August 2017

Project Report No. 577

Further development of “in field” tests for resting spores of clubroot and the development of control based on detection

Roy Kennedy[1*a,b], Mary Lewis[1a,b], Geoff Petch[1a,b], Angela Warren[1b], Emma Edwards[1b], Gary Keane[1a], Maude Proctor[1a,b] Simon John[1a] and Alison Wakeham[1*a,b]

[1] University of Worcester, Henwick Grove, Worcester, WR2 6AJ
*Current address Warwickshire College Group, Pershore College, Avonbank, Pershore, WR10 3JP
[a] Authors contributing to work jointly funded by AHDB Horticulture and AHDB Cereals & Oilseeds 2009-2013
[b] Authors contributing to work carried out under an AHDB Cereals & Oilseeds-funded extension 2013-2016

Cross reference: Research carried out 2009 to 2013 published by AHDB Horticulture under FV 349

This is the final report of a 78 month project (RD-2008-3525) which started in April 2009. The work was funded by AHDB and Syngenta and a contract for £167,533 from AHDB Cereals & Oilseeds.

While the Agriculture and Horticulture Development Board seeks to ensure that the information contained within this document is accurate at the time of printing, no warranty is given in respect thereof and, to the maximum extent permitted by law, the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

Reference herein to trade names and proprietary products without stating that they are protected does not imply that they may be regarded as unprotected and thus free for general use. No endorsement of named products is intended, nor is any criticism implied of other alternative, but unnamed, products.

AHDB Cereals & Oilseeds is a part of the Agriculture and Horticulture Development Board (AHDB).

CONTENTS

1. Abstract

Oilseed rape (OSR) is widely grown in the UK and has a high economic value for many arable farmers, frequently forming part of cereal rotations. As a member of the Brassica family, OSR is susceptible to the clubroot pathogen (*Plasmodiophora brassicae)*. Once soil has been contaminated, clubroot spores can remain viable for several years causing any subsequent oilseed or vegetable brassica crop to be at risk of infection. With the development of new detection methods based on identifying *Plasmodiophora brassicae* DNA levels in soils, the presence or absence of clubroot risk can be determined; however, these are limited by the need for processing in a laboratory.

The primary aim of this work was to validate a clubroot lateral flow device (LFD) for use in fields by OSR growers, therefore avoiding the need for specialised laboratories. Quantification of spores by lateral flow devices (protocol developed and reported earlier by Kennedy *et al*, 2013; AHDB Horticulture final report FV349) was tested against a molecular quantification method (quantitative PCR; also validated and reported earlier by Kennedy et al., 2013) to establish whether a commercially viable diagnostic test could be provided for growers. In addition, the effect of oilseed rape cultivar on disease development was investigated through pot-based bioassays, as was the effect of *P. brassicae* resting spore density on seedling infection levels. An additional objective was to study the effect of clubroot on yields of resistant and susceptible oilseed crops.

In a comparison of spore quantification by lateral flow device (LFD) and quantitative PCR, it was found that the LFDs overestimated the number of clubroot spores in soil samples. However, qPCR proved to be a reliable assay after appropriate soil sampling. In the pot-based bioassays, there was increased damage observed on OSR roots at higher soil spore concentrations in both a resistant and a susceptible cultivar; it was notable that galling (at very low levels) was observed during glasshouse trials on the main resistant cultivar of OSR currently used in the UK: cv. Cracker. Of other cultivars tested, a range of disease severity was observed.

The relationship between clubroot resting spore density at planting and plant infection at harvest and yield was examined by comparing OSR seed weight from fields with and without clubroot presence. There was a good relationship between the clubroot detection in soil at planting and subsequent infection on plants at harvest ($r^2 = 0.726$). This indicates that clubroot quantification at sowing in soil using a diagnostic test is a good indicator of subsequent plant infection. However, this does not necessarily allow for a determination of likely yield loss for oilseed rape. At sites where clubroot resting spores were detected at planting, there were consistently higher yields where clubroot resistant varieties were used in comparison to clubroot susceptible varieties.

2. Introduction

Oilseed rape is a widely grown crop in the UK and has a high economic value for many farmers, frequently forming part of arable rotations. As a member of the Brassica family, oilseed rape is susceptible to clubroot caused by *Plasmodiophora brassicae,* a soil-borne protozoan. Disease is initiated in the roots when the pathogen encysts on root hairs and penetrates the root tissue. It is here that maximum damage occurs because the disease causes root galls to form as it completes its reproductive cycle and this subsequently affects the uptake of water and nutrients by the plant resulting in crop damage.

Once *P. brassicae* has replicated inside host cells the roots decay and release resting spores into the soil. Resting spores can survive for many years in the soil without the presence of host plants for replication: the half-life of resting spores has been given at 3.6 years (Wallenhammer, 1996) and there are anecdotal reports of spores persisting for two decades after the last Brassica crop was grown. The longevity of the resting spores poses a problem for growers, because continuous Brassica cropping can lead to a build-up of high levels of resting spores in the soil, and higher levels of resting spores are associated with higher root damage and club formation.

Yield losses caused by *P*. *brassicae* are significant and equate to over 50% of potential yield in the most severely infected crops (Burnett et. al., 2013). In warm autumns, infection can be so severe that crops are abandoned and then ploughed in so that 100% losses are recorded. Vegetable growers will commonly test soil and select rented fields (over fixed periods) which are free of the disease, but for arable farmers avoiding infected fields is seldom an option (Burnett et al., 2013). A significant and rising proportion of arable land used for oilseed rape production in the UK is contaminated with clubroot and a number of farms continue to find clubroot for the first time each year. In areas of intense oilseed rape cropping such as the North East of Scotland the incidence of infected fields is over 50% (Burnett at al., 2013).

