; Burkard Manufacturing Ltd.). The samplers were preset for an automatic change of the trapping vessel (1.5-ml microfuge tube) each day at a preset time. The tubes within each sampler (one tube for each day) were replaced weekly. The samplers were operated for 12 h in each day between 5 A.M. and 5 P.M. 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 were 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). The remaining 10-μl suspension of each sample was examined in the laboratory under bright-field microscopy (×400) for the presence of *A. brassicae* conidia. Throughout the sampling period, Brassica leaf spot infection periods were monitored (Kennedy and Graham 1995; Wakeham and 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

Monoclonal antibody selection. Five hybridoma cell lines were identified by PTA-ELISA as producing antibodies with recognition sites to *A. brassicae* antigen. Three of the cell lines emanated from mice immunized 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 immunized 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* spp. was observed (Fig. 1). Although, with a 10-fold difference observed between *A. brassicae* and the other the *Alternaria* spp. tested, this was at a low level. Of those species tested outside of the genus, little or no reactivity with EMA 212 was observed.

Monoclonal antibody characterization. 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 to 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 (Tables 3 and 4). This would suggest that the antibody recognized both carbohydrate and protein moieties of a glycoprotein. 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 the 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 counterintuitive (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, approximately 50 *A. brassicae* spores per 100 μ l of sample volume are required for depletion of the test line. Test sensitivity was increased approximately fivefold 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., tested negative for *A. brassicae*).

Preliminary field trial study. During the 3-week monitoring period, an overwintered crop of Brussels sprouts inoculated with *A. brassicae*, five 12-h-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 six 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 10 days designated moderate risk (infection score 100 to 150) and 15 days designated high risk (infection score >151). Infection scores <100 were interpreted as low risk. With the weather-driven model for each week of the 6-week observation period, at least at least one daily *A. brassicae* infection risk period was identified (Fig. 5). In contrast, the CLFD assay performed daily on collected air samples detected *A. brassicae* on only 3 days (13, 14, and 23 August) 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 tagged Brussels sprout plants prior to the start of the experiment (3 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 6-week period (Fig. 7). The second phase of dark leaf spot symptom expression was recorded on the tagged plants after 26 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 <2 (EVL test reading at 1.6 for 9 to 10 September). For this period, *A. brassicae* conidia were identified by bright-field microscopy within the collected air sample (5 conidia/m³ of air sampled). Ten days later, dark leaf spot establishment (at a low level) was observed within nonfungicide-treated areas of the cauliflower crop.

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

Table 2. Absorbance values from plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) tests with heat-treated *Alternaria brassicae* antigens by using monoclonal antibody EMA 212

Time (min)	Temperature (°C)	PTA-ELISA absorbance (450 nm) ^a	
		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; n/a = not available.

Discussion

In this study, two preparation types of *A. brassicae* conidia were used for the production of antibody-producing hybridoma cell lines from female Balb C mice. IgM and IgG subclasses were produced by *A. brassicae*-positive cells lines regardless 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 (LF), 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, CLFD are often preferred because 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 an LF assay can be very different from that in ELISA, where long incubation times and lower surface concentration exist. For LF, an antibody with high affinity is required (O’Farrell 2013). Typically, IgM antibodies are considered to be of lower affinity than that of IgG preparations (Makela et al. 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 because 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 LF provides limited time for interaction between the antibody and analyte. A time of 1 and 6 s for binding has been reported. However, 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 ending after the conjugate passes the test line. This time is typically on the order of 10 to 20 s (Brown 2008). Malmberg et al. (1992) reported by surface plasmon resonance that IgM anti-Tn α antibodies showed one order of magnitude higher association rate constants, as compared with the IgG antibodies. Also, IgM multivalency (10 binding sites,

compared with 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 LF development. The ELISA format lends itself to high-throughput screening of multiple antigens and antibodies and provides a quick primary screen. Because the fungal structure is complex, variation in antigen concentration and type is likely to occur between species. Plant pathology articles often report fungal plate washings and adjustment by dilution

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

Table 3. Absorbance values from plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) tests with periodate-treated *Alternaria brassicae* antigens by using monoclonal antibody EMA 212

Time (h)	Temperature (°C)	PTA-ELISA absorbance (450 nm) ^a			Reduction (%)
		Periodate	Control		
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 plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) tests with protease-treated *Alternaria brassicae* antigens by using monoclonal antibody EMA 212

Time (h)	Temperature (°C)	PTA-ELISA absorbance (450 nm) ^a			Reduction (%)
		Protease	Control		
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.

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

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). Using spore concentration as a normalization factor provides a better measure when relating to the field situation (spores per cubic meter of air sampled). In the development of monoclonal antibodies for the detection of *Puccinia striiformis* urediniospores,

Skottrup et al. (2007) reported 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* spp. when tested by PTA-ELISA. However, 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 (Sutula

Approximate numbers of *A. brassicae* conidia applied in extraction buffer to a competitive lateral flow device

Fig. 4. Assessment by optical densitometry of competitive immunochromatographic lateral flow device assay strips for measurement of *Alternaria brassicae* (serial doubling dilution series of *A. brassicae* conidial spore concentration of 6,000 to approximately 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 (Ordnance Survey National Grid reference TF509615).

Number of *Alternaria brassicae* conidia m^3 air sampled in a Brussels sprout crop

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* conidia.

Fig. 7. Dark leaf spot disease development in two U.K. brassica commercial crops: Brussels sprouts (□) Lincolnshire (Ordnance Survey National Grid [OS] reference TF509615) and cauliflower (♦) Lancashire (OS reference SD430235) during 2005.

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* spp. tested, with a 10-fold difference by PTA-ELISA. No significant reactivity was observed for 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 *M. brassicicola* (ringspot) and *Pyrenopeziza brassicae* (light leaf spot). However, it is 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, with reactivity to the two types of *A. brassicae* antigen preparations used. Both isotypes are frequently reported in the development of monoclonal antibodies for fungal diagnostic assays (Skotttrup et al. 2007; Werres and Steffens 1994) and found to be 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. However, for widespread commercial application, the test may require refinement to reflect potential reactivity of the diagnostic probe with other spore types. The development of disruptive technologies over recent years provides application of DNA aptamers, molecular beacons, and quantum-dot LF 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. 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 at which infection occurs, cultivar, environmental conditions, and inoculum concentration are limiting factors for *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 days after inoculation (Duhan and Suhag 1990).

Risk assessments 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 (for example, on Brussels sprout buttons). In vegetable crops, the use of F₁ 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 because 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 may provide 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 *P. infestans* (Fall et al. 2015). In horticultural crops, there is considerable interaction between transplanting date and spatial location. Humpherson-Jones and Maude (1982) reported wind transport of *A. brassicae* conidia of up to 1.8 km. The epidemiology of *A. brassicae* on local horticultural brassica crops may relate to harvesting of *B. napus* (Skjth et al. 2012). Directly measuring airborne inoculum could improve the estimation of risk resulting from the interaction of arable and horticultural brassica crops. However, 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, in many cases, detecting very low numbers of spores may not be necessary. Studies for the ascosporic fungi *Pyrenopeziza brassicae* and *M. 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). Therefore, nonmolecular methods are adequate and can provide a low-cost approach when compared with the use of molecular methods (Shan 2011). It should be noted that considerable advances have been made toward the deployment of DNA-based systems to the field and in reducing analysis cost (Thiessen et al. 2015). However, because immunochromatographic devices do not require a preextraction or a DNA amplification stage, they have considerable advantages in both 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 in a CLFD reading system (Sangdaee 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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