

# plant disease

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

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

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

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

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3 41 immunochromatographic test strips are commercially available for the detection of viral, bacterial  
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5 42 and fungal plant pathogens (Danks and Barker 2000). These types of tests have been developed to  
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8 43 diagnose the presence or absence of individual pathogens on plants expressing disease symptoms.  
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13 45 There is the potential to apply this technology as an early warning system for the occurrence of  
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15 46 inoculum in air samples. This development would require a system to collect and concentrate  
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17 47 inoculum into a suitable sample vessel for further testing. Immunoassays have been developed to  
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19 48 quantify airborne inoculum captured by passive deposition on to plant surfaces (Jamaux and Spire  
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21 49 1994) or impaction using rotor rods (Schmechel et al. 1996). Alternatively, an MTIST (microtitre  
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23 50 immunospore air sampler, Burkard Manufacturing, Rickmansworth, UK) provides collection of air  
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25 51 samples by impaction across 32 microtitre wells (Kennedy et al. 2000). Quantification of trapped  
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27 52 particulates can then be subjected to enzyme-linked immunosorbent assay (ELISA) and with  
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29 53 suitable antibodies multiplex assays can be devised (Wakeham et al. 2004). Most of these tests  
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31 54 require laboratory processing facilities. A multi vial cyclone air sampler (Burkard Manufacturing  
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33 55 Ltd) which collects air samples in successive into 8 x 1.5ml collection vessels at pre-determined  
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35 56 time periods has been considered for use in PCR based methods (West et al 2008; West and  
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37 57 Kimber 2015). In contrast harnessing this sampling technology with immunochromatographic test  
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39 58 strips provides an opportunity to measure target inoculum in-situ at low cost by the end user e.g. a  
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41 59 grower or consultant.  
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51 61 The combined use of these two technologies (air sampling and immunological processing) could  
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53 62 prove a powerful tool across a range of disciplines. The predictive power of this approach could be  
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55 63 improved further if used in conjunction with meteorological models (Jones and Harrison 2004;  
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57 64 Zinc et al. 2012). For example, in horticulture reduced applications of fungicides has been  
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4 65 evaluated using a weather driven forecast model to predict risk of *Albugo candida* sporulation and  
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6 66 infection periods (Minchinton et al. 2013). Combining this approach with concentration of  
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8 67 inoculum in air sample could further improve the predictive outcome of the test and subsequent  
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10 68 control of the disease.  
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15 70 This study reports on the development and evaluation of an immunochromatographic test device  
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17 71 (CLFD) for risk assessment of the fungal plant pathogen *Alternaria brassicae* (dark leaf spot) in  
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19 72 air samples. *A. brassicae*, causes dark leaf and pod spot on Brassica spp. and is of worldwide  
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21 73 occurrence (Hong et al. 1996; Humpherson-Jones, 1992). A necrotroph, which on host tissue  
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23 74 produces asexual spores (conidia) for wind dispersal (McCartney et al 1998). Infection of crops by  
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25 75 *A. brassicae* can result in severe yield losses (Parada et al. 2008). The relationship between  
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27 76 important meteorological parameters affecting infection and sporulation of *A. brassicae* have been  
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29 77 incorporated into a disease forecast model (Kennedy and Graham, 1995). Integration of this  
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31 78 information with an in-field detection system (CLFD) for *A. brassicae* in air is evaluated for  
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33 79 improved control of dark leaf spot in *Brassica oleracea*.  
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## 45 82 MATERIALS AND METHODS