Oilseed rape varieties resistant to clubroot are available, but at sites where this resistance mechanism has been used previously, alternative clubroot strains which can overcome the resistance mechanisms have built up to potentially damaging levels. Liming has been used to reduce the problem in vegetable crops but in oilseed rape crops, and at the pHs common in arable production, control with coarse limes offers only partial control and may have negative impacts to other following crops in the arable rotation – for example potatoes. Fine limes (e.g. LimeX) offer more effective control and can be used just before sowing.

Defra (2013 crop statistics) estimates that clubroot and other soil borne pathogens are responsible for approximately £30m in crop losses per year in the UK and it is estimated that globally 11.6% of Brassicas are infected by this pathogen (Dixon, 2009). Therefore, it is of interest to growers for

research to be performed that will advance detection techniques and investigate control measures for clubroot.

As an area of common interest to both vegetable brassica and oilseed rape growers, the early stages of this project (2009-2013) were funded jointly by AHDB Cereals & Oilseeds and AHDB Horticulture. An extension to the project was subsequently funded by AHDB Cereals & Oilseeds from 2013-2016. This report will focus on the development and validation of the in-field tests for resting spores of clubroot for use by oilseed rape growers, with key areas of the lateral flow device development work from 2009-2013, previously reported in full under AHDB Horticulture report FV 349, summarised again here for completeness. Work carried out from 2013-2016 is reported here in full for the first time.

2009-2013

The aim of the initial work was to optimise and validate a clubroot lateral flow device (in-field test) on a range of soil types and incorporate changes in the lateral flow format, with a view to mass production and usage on all soil types, initially where vegetable Brassica are produced. Further studies were carried out to examine and strengthen the shelf life of the developed lateral flow test. An additional aim was to carry out a series of field experiments investigating clubroot control (not reported here, see Kennedy et al. 2013 for results) where a molecular test (quantitative PCR) would be used to determine variation in clubroot contamination. The description of the quantitative PCR test is included here.

Quantitative PCR has been an established procedure in the quantification of disease pathogens in many horticultural systems, including in the detection and quantification of the clubroot pathogen *Plasmodiophora brassicae*. The ability to quantify *P. brassicae* in field soils has useful applications in assessing the risk of clubroot ahead of planting Brassica crops and in management of the disease. In UK soils, Buczacki (1983) reported that a concentration of 100000 ($1x10^5$) clubroot resting spores /g soil are required for severe and uniform disease expression on plants. It is important also to consider environmental factors such as moisture, temperature and soil pH which will affect infection level irrespective of spore density i.e. depending on the suppressive or conducive nature of the soil to the disease.

Technology has also been developed that allows lateral flow devices (LFDs) to quantify clubroot resting spores in soil. This is a significant step forward for growers in the quantification of *P. brassicae* spore load, providing a rapid diagnostic tool which is portable and can be carried out quickly and relatively inexpensively in their own fields by non-scientific staff.

2013-2016

An extension to the project (2013-2016) was funded by AHDB Cereals & Oilseeds to validate the use of the in-field clubroot detection test (LFD) in a range of clubroot contaminated arable soils and to produce information on initial clubroot contamination in relation to risk of crop and yield loss in oilseed rape crops for a range of soil types. Initial clubroot contamination in relation to the usage of resistant varieties was also investigated in association with Syngenta. The objectives of the extension were to:

1) Validate the use of the “in field” clubroot detection test in a range of clubroot contaminated arable soils
2) Establish the relationship between resting spore concentration in arable soils, visible symptoms and potential yield loss
3) Evaluate and optimise risk assessments for clubroot in oilseed rape crops based on soil type, variety and initial clubroot resting spore numbers in the soil
4) Evaluate changes in clubroot resting spore concentrations in soil using Syngenta resistant varieties (objective funded by Syngenta)

3. Materials and methods

3.1. Laboratory clubroot test development (2009-2013)

A full description of the research carried out in 2009-2013 is reported in Kennedy et al 2010, 2012 and 2013. Key steps in the development and use of the lateral flow device (LFD) and the molecular test (qPCR) are reported here again.

3.1.1. Production of new *Plasmodiophora brassicae* antibody conjugates

A clubroot lateral flow test, based on the protocol previously developed in HDC project FV259 (Kennedy & Wakeham, 2007), was constructed. This time however a 5 x 5 mm sample filtration pad (www.whatman.co.uk) was inserted between the conjugate and membrane to inhibit blocking by soil particles (Figure 1). A control line of anti-mouse serum (0.5mg ml^{-1}) and a test line of a *Plasmodiophora brassicae* soluble antigen were applied to the membrane as described in HDC project FV259. Four batches of 50 conjugate pads each were prepared using Monoclonal antiserum EMA 3A5 (Batch 4) at a dilution of 1 in 200 in gold Warwick HRI conjugate buffer. To each 5 x 5 mm conjugate pad a 27 µl aliquot of the antibody bound gold buffer was applied. Following air drying each pad was incorporated in to a lateral flow device. Ten fold concentrations, ranging from $1x10^8$ to $1x10^2$ resting spores of *P. brassicae* in lateral flow sample buffer (Kennedy & Wakeham, 2008), were applied in 100µl aliquots to sample pads of seven constructed lateral flows. After ten minutes optical density readings (OD) were made using a Quadscan lateral flow reader (www.biodot.com). A control of extraction buffer alone was added to an additional lateral flow at 100 µl volume. This process was repeated for each of the prepared conjugate batches.

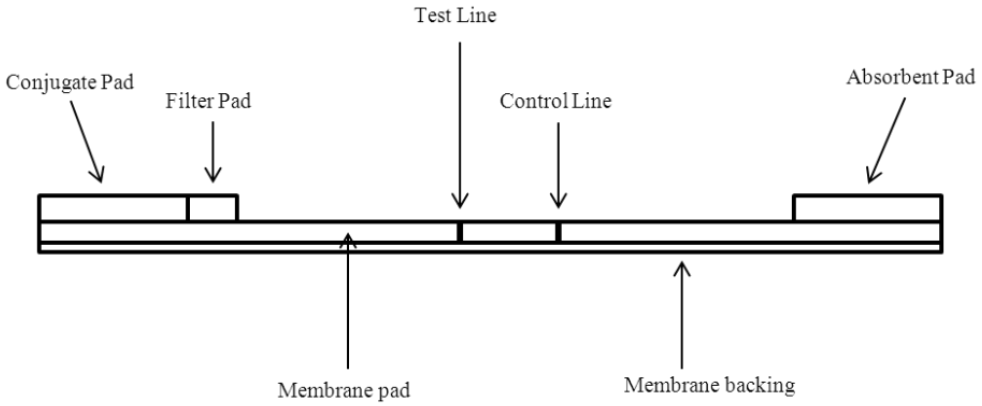

Figure 1: Lateral flow device for semi-quantitation of clubroot resting spores

3.1.2. **Investigation of lateral flow device test line stability**

A 10ml filtered $1x10^8$ *P. brassicae* resting spore suspension in chilled phosphate buffered saline ((PBS) 0.01M phosphate buffer, 0.0027M potassium chloride, 0.137M sodium chloride, pH 7.4), was processed with Ballotini glass beads in a Fast Prep 120 machine (www.qbiogene.com), at a speed setting of 5 for 30 seconds. The sample was held on ice for 5 min. and the process was repeated twice. The liquid phase was microfuged at 5000 rpm for 5 min. and the pellet discarded. The retained liquid phase was adjusted to 10mg ml^{-1} in PBS and a series of lateral flow test line samples were prepared (Table 1). Each test line solution contained 1mg ml^{-1} of *P. brassicae* soluble resting spore fraction.

Table 1 Test line solution types for lateral flow application

Test line
P. brassicae soluble antigen in 0.0025M PBS
P. brassicae soluble antigen in 0.0025M PBS, 1mg ml^{-1} Poly-L-Lysine
1mg ml^{-1} Poly-L-Lysine in 0.025M PBS overlaid with *P. brassicae* soluble antigen in 0.0025M PBS
P. brassicae soluble antigen in 0.0025M PBS, 0.5% Bovine Serum Albumin
P. brassicae soluble antigen in 0.0025M PBS, 0.5% Casein

A control line of an anti-mouse serum at 0.5 mg ml^{-2} (www.sigmaaldrich.com) was sprayed directly on to the membrane surface of a lateral flow (Figure 1) using a flat bed air jet dispenser (Biodot Ltd, West Sussex, UK) at a constant rate of 50 m/s. The *P. brassicae* (clubroot) test line fractions were individually applied to lateral flow membranes (Figure 1) at a rate of 25 m/s. The lateral flow membranes were air-dried overnight at 18 to 20° C, sectioned in to 5mm strips and a conjugate pad inserted before being individually housed in a plastic case (EVL, Netherlands). Using ten fold concentrations of *P. brassicae* resting spores in sample buffer, 100µl aliquots were applied to the competitive lateral flows (*clfd*) of each test line type. Optical density readings were made 10 min. after application using a Quadscan lateral flow reader. A control comprising of extraction buffer alone was included for each test line type at 100 µl volume. This process was repeated twice.

3.1.3. **Standard curve for quantification of *P. brassicae* in artificially infested soils by lateral flow device**

Lateral flow devices were constructed as previously described (section 3.1.1). A standard curve was created for each batch of lateral flow devices prepared. A soil which had previously been identified as free of clubroot spores was artificially inoculated to provide a 10 fold serial dilution of *P. brassicae* resting spore concentrations ranging from $1x10^9$ to $1x10^3$ resting spores g^{-1} soil. A control soil of zero

resting spores was also included. These samples were processed by lateral flow assay, as described below, to provide a standard curve.

Lateral flow assay

For each soil, 0.26g was weighed into an individual microtube and 400µl of B2 buffer was added. The soil was shaken vigorously and the suspended soil particulates then allowed to settle over a 1 min. period. A 100µl aliquot of the resulting liquid phase was removed and transferred to a lateral flow device. A reading of optical density (OD) was taken after 30 minutes using an ESE reader device. The OD values recorded for each of the artificially infested soils were then used to generate a standard curve.

3.1.4. Molecular quantification of *P. brassicae*

DNA extraction from soil samples

Soil DNA was extracted using the MOBIO UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc. Carlsbad, CA) following the manufacturers protocol. The vortex step was replaced with three repeats on a FastPrep® Instrument (QBiogene, Irvine, CA) at speed 5.5 for 25s with samples resting on ice in between runs. A Polyvinylpolypyrrolidone (PVP) DNA cleanup was subsequently performed on all DNA extracts (Klemsdal *et al*., 2008) and DNA was stored at -20^{o}C.