46 83 **Monoclonal antibody production.** Nine *Alternaria brassicae* isolates (Warwick HRI, University  
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48 84 of Warwick, UK (Maude and Humpherson-Jones 1980)) were cultured on V8 juice agar (3g  
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50 85 technical agar, 0.4g calcium carbonate, 20ml V8 juice (Campbell Soup Company, Camden, N.J.)  
51  
52 86 for one week in darkness at 25° C. A 5cm square of mycelium from each inoculated plate was then  
53  
54 87 removed, homogenised in 5 ml of sterile distilled water (SDW) and transferred in 500µl aliquots to  
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56 88 10 x 5ml sterile clarified V8 juice medium (Johnston and Booth 1983). The V8 mycelial  
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3 89 suspensions were mixed and then incubated in darkness at 25° C. At a magnification of 100x,  
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5 90 conidial production was **observed** seven days later. Using the method described by Lawrie (2002),  
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7 91 conidia of *A. brassicae* were collected from liquid culture and suspended in phosphate buffered  
8  
9 92 saline (PBS, pH 7.2) **at a** concentration of 10<sup>5</sup> conidia ml<sup>-1</sup>. Using a Fast Prep device (Qbiogene  
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11 93 **FP120, Anachem Ltd, Luton, UK**) the conidia were mechanically disrupted according to the  
12  
13 94 manufacturer's guidelines (3 x 25 seconds at a speed setting of 5.5) and then aliquoted in to 50µl  
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15 95 lots; this preparation was labelled 'antigen-A'. The **fungal culturing** procedure was repeated and *A.*  
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17 96 *brassicae* conidia were suspended in a 0.1 % glucose SDW solution. Following agitation for 1  
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19 97 hour on a wrist action shaker, the conidial suspension was sprayed directly on to healthy Brussels  
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21 98 sprout plants (*Brassica oleracea* var. *gemmifera*) c.v. Golfer. Inoculated plants were exposed to a  
22  
23 99 relative humidity of 100% for 48 hours and thereafter retained in a **greenhouse held at 18 °C**. Two  
24  
25 100 weeks after inoculation *A. brassicae* conidia were **observed on lesions**. **Leaf sections bearing**  
26  
27 101 **sporulating lesions** were detached and agitated in PBS for a period of 30 minutes. **Leaf material**  
28  
29 102 was removed by filtering the suspension through a membrane of 97µm pore size. Conidia of *A.*  
30  
31 103 *brassicae* were recovered on a 37µm pore size membrane and, resuspended in 5ml PBS (10<sup>5</sup>  
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33 104 conidia ml<sup>-1</sup>). Following mechanical disruption, as described above, the *A. brassicae* suspension  
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35 105 was aliquoted in to 50µl lots; **this preparation was labelled 'antigen-B'**. **Three female Balb C/Cj**  
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37 106 **substrain mice were each immunised for induction of antibody secreting spleen cells (Kohler and**  
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39 107 **Milstein, 1976) with 50µl of antigen-A mixed with an equal volume of Titermax adjuvant (Sigma-**  
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41 108 **Aldrich T-2684). Three additional mice were injected with 50µl of antigen-B. The same mice were**  
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43 109 **immunized on two further occasions at 14-day intervals without adjuvant.**  
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55 111 **Collected tail bleeds (Kohler and Milstein, 1975) were titrated against their respective homologous**  
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57 112 **antigen preparation by plate trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA)**  
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3 113 (Kemeny 1991). For each tail bleed, 10 paired wells of a 96 well Nunc Immunosorbent Polysorp  
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6 114 flat-bottomed microtitre plate (model no. 475094, Life Technologies, Paisley, Scotland) were  
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8 115 coated with  $100\mu\text{l}^{-1}$  well of the homologous antigen type in PBS. Ten paired wells received  $100\mu\text{l}^{-1}$   
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10 116  $^{-1}$  well of PBS alone for each tail bleed as a control. After overnight incubation at  $18^{\circ}\text{C}$ , unbound  
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12 117 antigen was removed by inverting the individual microtitre plates and tapping them down on to  
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14 118 absorbent towelling. The plates received four one-min washes of  $200\mu\text{l}^{-1}$  well PBS. Wells were  
15  
16 119 blocked with  $200\mu\text{l}$  3% casein buffer (3%[wt/vol] casein in PBS) and incubated in a Wellwarm  
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18 120 shaker incubator (model no. W1031B, Denley Instruments Ltd., Sussex, UK) for 30 mins at  $30^{\circ}\text{C}$ .  
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20 121 Residual blocking buffer was removed and wells washed four times for one min each with  $200\mu\text{l}^{-1}$   
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22 122 well PBS 0.05% Tween 20 (PBST). Mice tail bleeds were diluted 1:100 in PBST and doubling  
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24 123 dilutions made to 1:25600. A diluted tail bleed was applied ( $100\mu\text{l}^{-1}$  well) respectively to  
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26 124 homologous antigen coated paired wells. The remaining homologous antigen paired well of each  
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28 125 10 paired well set received PBST alone. This process was repeated but with microtitre wells which  
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30 126 had not been coated with *A. brassicae* antigen. After incubation in a Wellwarm 1 shaker incubator  
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32 127 as previously described unbound material was removed and wells were washed as previously  
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34 128 described four times for one min with PBST. Aliquots of  $100\mu\text{l}$  of anti-mouse IgG (whole  
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36 129 molecule) biotinylated antibody produced in goat (model no. B7264, Sigma-Aldrich  
37  
38 130 Company Ltd, London, UK) diluted 1 in 500 PBST, were added to each well and incubated for 45  
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40 131 min. as above. After washing as previous,  $100\mu\text{l}^{-1}$  well of Streptavidin peroxidase (model no.  
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42 132 SS512, Sigma-Aldrich Company) diluted 1:10000 PBST was added to each well and incubated as  
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44 133 above for 45 min. The microtitre wells were washed as previously described and each well  
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46 134 received  $100\mu\text{l}$  of 3,3',5,5'-tetramethylbenzidine substrate (model no. T-3405 and P-4922, Sigma-  
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48 135 Aldrich). The reaction was stopped by adding  $25\mu\text{l}$  of  $2\text{M H}_2\text{SO}_4$ . Absorbance was recorded at  
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50 136 450nm in an HT11 (Anthos Labtec Instruments, Salzburg, Austria) ELISA plate reader.  
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6 138 Mice with high titre tail bleeds (end point > 1:32000) to *A. brassicae* were identified. Following a  
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8 139 final pre-fusion boost, the spleen was removed four days later. Spleen cell fusions were carried out  
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10 140 according to a modified protocol (Kennett et al. 1978) with cell hybrids fed on days 3, 6, and 10.  
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12 141 By PTA-ELISA, the cell culture supernatants were screened 14 days after cell fusion to the  
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14 142 homologous antigen preparation as previously described.  
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20 144 Hybridoma cell lines identified as producing antibodies positive to *A. brassicae* antigen were  
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22 145 screened for reactivity to other fungal species (Table 1) by PTA-ELISA. For this process, fungal  
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24 146 plate washings were prepared on Supor 450 Membrane filter (Model no. HPWP09050;  
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27 147 MERKMillipore, Darmstadt, Germany) covered inoculated agar plates (Wakeham et al. 1997).  
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29 148 Spore suspensions of each fungal isolate were collected and adjusted to  $10^5$  spores  $\text{ml}^{-1}$  (Skottrup  
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31 149 et al. 2007). The collected spores were mechanically disrupted using a Fast Prep device as  
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34 150 described previously. By centrifugation for 5 minutes at 13,000g the particulate fraction was  
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36 151 removed and the soluble spore fraction was retained for PTA-ELISA. Paired wells of a 96 well  
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39 152 Nunc Immunosorbent Polysorp flat-bottomed microtitre plate (Life Technologies, Paisley,  
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41 153 Scotland; model. no. 475094 A) were coated with  $100\mu\text{l}^{-1}$  well of each fungal washing (Dewey et  
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43 154 al. 1989). Unbound antigen was removed after overnight incubation at  $18^\circ\text{C}$  (Wakeham et al.  
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46 155 1997) and the PTA ELISA process carried out as previously described with an *A. brassicae*  
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48 156 positive cell culture supernatant. The process was repeated twice for each of the *A. brassicae*  
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50 157 positive cell culture supernatants.  
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55 159 The immunoglobulin class produced by each *Alternaria* positive cell line was determined using an  
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57 160 Isostrip mouse monoclonal isotyping kit (model no. 11-493-027 001, Roche Diagnostics, Burgess



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3 161 Hill, West Sussex, UK). One of the cell lines (coded EMA 212) was selected and the tissue culture  
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5 162 supernatant purified using a High Trap™ IgM purification HP column according to the  
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8 163 manufactures instructions (model no. 17-5110-01, GE Healthcare Little, Chalfont). The effects of  
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10 164 antigen modification with protease and periodate on antibody binding (EMA 212) was determined  
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12  
13 165 by the method of Bossi and Dewey (1992). To determine antigen site expression, *A.brassicae*  
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15 166 conidia (Maude and Humpherson-Jones, 1980) were collected using the method described by  
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17 167 Lawrie (2002). Conidia were germinated in 0.01% glucose solution on multiwell glass slides  
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20 168 (model no. MIC3412, Scientific laboratory supplies, Nottingham, UK) and by  
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22 169 immunofluorescence probed with antibodies produced by EMA 212 cell line (Kennedy et al.  
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24 170 1999b).

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29 172 **Competitive immunochromatographic test device.** Immunochromatographic test strips  
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31 173 consisted of a carrier material containing dry reagents that are activated by applying a liquid  
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33 174 sample. Movement of this liquid allows passage across various zones (test framework) where  
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35 175 molecules are attached and exert specific interactions with target analytes. Results are generated  
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37 176 within 5 – 10 minutes by the formation of a control and test lines as appropriate to the sample and  
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39 177 the test type. The development of a control line provides confirmation that the test is valid. In a  
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41 178 competitive format (CLFD) the test line result is counter intuitive i.e. as the target analyte in a  
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43 179 sample increases test line colour intensity decreases. At a high concentration of target analyte no  
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45 180 test line is visible and a positive result is recorded. The test can be made semi-quantitative with the  
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47 181 use of a reader (Wong and Tse 2009).

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53 183 The test framework was constructed using a Millipore 135 HiFlow™ cellulose ester membrane  
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55 184 direct cast on to a Mylar backing (model no. SHF2400225, Millipore Corp., Bedford, MA.)  
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3 185 attached at either end to an absorbent pad (model no. GBOO4, Schleicher and Schuell, Dassel,  
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5 186 Germany) and a sample pad (model no. T5NM, Millipore Corp., Bedford, MA.). A control line of  
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8 187 a non-immune anti-mouse serum and a test line of *A. brassicae* (antigen-A), each adjusted to  
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10 188 0.5mg ml<sup>-1</sup> in PBS were independently applied to the cellulose ester membrane surface using a  
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12 189 flat-bed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK) operating at a line  
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15 190 travel speed of 50m s<sup>-1</sup>. The sprayed membranes were air dried overnight at room temperature (18  
16  
17 191 to 20°C) and cut in to 5 mm strips. A 500µl volume of EMA 212 (1mg ml<sup>-1</sup>) was mixed with  
18  
19 192 375µl of a goat anti-mouse IgM 40nm gold conjugate (Code BA GAMM 40, British Biocell,  
20  
21 193 International, Cardiff, UK) made to 2 ml in PBS and incubated on a roller incubator for 3 hours.  
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23 194 EMA 212 antibody bound gold particles were then collected by centrifugation (4000 xg) and  
24  
25 195 resuspended to a final volume of 1.625ml in application buffer (20mM sodium phosphate buffer,  
26  
27 196 100mM sodium chloride, 0.25% trehalose, 0.1% sucrose, pH 7.2). Sixty µl of the EMA 212  
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29 197 antibody gold conjugate solution was then pipetted on to individual CLFD sample pads and air  
30  
31 198 dried at 37°C for 30 min. Each pad was attached to the CLFD test strip. The CLFDs were mounted  
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33 199 within a plastic housing device (model no. SH 003, European Veterinary Laboratory, Woerden,  
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39 200 Netherlands).