Quantitative PCR

Quantitative PCR was performed using set of primers PbITS3 and PbITS4 and as a 20µl reaction mix comprising 10µl Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX (Invitrogen Ltd. Carlsbad, CA), 0.4µl each primer (final concentration 4nM), 7.7µl ROH_20 and 1.5µl DNA. To generate standards for real time quantification, total DNA was extracted and cleaned from eight 0.26g samples of a soil which had tested negative for clubroot presence by PCR and bait planting. A further aliquot of this soil was inoculated with $1x10^9$ spores g^{-1} *P. brassicae* suspension prior to DNA extraction and clean up. The spore suspension used for inoculating soil was obtained from gall tissue by filtration under vacuum. A dilution series of the $1x10^9$ spores g^{-1} *P. brassicae* soil DNA extract was then made into the uninoculated soil DNA extracts, providing a set of standards for quantification. Absolute quantification analysis based on the second derivative maximum method was used to quantify the levels of *P. brassicae* DNA by qPCR. Using this method the generation of the standard curve is based on non-linear regression.

Comparison of different primers for quantification by qPCR

A total of 37 soil samples previously collected from various locations around the UK (identified as series 7 and 10) were used in this small comparative study. Each soil was dried then ground and milled using gradients of wire mesh sized down to 2mm before weighing into 0.26g aliquots in preparation for DNA extraction.

Soil DNA was extracted and cleaned up as described above and standards for quantification by qPCR were as previously described. A second set of *P. brassicae* specific primers were developed called PbML1F and PbML1R and these were supplied by Sigma, and were designed to amplify within 18S and ITS1 regions of *P. brassicae* rDNA. Tailored quantitative PCR reactions were carried out for each primer set.

3.1.5. Determining the lower limits of quantification

The MoBio DNA extraction kit has shown itself to be extremely effective at isolating DNA from soil but due to the amount of starting material incorporated per isolation (0.25g soil – following manufacturer's recommendations) this ultimately places limitations on the lower levels of detection of *P. brassicae* spores in soil that could be reported on. Moreover by incorporating just 0.25g of soil inoculated with 1000 spores g^{-1} of soil, only the equivalent of two hundred and fifty spores are actually being extracted from. Final elution volume of 100ul allows a maximum average (theoretically) of two and a half spores per microlitre, though one should bear in mind a small percentage loss due to the extraction and clean up procedures. To follow on only two microlitres of the resultant 100µl elution volume per sample is incorporated into each qPCR reaction. While it may be possible to detect a minimum of approximately $2x10^2$ spores g^{-1} soil, the reliability of quantification is lower at this level due to the high number of qPCR cycles required. To increase sensitivity it would be necessary to either increase the size of the reaction or increase testing frequency, each of which would increase the cost. As below $1x10^3$ spores g^{-1} would generally be expected to indicate a low risk of severe and uniform clubroot disease occurrence the 'not detected' threshold is used to describe levels of *P. brassicae* DNA that fall at or below $1x10^3$ spores g^{-1} of soil.

3.1.6. Evaluation of 2012 soil samples for *P. brassicae* resting spore concentration by using lateral flow devices and qPCR and comparisons between the two methodologies

Note that vegetable brassica sites were used for this evaluation in 2012.

Methods of simple extraction of resting spores in soils were explored to enable growers to quickly sample and evaluate field soils for clubroot infestation. Prototype lateral flow devices were then distributed to a Brassica grower to trial the system. The grower was supplied with an ESE reader and a simple protocol for processing of the collected field soils by lateral flow. The soils collected by the grower (and identified as Series 12) were sent to the National Pollen and Aerobiology Research Unit for independent assessment of *P. brassicae* resting spore concentration by qPCR and lateral flow test. The results of the study were compared and the two techniques evaluated for the detection and quantification of resting spores in soil.

Lateral flow device testing

An ESE Reader, the computer software and a 2012 batch of optimised LFDs were sent to the East of Scotland Growers for use in the evaluation of commercial soil samples for clubroot resting spore infestation. A simple protocol was followed: individual collected soil samples were each shaken in a small volume of lateral flow buffer, allowed to stand for 1 minute and a small volume of the liquid phase of each field sample was transferred to the sample pad of individual lateral flow devices. A negative (soil with 0 resting spores) and positive samples (calibration series of artificially infested soil) were also processed by LFD and for each OD measurements were made. In total, 53 commercial grower field soils were tested for clubroot resting spore presence and the subsequent OD values generated were compared and evaluated against laboratory generated qPCR results.

Molecular qPCR

DNA extraction and qPCR analysis were carried out on all 53 commercial soils as described previously. The results from each assay were compared using 4 parameter logistic regression to model the relationship between them, and the fit of predicted spore g^{-1} soil values generated from LFD quantification were tested against the actual spore g^{-1} soil values obtained by qPCR.

3.1.7. **Lateral flow shelf life: Antibody and antigen stability**

In previous testing it was observed that over time assay sensitivity could be compromised. Two areas of concern were highlighted: stability of the immunological complex retained in the lateral flow conjugate pad and application of the antigen at the test line (Figure 1). For each conjugate pad, a solution containing 30µl of 1:40 UW249 monoclonal antiserum in conjugate buffer (0.0025M PBS, 2% trehalose, 2% BSA, 2% sucrose) with 5µl anti-IgM gold beads) (BBI International) was prepared. To this solution, different concentrations of sucrose (0%, 5%, 10%, 15% & 20%) were then added. The pads were dried at 37°C for 20 minutes. The conjugate pads were stored in a dry state at room temperature prior to assessment at twelve consecutive time points. At each selected time point three conjugate pads were removed from storage and each inserted within an LFD complex (Figure 1). Each LFD had 100µl B2 buffer added and an ESE reading was taken after 30 mins run time.

A solution of whole spores of *P. brassicae* resting spores ($1x10^9$ spores ml^{-1} water) were prepared in a) water b) 0.05% trehalose and 1% isopropanol and c) 0.05% trehalose , 1% isopropanol and 2% sucrose. These solutions were applied as antigen test lines to a series of lateral flow membranes at a speed of 10mm s^{-1}, and allowed to air-dry at room temperature overnight. The membranes were sectioned in to 5mm strips and assembled within a LFD format. The LFDs were stored at room temperature in foil pouches. At selected time intervals the LFDs were opened and, using newly prepared conjugate pads, a solution of *P. brassicae* resting spores was aliquoted to each sample pad and OD readings were taken after 30 min LFD run time.

3.2. Validation of the in-field tests in arable soils (2013-2016)

The finalised format for the LFD device as given in section 3.1.1. and reported by Kennedy et al. 2013 was used in work carried out in 2013-2016 (AHDB Cereals & Oilseeds-funded project extension).

3.2.1. Collection of soils with a history of clubroot from arable farms in Scotland and England

In Scotland seven commercial sites were visited over two consecutive years (2013 and 2014), and soil samples were taken from fields suspected to have clubroot disease based on farmers' experience/knowledge. All but one of these fields were planted with a clubroot resistant variety (cvs. Cracker or Mentor); the exception was planted with cv. Temple. In England, four commercial sites were sampled in 2014, and in 2015 four further sites were sampled. All were planted with either cv. Cracker, Picto or Extrovert. In addition, two variety trial sites planted with a wide range of cultivars (courtesy of Scottish Agronomy Ltd.) were also sampled in 2013, 2014 and 2015. Thus, a total of eight commercial farm fields were sampled in England and 14 in Scotland, plus a further two fields involved in variety trials (Figure 2).

All sites were sampled as soon as possible after planting (October/November) and just prior to time of harvest (July/August). The precise planting and harvesting dates were dependent on local weather conditions and farmers' schedules so all soil sampling was arranged not longer than four weeks after the planting or three weeks before harvesting.

At all sites five soil samples were taken at 20m intervals along a 100m transect and were analysed independently of each other. Soil samples were taken at a depth of 15-25cm, and marked with flags to ensure the same location was sampled at harvest. Once collected soil samples were air-dried, milled and sieved to <2mm. DNA extraction was performed in duplicate on each soil sample, using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Solana Beach, CA) with amendments and clean-up as described in 3.1.4.

3.2.2. Comparison of *P. brassicae* spore numbers in soil by qPCR and LFD

Quantitative PCR (qPCR) was set up as previously described (section 3.1.4). The primers used were PbITS3 and PbITS4 (Kennedy and Wakeham 2007; Faggian and Parsons 2002). Previous results had showed the qPCR to be a good measure of the number of *P. brassicae* spores g^{-1} soil (Kennedy et al., 2013). LFDs were prepared as detailed in section 3.1.1 (see also Kennedy et al. 2010, 2012, 2013, and Wakeham et al. 2015). Out of the total soils sampled across commercial and trial sites, the qPCR and LFD results from 137 soils were compared.

Figure 2. Sampled arable farms in Scotland and England. Closed circles represent sites that were sampled at time of planting and time of harvest, open circles represent cultivar trial sites. The location identified near Edinburgh contained two sampling sites.

3.2.3. The relationship between resting spore concentration and visible symptoms on oilseed rape cultivars (glasshouse)

Experiments were established to examine the extent of clubroot disease observed on plant roots within a range of spore concentrations in the soil, using a resistant and a susceptible oilseed cultivar. Soil from field 6 (Scotland) was diluted in a non-infected soil to provide a gradient of spore concentrations from 10^5 to 10^2 spores g^{-1} soil. Cracker was used as the resistant cultivar and cv. DK Cabernet was used as the susceptible cultivar. Seeds were sown in compost under 20°C and 8h photoperiod. On emergence, seedlings were transferred to a cold frame and grown for two weeks prior to transplantation into clubroot infested soil. Seedlings were grown in a polytunnel for 14 weeks, then roots were washed and the number of visible clubroot galls counted. This experiment was repeated in English soil collected from a known infected site in Warwickshire (which was not included as a commercial site due to no OSR cultivation) using a dilution series from 10^6 to 10^3 and 0 spores g^{-1} soil.

3.2.4. Evaluation of the effect of oilseed rape cultivar on clubroot disease development (glasshouse)

Fifteen cultivars were grown in two soils, a Scottish soil from field 6 of the commercial sites detailed in 3.2.1 and an English soil from a known infected site in Warwickshire. The Scottish soil contained $1x10^5$ spores g^{-1} and the English soil contained $1x10^6$ spores g^{-1} soil. For each cultivar there were 18 replicate pots with two plants per pot. Plants were grown in a polytunnel for 14 weeks, after which the plants removed from the soil and the roots washed. Clubroot disease index (DI) was assessed for each plant by the method of Dixon and Robinson (1986).

3.2.5. The relationship between clubroot spore number in soil and yield in oilseed crops

In Scotland, seven fields growing cv. Cracker in 2013 (two in Aberdeenshire, two in Fife, one in East Lothian and two in Roxburghshire; independent of the previously described sampling sites), were sampled for soil in April, and for soil plus plants in August (time of harvest). Plants were collected from $1m^2$ quadrats, by removing the aerial components and sealing them in large paper sacks. The plants were transported to the laboratories, where they were air-dried for one week prior to threshing and seed collection. The seed weight was calculated from the sampled $1m^2$ quadrat then extrapolated to t Ha^{-1}.