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43 202 *Alternaria brassicae* conidia (Warwick HRI, University of Warwick; AA3, AA4 isolated from  
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45 203 Brussels sprouts and AA10-1M isolated from Khol rabi) were produced in planta as described  
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47 204 above (antigen-B) and collected in extraction buffer (0.05M Tris HCL, 0.15M NaCl, 0.4% Triton  
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49 205 X100, 0.2% Tween 20, 0.2% BSA, 0.12% Geropan). For each isolate, a doubling dilution series  
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51 206 was made to provide 100µl aliquots of *A. brassicae* ranging from 6x10<sup>4</sup> conidia per aliquot.  
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53 207 Aliquots of each conidial suspension were applied drop wise to the sample pads of individual  
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55 208 CLFD. A negative control of three CLFD received extraction buffer alone. After an assay time of  
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3 209 6 min the development of test and control lines were recorded visually by eye and then by optical  
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5 210 densitometry using a laboratory-based Biodot Quadscan device (BioDot Ltd, Chichester, Sussex,  
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8 211 UK).

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12 213 The fungal species tested previously by PTA-ELISA were grown in sterile culture as described  
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15 214 previously but were collected in CLFD extraction buffer. A culture of *M. brassicicola* (single-spore  
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17 215 isolate CH195001) was maintained on sprout decoction agar with illuminated low intensity sub-stage  
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19  
20 216 fluorescent lighting for production of pseudothecia (Kennedy et al. 1999b). Ascospores were  
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22 217 identified on the underside of each Petri dish lid (bright field microscopy 100x) and removed in  
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24  
25 218 CLFD buffer by gentle agitation with a glass spreader (Kennedy et al. 1999b). Similarly,  
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27 219 *Pyrenopeziza brassicae* mating types MAT-1 and MAT-2 (Foster et al., 2002) were cultivated on  
28  
29 220 compost agar for apothecial development and the production of ascospores (Gilles et al., 2001). For  
30  
31 221 each fungal species spore concentrations were adjusted to  $< 10^5$  ml<sup>-1</sup> with CLFD buffer. A 100µl  
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33 222 aliquot of each fungal sample was applied drop wise to the sample pad of individual *Alternaria*  
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36 223 CLFD. After an assay time of 6 min data the CLFD was recorded visually for test line development  
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39 224 and using a Quadscan reader.

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43 226 **Preliminary field trial study.** For a 3-week period an over-wintered, heavily infected (dark leaf  
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45 227 spot, ringspot and white blister) field plot (20 m x 10 m) of Brussels sprouts (c.v. Golfer) was  
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47 228 monitored continuously, for the presence of *Alternaria brassicae* spores in the air, using a Burkard  
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49 229 cyclone sampler (model no. MEI0073, Burkard Manufacturing, Rickmansworth, Hertfordshire,  
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51 230 U.K.) at a site in Warwickshire, U.K. (OS grid reference SP278552). After each sampling period (1  
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54 231 or 3 days) the 1.5 ml sample collection vessel from the cyclone spore trap was removed and taken to  
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57 232 the laboratory where 110µl volume of extraction buffer was then added. The contents of the  
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3 233 collection vessel were gently mixed using a disposable micro pipette (model no. 50504NU, Alpha  
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6 234 Lab Ltd, Eastleigh, UK) and a 100 µl aliquot transferred to a sample pad of an *Alternaria* CLFD  
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8 235 with assay time and data collection as described above. The remaining 10µl of extraction buffer was  
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10 236 removed from the sample collection vessel and examined using a microscope for the presence of *A.*  
11  
12 237 *brassicae* conidia (expressed m<sup>3-1</sup> of air sampled). The risk of *A. brassicae* infection for each of the  
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14 238 field sampling periods was assessed by placing six healthy, greenhouse-grown *B. oleracea* trap  
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16 239 plants (c.v. Golfer, 10 true leaves) in the field plot adjacent to the spore trap. After each air sampling  
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18 240 period trap plants were removed and placed in an isolated environment of 100% humidity for 48 hrs  
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20 241 at 16° C to fulfil the environmental requirements for infection by *A. brassicae* (Kennedy et al.  
21  
22 242 1999a). Additionally, six healthy, greenhouse-grown *B. oleracea* plants (which were not exposed to  
23  
24 243 the field) were included as controls. The plants were then air-dried and retained in a glasshouse at a  
25  
26 244 temperature of 12 to 14°C for 21 days. Plants were visually examined for the expression of dark leaf  
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28 245 spot on leaves. Confirmatory isolations from these lesions were recorded on sprout leaf decoction  
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30 246 agar to confirm the presence of *A. brassicae* (Kennedy et al. 1999b).  
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39 248 **Field risk assessment studies.** In conjunction with a weather driven infection model (Kennedy and  
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41 249 Graham, 1995; Wakeham and Kennedy, 2010) *A. brassicae* CLFD assays were evaluated in  
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43 250 commercial fields of Brussel sprouts (OS grid reference TF509615, Lincolnshire, UK) and  
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45 251 cauliflower (OS grid reference SD430235, Lancashire, UK). Within the fields, three trial plots (15  
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47 252 x 15 m) were marked. No fungicide application or treatments were made to the crop in these areas.  
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49 253 During a six week period, daily air samples were collected using a Burkard multi-vial cyclone  
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51 254 sampler adapted for field use (model no. ME10029; ME10031; ME10034, Burkard Manufacturing,  
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53 255 Rickmansworth, Hertfordshire, U.K.). The samplers were pre-set for an automatic change of the  
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55 256 trapping vessel (1.5 ml microfuge tube) each day at a pre-set time. The tubes within each sampler  
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3 257 (one tube for each day) were replaced weekly. The samplers were operated for 12 hours in each day  
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5  
6 258 between 5 am and 5pm to match the diurnal periodicity of *A. brassicae* conidial dispersal (Kennedy  
7  
8 259 et al. 1999a). At collection, a 110 µl volume of extraction buffer was added to each of the tubes and  
9  
10 260 the contents mixed as previously described. A 100 µl aliquot was transferred from each tube to a  
11  
12 261 sample pad of an *A. brassicae* CLFD. After 6 min, data on the CLFD were observed visually and  
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15 262 with a field portable one-step digital immunochromatographic test strip reader (European Veterinary  
16  
17 263 Laboratory, Woerden, Netherlands). The remaining 10 µl suspension of each sample was examined  
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19  
20 264 in the laboratory under bright field microscopy (x 400) for the presence of *A. brassicae* conidia.  
21  
22 265 Throughout the sampling period, Brassica leaf spot infection periods were monitored (Kennedy &  
23  
24 266 Graham 1995; Wakeham & Kennedy 2010). For this purpose, canopy positioned wetness sensors  
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26  
27 267 and temperature and humidity environmental conditions were monitored with a SKYE Datahog II  
28  
29 268 weather station (Skye Instruments Ltd, Powys, UK) at intervals of 30 min. Throughout the trial  
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32 269 period, visual dark leaf spot disease assessments were taken in the unsprayed plots of each crop.  
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34 270 Leaves of 10 plants in each plot were tagged and numbered. Recordings were made weekly and  
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36 271 isolations were taken from identified dark leaf spot lesions as previously described.  
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## 274 RESULTS