Scottish Agronomy sites

Additionally, from 2013 to 2015, fields that were part of cultivar trials had been tested annually for clubroot disease by qPCR in order to examine varietal differences in yield in response to clubroot disease, if present. In 2013 and 2014 soil sampling was performed at a rate of three samples per

cultivar, with three cultivars tested: Cracker, DK Cabernet and Catana, at the time of planting and at the time of harvest. Each cultivar was grown in a 3 by 6 m area; two plants were also removed to examine the root tissue for the presence of galls. Duplicate DNA extracts were performed on each soil sample and quantified by qPCR as described previously. For these years there were no spores detected and with no evidence of clubroot disease in the crop. In the third year of sampling (2015), only cv. Cracker was tested; however, two fields were identified as having low clubroot load at time of planting, although the spore load of the soil had decreased by the time of harvest. From these trials, yield data was collected from ten cultivars, of which seven which had been consistently included in all years of trials; so these seven were used to assess the effect of clubroot disease on yield.

Syngenta-funded trials

A clubroot resistant and susceptible oilseed rape variety were used in yield trials at nine clubroot infected sites (Syngenta yield trials). At each location there were two separate yield comparisons. Yield comparisons were conducted on areas where there were high and low clubroot concentrations at each location (18 comparisons possible). Four replicated plots were sited as 20 X 20 metre plots within larger oilseed rape growing areas (fields). Clubroot infection at each site was measured initially prior to planting using the plant biotest to confirm infection at each site. The qPCR test was also carried out on samples from each site prior to planting. Yield measurement was taken using a trials harvester with yield measurement attached. Clubroot concentration per plot was assessed using 4 soil samples taken per plot in a randomised design. Plant assessment was taken at the same point as soil samples. Field index on plant samples was after the method of Dixon & Robinson (1986). Only comparisons where clubroot was detected on the plant were used in the subsequent analysis.

3.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism v.7, GraphPad Software, La Jolla California USA. For analysing more than one time point or parameter, paired t-tests or repeated measures ANOVA was used. Linear regression and non-linear regression were used to examine relationships between variables.

4. Results

4.1. Laboratory clubroot test development (2009-2013)

A full description of the research carried out in 2009-2013 is reported in Kennedy et al 2010, 2012 and 2013. Key steps in the development and use of the lateral flow device (LFD) and the molecular test (qPCR) are reported here again.

4.1.1. Production of new *Plasmodiophora brassicae* antibody conjugates

A linear correlation of 0.9878 was recorded between the *P. brassicae* resting spore numbers applied and the corresponding competitive lateral flow device (*clfd*) OD value generated (Figure 3). Visual observation of each of the *clfd* conjugate batches tested, recorded test line depletion of the *clfd* to occur in the region of $1x10^5$ resting spores ml^{-1}.

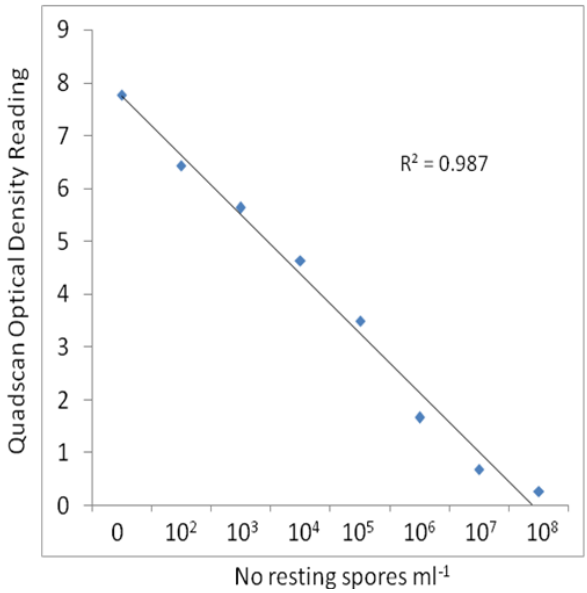

Figure 3: Lateral flow device for semi-quantitation of clubroot resting spores in liquid phase

4.1.2. Investigation of lateral flow device test line stability

Addition of test line material and / or pre striping the *clfd* membrane with Poly-L-Lysine did not improve the interaction of monoclonal antibody / antigen binding at the clubroot *clfd* test line (Figure 4a). In addition, these components gave no benefit in retaining test line activity over time (Figure 4b). Degradation of the test line was observed for all *clfd* test line applications over time. Sensitivity and quantitative loss of the test was observed three months post test line application.