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

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3 281 preparation A) provided an antibody type which could be used to detect conidial preparations of *A.*  
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5 282 *brassicae* in a CLFD format (data not presented). When this hybridoma cell line was assessed by  
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8 283 PTA-ELISA some reactivity to other *Alternaria* species was observed (Fig 1). Although, with a 10  
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10 284 fold difference observed between *A. brassicae* and the other the *Alternaria* species tested this was  
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12 285 at a low level. Of those species tested outside of the genus little or no reactivity with EMA 212  
13  
14 286 was observed.  
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20 288 **MAb characterisation.** Effects of heat treatment, chemical and enzymatic effect of periodate  
21  
22 289 oxidation and protease digestion on antigen binding by the antibody type produced by hybridoma  
23  
24 290 cell line EMA212 are shown in Tables 2-4. Antibody binding was not affected by heat treatment of  
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26  
27 291 the *A. brassicae* conidial antigen (Table 2). Both periodate and protease reduced antibody binding  
28  
29 292 at each of the temperatures and time periods tested (Table 3,4). This would suggest that the  
30  
31 293 antibody recognised both carbohydrate and protein moieties of a glycoprotein. As heat treatment of  
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33  
34 294 the protein did not diminish the reactivity by the monoclonal antibody this would suggest linear  
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36 295 epitopes in the protein molecule. Differential sensitivity to chemical and enzymatic modification  
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39 296 illustrates the need to study a range of incubation periods and temperatures. The antibody  
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41 297 produced by EMA 212 cell line was observed by immunofluorescence to bind to an epitope  
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43 298 associated with the germ tube of the conidium which dissipated with mycelial growth (Fig. 2).  
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48 300 **Competitive immunochromatographic test (CLFD).** Using a monoclonal antiserum (produced  
49  
50 301 by hybridoma cell line EMA 212), an immunochromatographic assay was developed to provide a  
51  
52 302 visual indicator of *A. brassicae* presence within an air sample. This is a competitive assay and is  
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54  
55 303 counter intuitive i.e. test line depletion is dependent on increasing *A. brassicae* concentration (Fig.  
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57 304 3). The test is recorded positive for *A. brassicae* when no test line is observed by visual eye  
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3 305 observation. With an assay time of 6 min. approx. 50 *A. brassicae* spores 100  $\mu\text{l}^{-1}$  sample volume  
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5 306 are required for depletion of the test line. Test sensitivity was increased approx. five-fold with a  
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8 307 laboratory based optical density (OD) reader (Fig. 4) and provided potential for quantitative  
9  
10 308 measurement of *A. brassicae* in air samples. When other fungal species were tested with the *A.*  
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12 309 *brassicae* CLFD a visible test line was evident and OD test line values for each recorded  $> 5.0$  i.e.  
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14 310 test negative for *A. brassicae*.  
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20 312 **Preliminary field trial study.** During the 3 week monitoring period an over-wintered crop of  
21  
22 313 Brussels sprouts inoculated with *A. brassicae*, five 12 hour day periods of air sampling were  
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24 314 identified by the CLFD-assay as positive for conidia of *A. brassicae*. For each of these periods, no  
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26 315 visible CLFD test line was recorded and the OD test line values recorded by the Quadsan reader  
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28 316 gave a value of  $< 2$  OD. On each of these dates the total numbers of dark leaf spot lesions on the 6  
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30 317 trap plants exceeded 13. For all other sampling periods, all trap plants were negative for dark leaf  
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32 318 spot lesions (*A. brassicae*).  
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39 320 **Field risk assessment studies.** Daily *A. brassicae* infection score ratings over the 6-week  
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41 321 observation period resulted in ten days designated 'moderate risk' (infection score 100 to 150) and  
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43 322 15 days as 'high risk' (infection score  $>151$ ). Infection scores  $< 100$  were interpreted as 'low risk'.  
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45 323 With the weather-driven model for each week of the six week observation period, at least at least one  
46  
47 324 daily *A. brassicae* infection risk period was identified (Figure 5). In contrast, CLFD assay performed  
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49 325 daily on collected air samples detected *A. brassicae* on only three days (13<sup>th</sup>, 14<sup>th</sup> and 23<sup>rd</sup> Aug) by  
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51 326 both visual and field-portable strip reader. Microscopic examination of each daily collected field air  
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53 327 sample showed a correlation ( $r^2=0.8081$ ) with the number of *A. brassicae* conidia trapped and the  
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55 328 corresponding field portable EVL immunochromatographic test strip reading (Fig. 6).  
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6 330 For the three dates where the CLFD test determined *A. brassicae* to be at an air sample threshold risk  
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8 331 in the crop, the environmental model recorded a moderate or high risk of *A. brassicae* infection.  
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10 332 Evaluation of the Brussels sprout tagged plants prior to the start of the experiment (3rd August)  
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12 333 revealed a level of dark leaf spot incidence already within the crop (10 lesions plant<sup>-1</sup>). Thereafter  
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15 334 two distinct phases of dark leaf spot development occurred on the tagged Brussels sprout plants over  
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17 335 the six week period (Fig. 7). The second phase of dark leaf spot symptom expression was recorded  
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19 336 on the tagged plants after the 26<sup>th</sup> August.  
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24 338 At the second commercial site (cauliflower crop) the weather driven model identified 16 days when  
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26 339 the crop was at risk of *A. brassicae* infection. However, visual assessment of the CLFD assay  
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28 340 indicated that *A. brassicae* inoculum was either absent or at a low level. Using the portable in-field  
29  
30 341 reader, a single CLFD assay showed a digital reading below 2 (EVL test reading at 1.6 for the 9-10<sup>th</sup>  
31  
32 342 September). For this period, *A. brassicae* conidia were identified by bright field microscopy within  
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34 343 the collected air sample (5 conidia m<sup>-3</sup> air sampled). Ten days later dark leaf spot establishment (at a  
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36 344 low level), was observed within non-fungicide treated areas of the cauliflower crop (Fig. 8).  
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## 47 **DISCUSSION**

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50 349 **In this study two preparation types of *Alternaria brassicae* conidia were used for the production of**  
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52 **antibody producing hybridoma cell lines from female Balb C mice. IgM and IgG subclass were**  
53 350 **produced by *A. brassicae* positive cells lines irrespective of the growth medium used for conidial**  
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55 351 **antigen (V8 juice agar culture (antigen A) or Brussels sprout plants (antigen B)). When tested by**  
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3 353 ELISA or lateral flow, none of the *A. brassicae* positive cell lines produced antibodies which  
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5 354 could be used in a double antibody sandwich (DAS) (data not supplied). A DAS assay requires  
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8 355 target analyte (antigen) to bind simultaneously to both the reporter and an immobilized ‘capture’  
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10 356 antibody. This cannot be accomplished with small analyte molecules that may have a single  
11  
12 357 antigenic determinant. Additionally, steric hindrance may prevent simultaneous binding. When the  
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14 358 target analyte consists of a small molecule, CLFDs are often preferred as they do not require an  
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16 359 analyte to provide the linkage between the capture and reporter antibody (Qian and Bau, 2004).  
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18 360 Similarly, this is the case with PTA-ELISA and the processes by which each of the cell lines were  
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20 361 initially screened for antibodies reactive to *A. brassicae* antigen.  
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27 363 Interestingly, of the *A. brassicae* positive hybridoma cell lines selected by this method only one  
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29 364 produced an antibody type (Antigen Type A, IgM producing cell line) which could be used within  
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31 365 a CLFD assay format. Antibody performance in a lateral flow assay can be very different to that in  
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33 366 ELISA where long incubation times and lower surface concentration exist. For lateral flow (LFD)  
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35 367 an antibody with high affinity is required (O’Farrell, 2013). Typically, IgM antibodies are  
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37 368 considered to be of lower affinity to that of IgG preparations (Makela, 1997). For this reason it was  
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39 369 surprising that the IgG cell lines did not prove suitable for use. However, high affinity may not be  
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41 370 the sole driver in this type of assay as affinity needs to be driven by a fast on-rate ( $k_{on}$  or  
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43 371 association rate constant). Unlike the ELISA, the test line zone of the LFD provides limited time  
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45 372 for interaction between the antibody and analyte. A time of 1 and 6s for binding has been reported.  
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47 373 Although a little more contact time exists between antibody and analyte from the conjugate pad  
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49 374 with the “effective” binding reaction starting with resolubilization of the conjugate and ends after  
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51 375 the conjugate passes the test line. This time is typically on the order of 10-20 s (Brown 2009).  
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53 376 Malmberg (1992) reported by surface plasmon resonance that IgM anti-Tn alpha antibodies  
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3 377 showed one order of magnitude higher association rate constants, as compared with the IgG  
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5 378 antibodies. Also, IgM multi-valency (10 binding sites compared to 2 of IgG) leads to a large effect  
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8 379 on the dissociation rate resulting in high binding avidity (King, 1998). The IgM antibody used in  
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10 380 this study (EMA 212 hybridoma cell line) provided clear test line development in the CLFD  
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12 381 format within 6 min. By eye a detection threshold of approximately 50 *A. brassicae* conidia was  
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15 382 achieved. Assessment using a portable reader (optical densitometry) improved test sensitivity.  
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20 384 Initial reactivity screening of the hybridoma cell lines to other spore types was carried out by PTA-  
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22 385 ELISA prior to lateral flow development. The ELISA format lends itself to high throughput  
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24 386 screening of multiple antigens and antibodies and provides a quick primary screen. As the fungal  
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27 387 structure is complex variation in antigen concentration and type is likely to occur between species.  
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29 388 Plant pathology papers often report fungal plate washings and adjustment by dilution or weight for  
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31 389 immunoreactivity studies rather than protein concentration (Dewey et al 1990, Bermingham et al.  
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33 390 1995, Kennedy et al. 2000, Meyer et al. 2000). For air sampling it is standard to relate pollen and  
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36 391 fungal spore number by enumeration (British Aerobiology Foundation, 1995). By using spore  
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39 392 concentration as a normalisation factor provides a better measure when relating to the field  
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41 393 situation (spores cubic metre<sup>-3</sup> air sampled). Skottrup (2007), reported in the development of  
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43 394 monoclonal antibodies for the detection of *Puccinia striiformis* urediniospores the adjustment of  
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45  
46 395 fungal spores for ELISA reactivity testing to 10<sup>5</sup> ml<sup>-1</sup> rather than by protein concentration.  
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50  
51 397 Using this methodology, the IgM antibody produced by cell line EMA 212 showed some reactivity  
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53 398 to other *Alternaria* species when tested by PTA-ELISA. Although, interpreting ELISA data to  
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55 399 establish useful thresholds for antibody specificity and sensitivity is arbitrary and dependent on the  
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58 400 test requirement. Frequently, positive thresholds used are multiples from two to four of the control  
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3 401 mean (Sutulu et al. 1986). With negative controls replicating the solution containing the antigen  
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5 402 (Dewey et al. 1997). Using these parameters, the PTA- ELISA test provided a good measure of  
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7 403 discrimination between *A. brassicae* and the other *Alternaria* species tested with a 10 fold  
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9 404 difference by PTA-ELISA. No significant reactivity was observed to the species tested outside of  
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11 405 the *Alternaria* genus. This was also observed when EMA 212 was incorporated within a CLFD  
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13 406 format and additional vegetable brassica pathogen tests included (*Mycosphaerella brassicicola*  
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15 407 (ringspot) and *Pyrenopeziza brassicae* (light leaf spot). It is however important to note the low  
16  
17 408 number of fungal species tested in this study. High throughput sequencing methods suggest that as  
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19 409 many as 5.1 million fungal species exist (Blackwell, 2011). A weakness of antibody reactivity  
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21 410 testing is the number of isolates that can be accessed and screened easily. Unlike PCR based  
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23 411 technologies there is no bioinformatics tool available to quickly and remotely screen hybridoma  
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25 412 culture supernatants for specificity to target organisms (Ye et al. 2012).  
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34 414 In this study, both IgG and IgM- producing hybridoma cell lines were selected and with reactivity to  
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36 415 the two types of *A. brassicae* antigen preparations used. Both isotypes are frequently reported on in  
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38 416 the development of monoclonal antibodies for fungal diagnostic assays (Werres and Steffens, 1994;  
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40 417 Skottrup et al 2007) and found equally cross-reactive to the fungal species tested (Dewey et al.  
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42 418 1989). The structurally complex nature of fungi, often with shared immunodominant antigens,  
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44 419 makes the development of species specific antibodies difficult (Drouhet, 1986; Notermans and  
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46 420 Soentoro, 1986; Priestley and Dewey, 1993). No value of improved specificity or sensitivity could  
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48 421 be drawn from the use of different *A. brassicae* conidial antigens (spores produced in culture or in  
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50 422 planta) for monoclonal antibody production.  
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3 425 Nevertheless, in combination with prediction of infection, the *A. brassicae* CLFD assay  
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6 426 demonstrated potential for 'in-field' risk assessment to identify periods when brassica crops were at  
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8 427 risk from *A. brassicae* infection. Although for widespread commercial application, the test may  
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10 428 require refinement to reflect potential reactivity of the diagnostic probe with other spore types. The  
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12 429 development of disruptive technologies over recent years provides application of DNA aptamers,  
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15 430 molecular beacons and quantum dot LFD assays which, if improved specificity is required, could  
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17 431 prove useful (Bruno 2014; Sajid et al. 2015; Wang et al. 2014).  
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21  
22 433 Developing accurate risk assessments for plant (and animal) diseases is an important area within  
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24 434 epidemiological research. Improving risk assessments for plant diseases in agricultural cropping  
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27 435 systems has been useful in the reduction of pesticide applications in the environment (Bugiani et al.  
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29 436 1995; Fry and Fohner 1985; Kennedy and Graham 1995; Wakeham and Kennedy 2010). However,  
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31 437 the use of direct detection and quantification of pathogenic inoculum in risk assessments for plant  
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34 438 diseases in agricultural production and biosecurity is not routine. One reason for the low usage of  
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36 439 direct detection systems has been the cost and speed at which results can be processed and obtained  
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39 440 by end users. Detection systems often rely on laboratory facilities in conjunction with specialist  
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41 441 knowledge. For these reasons infection risk (based on environmental conditions) is often used as a  
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43 442 risk criteria for controlling plant pathogens in agricultural production systems (Gilles et al. 2004;  
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45  
46 443 Magarey et al. 2005). However, although infection is an important part of the plant pathogens life  
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48 444 cycle it does not adequately estimate the real risk of the development of crop diseases. For example,  
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50 445 in the present study infection of *A. brassicae* based on temperature and wetness duration (two major  
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52 446 criteria) gave an infection risk on most days within a susceptible crop. By incorporating information  
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55 447 on inoculum availability (CLFD test) reduced the number of days when the crop was identified as at  
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3 448 risk of infection. The study demonstrates the potential to forecast disease risk in the field at a  
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6 449 relatively low cost.  
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10 451 Development of the disease in the field will depend on a number of factors. The plant growth stage  
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12 452 that infection occurs, cultivar, environmental conditions and inoculum concentration are limiting  
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14 453 factors of *A. brassicae* disease development on oilseed rape (Hong and Fitt 1995). In this study, *A.*  
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16 454 *brassicae* conidial concentrations were identified as being above a disease risk threshold on three  
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18 455 occasions in a Brussels sprout crop using on-site CLFD assays. After the first risk period had been  
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20 456 identified an increase in dark leaf spot symptoms occurred between 14 and 21 days later in the crop.  
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22 457 Thereafter increasing lesion development was observed in the crop. In a cauliflower crop, the disease  
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24 458 was first observed on plants 10 days after a CLFD assay predicted a risk of *A. brassicae*. Under  
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26 459 optimal conditions in a controlled glasshouse very small lesions on cauliflower were first observed 6  
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28 460 day after inoculation (Duhan and Suhag 1990).  
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36 462 Risk assessment which include direct measurement of numbers of plant pathogenic spores in the air  
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38 463 should prove useful in predicting the occurrence of pathogens in crops. This is particularly important  
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40 464 in systems where pathogen infection causes cosmetic damage and downgrades value by its  
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42 465 occurrence on for example Brussels sprout buttons. In vegetable crops the use of F<sub>1</sub> hybrids means  
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44 466 that pathogen occurrence is usually uniform within the production area. The occurrence of  
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46 467 pathogenic inoculum results from interactions with other crops in the area produced under a different  
47  
48 468 production schedule. For example, oilseed rape which *A. brassicae* infects, is often unsprayed and  
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50 469 grown in proximity to vegetable brassica production. Freshly transplanted summer vegetable  
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52 470 brassica crops are often produced adjacent to overwintered and unsprayed vegetable brassica crops.  
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54 471 Detecting inoculum will be useful as many of the approved fungicides which control *A. brassicae*  
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3 472 infection have systemic activity allowing good control even when applied after infection has  
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6 473 occurred.