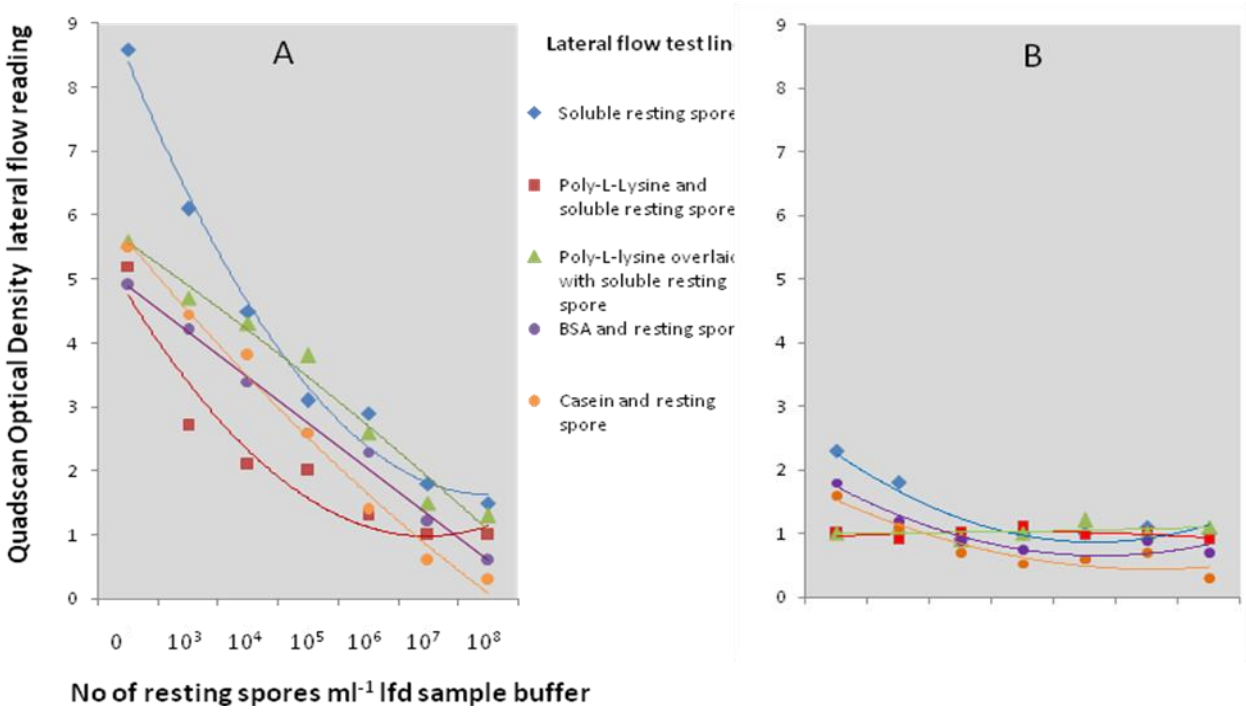

Figure 4: Quantitative measurement of clubroot resting spores using lateral flows prepared 48 hours after test line application (A) and at three months (B)

4.1.3. Standard curve for quantification of *P. brassicae* in artificially infested soils by lateral flow device

A correlation (r2=0.987) was observed between the optical density values of the lateral flow test device (LFD) readings and the number of clubroot resting spores in the artificially inoculated soils.

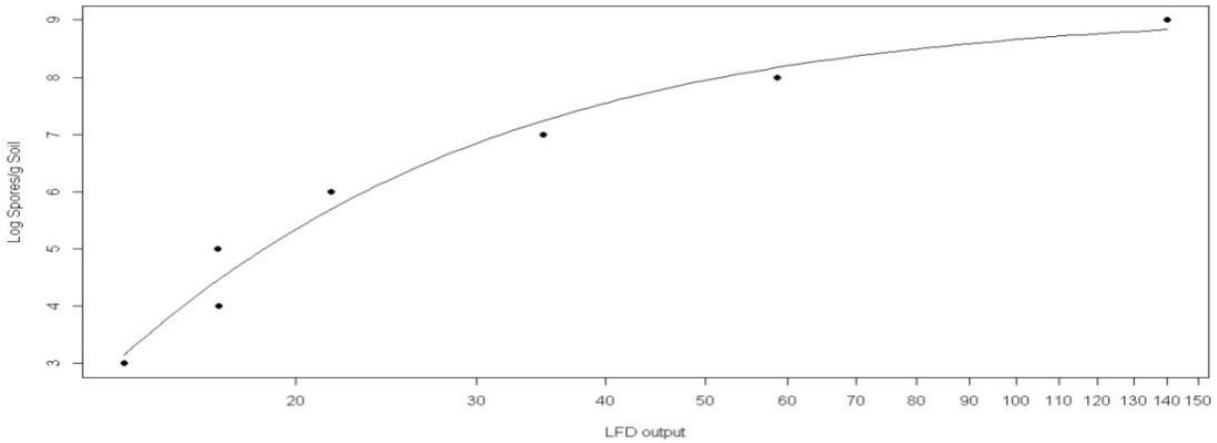

Figure 5 Standard curve generated using soil samples inoculated with known concentrations of *P. brassicae* resting spores g^{-1} soil.

4.1.4. **Molecular quantification of *P. brassicae***

Comparison of different primers for quantification by qPCR

Sixteen out of 37 soil samples tested showed successful amplification of *P. brassicae* DNA, whilst 21 of the samples fell below the detection threshold suggesting no presence or very low levels of *P. brassicae*. Matching results were observed for 19 of the 21 samples with both the PBITS3/4 and the PBML1F/R primers. Both primer sets indicated some soils with *P. brassicae* levels below the detection threshold, however only two samples gave a difference between a positive compared to a below threshold result. Of these two, one sample suggested a result of just below the cut-off threshold indicating a borderline result and the other gave an increased deviation in the cross-over point (Cpd) value suggesting that with an increase in replicates these results may be more defined.

For the remaining 16 samples showing positive values for both primer sets, The Welch two sample t-test showed no significant difference between the means of the samples from the PbML1F/R and PbITS3/4 primer sets, with t= 0.2615, df = 29.733 and p-value = 0.7955. Overall R=0.0995, thus showing a strong correlation between primer sets PbML1F/R and PbITS3/4 when using Pearson's Product Moment Correlation Coefficient (Figure 6).