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10 475 It has been reported that one or two peaks in sporangial concentration in the air of the potato blight  
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12 476 pathogen *Phytophthora infestans* preceded the first observed symptoms of the disease in the field  
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14  
15 477 (Bugiani et al. 1998). Similarly the occurrence of detected inoculum was shown before increased  
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17 478 disease in the field in the two cropping systems reported on in this study. Recently it has been shown  
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20 479 that spore sampling network devices maybe a suitable approach for early detection of incoming  
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22 480 inoculum (Skelsey et al. 2009). When combined with decision support systems this approach  
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24 481 represents a potential aid for targeting the optimal timing of disease control products against  
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27 482 *Phytophthora infestans* (Fall et al. 2015). In horticultural crops, there is considerable interaction  
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29 483 between transplanting date and spatial location. Humpherson-Jones (1982) reported wind transport  
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31 484 of *A. brassicae* conidia of up to 1.8km. The epidemiology of *A. brassicae* on local horticultural  
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34 485 brassica crops may relate to harvesting of *B. napus* (Skjoth et al. 2012). Directly measuring airborne  
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36 486 inoculum could improve the estimation of risk resulting from the interaction of arable and  
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39 487 horticultural brassica crops. Although, the number of air samplers and siting of these within a  
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41 488 locality requires further research (West and Kimber 2015).

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46 490 Immunochromatographic tests provide a suitable test format to detect and quantify inoculum 'in-situ'  
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48 491 (Kennedy and Wakeham, 2008; Thornton et al. 2004). The presence of relatively high spore  
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50 492 concentrations as a prerequisite for infection means that detecting very low numbers of spores in  
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53 493 many cases may not be necessary. Studies for the ascosporic fungi *Pyrenopeziza brassicae* and  
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55 494 *Mycosphaerella brassicicola* (Brassica light and dark leaf spot) have demonstrated that spore  
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58 495 inoculum occurs at high concentrations in the air before crop-to-crop transport is possible (Gilles et

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3 496 al. 2004; Wakeham and Kennedy, 2010). Non-molecular methods are therefore adequate and can  
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6 497 provide a low-cost approach when compared to the use of molecular methods (Shan 2011).

7  
8 498 Although, it should be noted that considerable advances have been made towards the deployment of  
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10 499 DNA based systems to the field and in reducing analysis cost (Thiessen et al. 2015). However, as  
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12 500 immunochromatographic devices do not require a pre-extraction or a DNA amplification stage they  
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14 501 have considerable advantages both in cost and simplicity of use. Also, the cross-contamination of  
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16 502 spores from collected field air samples are not likely to prove problematic as has been reported with  
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18 503 the deployment of molecular methodologies in the field (Reiger 2013).

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23 505 The improvement of immunochromatographic readers since the study was carried out also provides  
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25 506 the potential of smartphones for use as a CLFD reading system (Sangdae et al. 2013). The  
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27 507 synergistic use of these two technologies demonstrate the potential to help the agri-food industry to  
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29 508 assess and predict disease potential in a cost effective way.  
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43 514 CV35 9EF, UK. Kath Phelps (University of Warwick, HRI) provided advice on statistical analyses.  
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### 50 517 **LITERATURE CITED**

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15 806 **Fig. 1.** Reactivity of monoclonal antiserum (hybridoma cell line coded EMA 212) to fungal  
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17 807 species : *Alternaria dauci*, *Alternaria alternata*, *Alternaria cheranthi*, *Alternaria brassicae*,  
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20 808 *Penicillium waksmani*, *Phoma lingam*, *Stemphyllium lycopersici*, *Pyrenophora dictyoides*,  
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22 809 *Botrytis squamosa*, *Aschochyta fabae*, *Aureobasidium pululans*, *Fusarium solani* by plate-  
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24 810 trapped enzyme-linked immunosorbent assay.  
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27 811  
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29 812 **Fig. 2.** Visualization of *Alternaria brassicae* on a glass microscope slide with monoclonal  
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31 813 antiserum (hybridoma cell line coded EMA 212) labelled with a fluorescein conjugate as  
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33 814 viewed by (a) bright field microscopy (b) UV episcopic fluorescence (filter wavelengths at  
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35 815 450–560 nm).  
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41 817 **Fig. 3.** Visual assessment (by eye) of competitive immunochromatographic assay strips  
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43 818 (CLFDs) for risk of *Alternaria brassicae* inoculum in air samples: A, test line development  
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45 819 indicates low or no risk; B, no test line development – risk of *Alternaria brassicae*  
46  
47 820 inoculum.  
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50 821  
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53 822 **Fig. 4.** Assessment by optical densitometry of competitive immunochromatographic assay  
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55 823 strips (CLFDs) for measurement of *Alternaria brassicae* (serial doubling dilution series of  
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57 824 *A. brassicae* conidial spore concentration (6000 to aprox. 6 conidia)).  
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6 826 **Fig. 5.** Daily infection score ratings generated with a weather-based model that identifies  
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8 827 infection periods of the dark leaf spot brassica pathogen (*Alternaria brassicae*),  
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10 828 Lincolnshire, UK (OS grid reference TF509615).

11 829  
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13 830 **Fig. 6.** Relationship between the number of daily *Alternaria brassicae* conidia sampled by  
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15 831 a multivial cyclone sampler and the corresponding value generated by  
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17 832 immunochromatographic strip-type assay for presence of *A. brassicae* conidia.  
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19 833

20 834 **Fig. 7.** Dark leaf spot disease development in two UK brassica commercial crops: Brussels  
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22 835 sprouts (□) Lincolnshire (OS grid reference TF509615) and cauliflower (♦) Lancashire (OS  
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24 836 grid reference SD430235) during 2005.  
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34 838 **Table 1.**

35 839	Species	Growth Media	Morphological Classification	Host isolated
36 840	<i>Alternaria brassicae</i>	V8 agar	Ellis, 1971	<i>B.oleracea</i>
37 841	<i>Alternaria dauci</i>	V8 agar	Ellis, 1971	<i>D. carota</i>
38 842	<i>Alternaria cheranthi</i>	V8 agar	Ellis, 1971	<i>C. cheiri</i>
39 843	<i>Alternaria alternata</i>	V8 agar	Ellis, 1971	<i>F. vespa</i>
40 844	<i>Penicillium waksmani</i>	PDA	Pitt, 1988	Soil
41 845	<i>Phoma lingam</i>	PDA	Punithalingam and Holliday, 1975	<i>B. napus</i>
42 846	<i>Stemphyllium lycopersici</i>	PDA	Ellis, 1971	<i>S. lycopersicum</i>

849	<i>Pyrenophora dictyoides</i>	PDA	Ellis, 1971	<i>L. perenne</i>
850			(Drechslera state)	
851	<i>Botrytis squamosa</i>	PDA	Ellis, 1971	<i>A. cepa</i>
852	<i>Aschochyta fabae</i>	PDA	Punithalingam	<i>V. faba</i>
853			and Holliday, 1975	
854	<i>Aureobasidium pululans</i>	PDA	Ellis, 1971	Air
855	<i>Fusarium solani</i>	PDA	Booth, 1971	<i>A. cepa</i>
856			Nelson et al., 1983	
857	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**

<u>PTA-ELISA Absorbance (450nm)<sup>a</sup></u>				
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	

<sup>a</sup>Each value represents the mean of three replicate values ± standard errors.

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874 **Table 3**

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

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878 PTA-ELISA Absorbance (450nm)<sup>a</sup>

879	Time (h)	Temperature (°C)	Periodate	Control	Reduction (%)
880	5	4	0.223 ±0.012	0.371±0.005	40
881	5	37	0.150±0.006	0.324±0.00	54
882	24	4	0.133±0.014	0.409±0.034	67
883	24	37	0.192±0.038	0.263±0.028	27

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885 <sup>a</sup>Each value represents the mean of three replicate values ± standard errors.

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888 **Table 4**

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

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892 PTA-ELISA Absorbance (450nm)<sup>a</sup>

893	Time (h)	Temperature (°C)	Protease	Control	Reduction (%)
894	5	4	0.119 ±0.009	0.332±0.005	64
895	5	37	0.135±0.016	0.341±0.004	60
896	24	4	0.104±0.003	0.453±0.034	77

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3	897	24	37	0.090±0.023	0.398±0.028	100
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<sup>a</sup>Each value represents the mean of three replicate values ± standard errors.

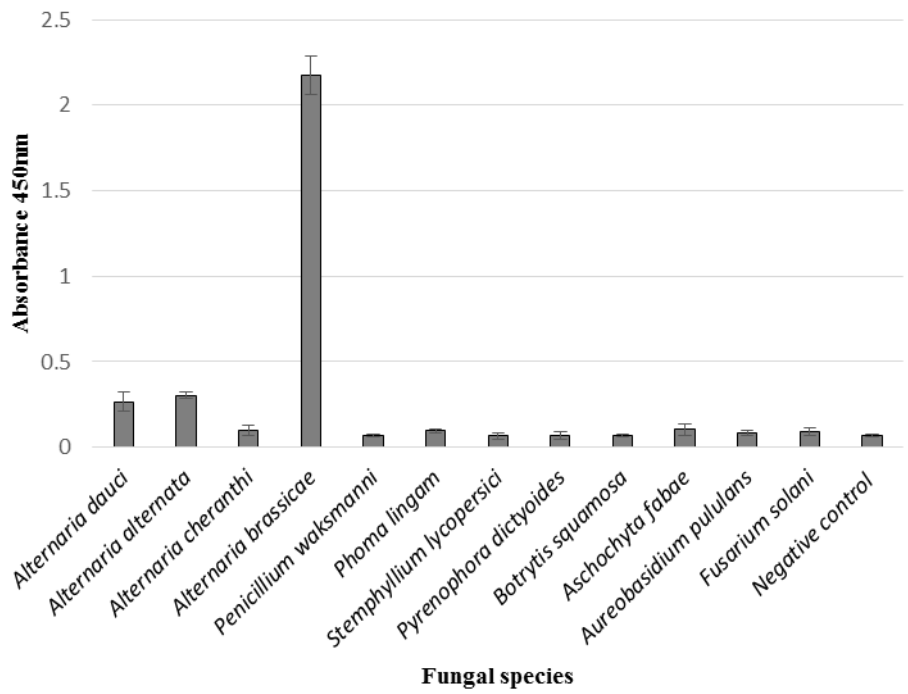
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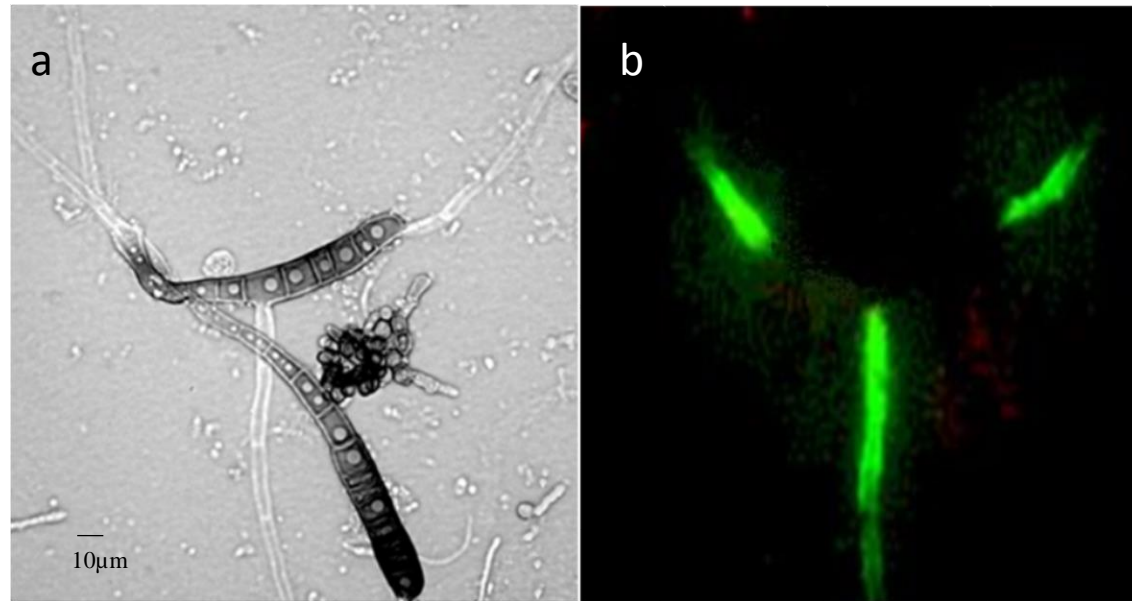
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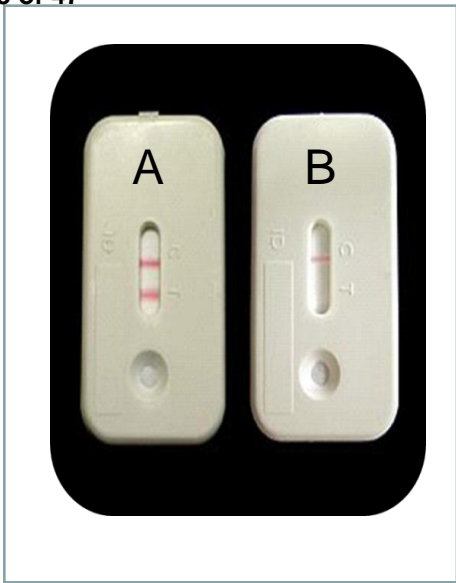
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**Fig. 1.** Reactivity of monoclonal antiserum (hybridoma cell line coded EMA 212) to fungal species : *Alternaria dauci*, *Alternaria alternata*, *Alternaria cheranthi*, *Alternaria brassicae*, *Penicillium waksmani*, *Phoma lingam*, *Stemphyllium 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).



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

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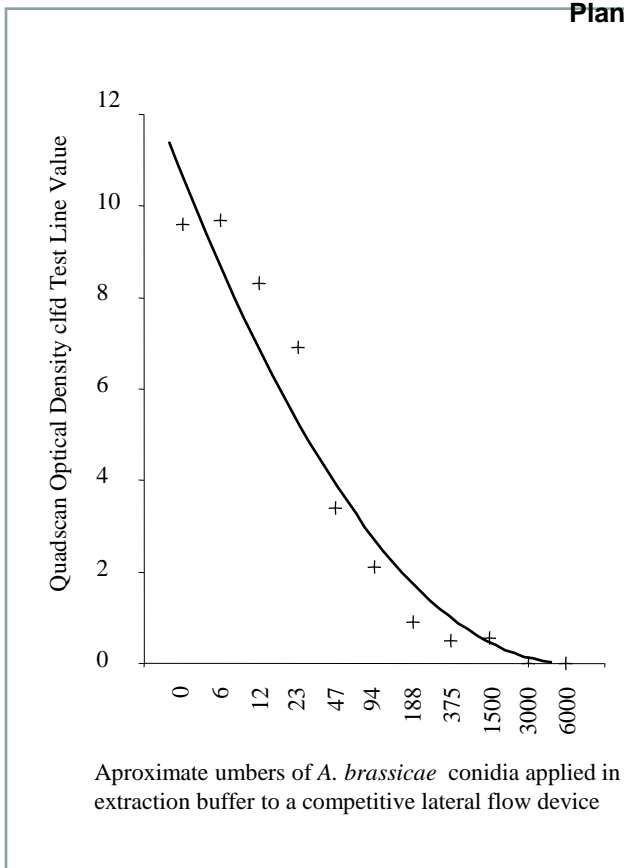
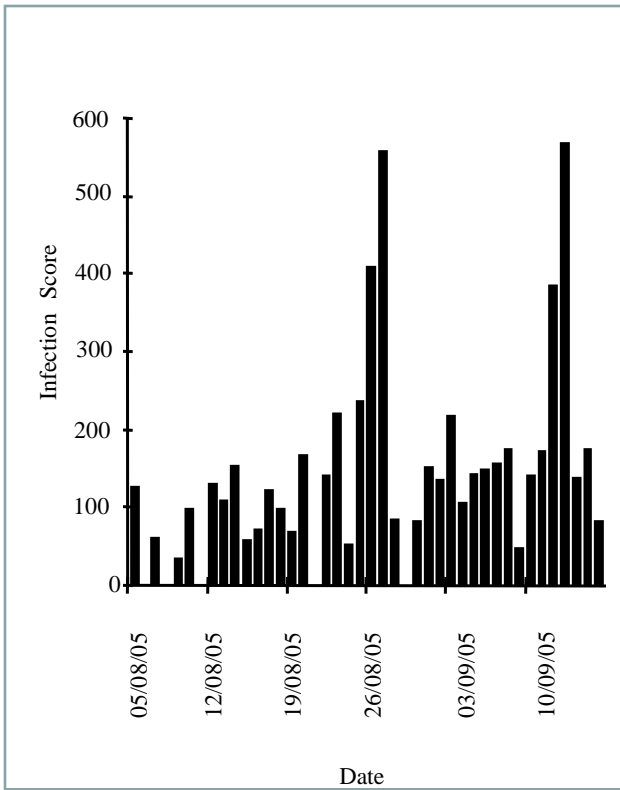


Fig. 4. Assessment by optical densitometry of competitive immunochromatographic assay strips (CLFDs) for measurement of *Alternaria brassicae* (serial doubling dilution series of *A. brassicae* conidial spore concentration (6000 to aprox. 6 conidia).



**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).

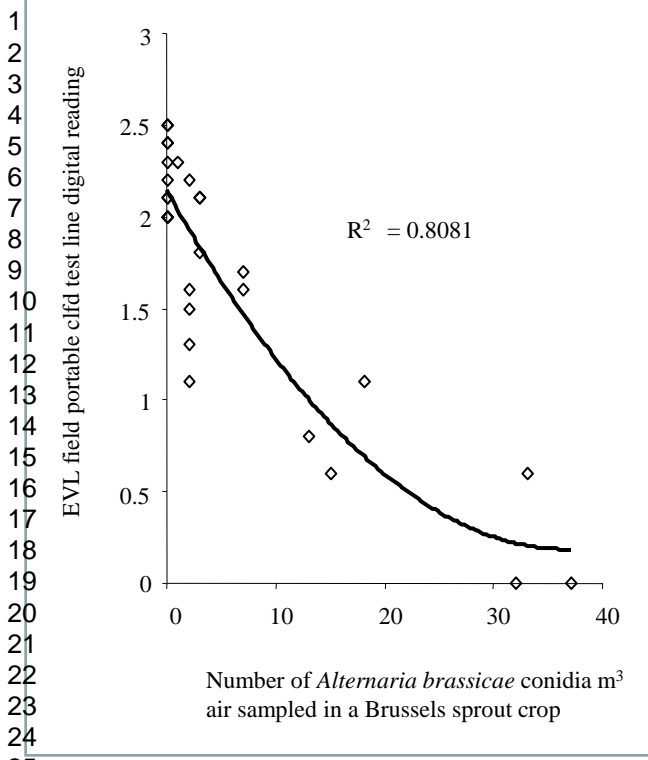


Fig 6. Relationship between the number of daily *Alternaria brassicae* conidia sampled by a multivial cyclone sampler and the corresponding value generated by immunochromatographic strip-type assay for presence of *A. brassicae* conidia.

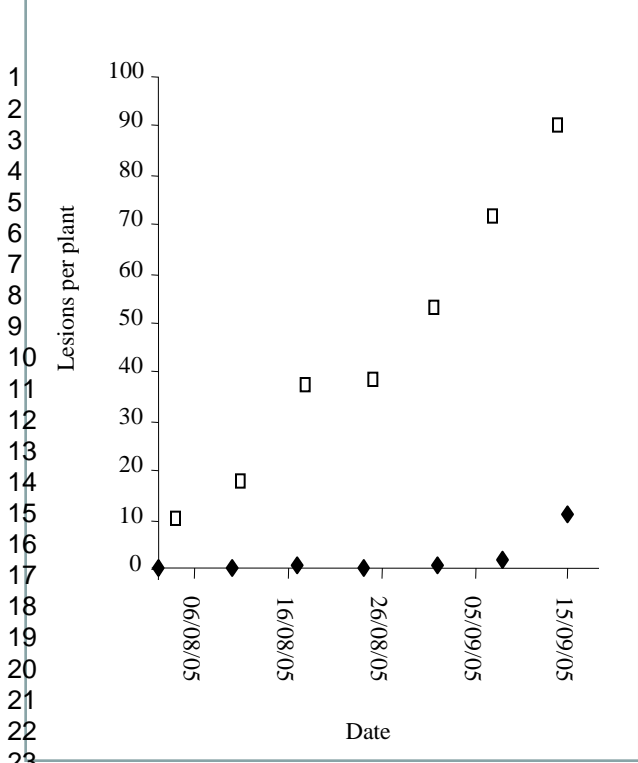


Fig. 7. Dark leaf spot disease development in two UK brassica commercial crops: Brussels sprouts (□) Lincolnshire (OS grid reference TF509615) and cauliflower (◆) Lancashire (OS grid reference SD430235) during 2005.