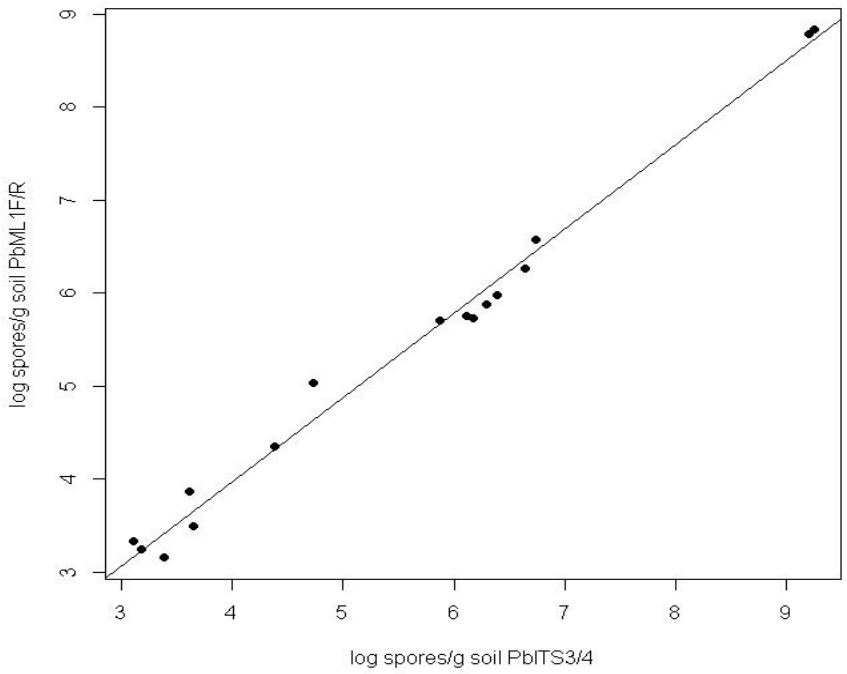

Figure 6. Correlation of *P. brassicae* quantification from soil by PbITS3/4 and PbML1F/R.

4.1.5. Evaluation of 2012 soil samples for *P. brassicae* resting spore concentration by using lateral flow devices and qPCR and comparisons between the two methodologies

Lateral Flow

Of 53 commercial vegetable field soils tested, 32 would be considered at high risk for sown Brassica crops, developing severe and uniform disease expression when grown under environmental conditions conducive to the disease development. All soils for this group were recorded at a *P. brassicae* resting spore concentration of $1x10^6$ g^{-1} soil or greater (Figure 7). Twenty three of these soils recorded resting spore concentrations between $1x10^7$ and $1x10^9$ resting spores g $soil^{-1}$ which in terms of disease risk is exceptionally high. Six field soil samples were considered to be at moderate to high risk of the disease with resting spore concentration circa $1x10^4$ to $1x10^5$ resting spores g^{-1} soil. Soil samples which recorded a resting spore concentration between $1x10^3$ and $1x10^4$ resting spores g^{-1} soil were graded at low to moderate risk of clubroot disease development. The remaining five samples recorded values of less than $1x10^3$ spores g^{-1} and are considered to be free / low risk of the disease. Ultimately the development and expression of the disease will be dependent on a number of factors: the resting spore concentration, the conducive or suppressive nature of the soil type, the environmental conditions over the growing season and the vegetable Brassica cultivar planted.

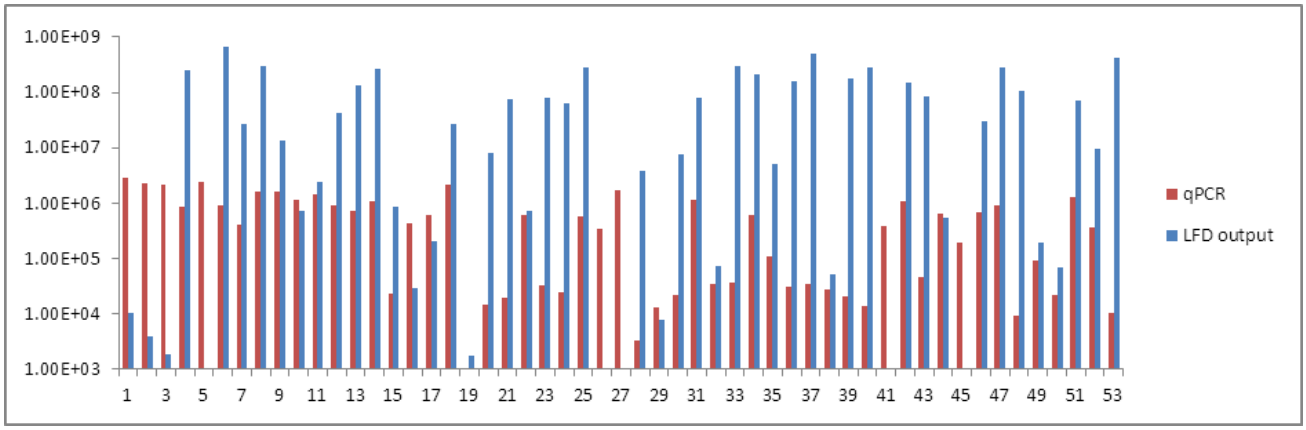

Figure 7: Lateral flow and qPCR results for the "Series 12" collection of 53 UK vegetable soil samples

qPCR

None of the 53 commercial vegetable field soils when tested by molecular quantification were considered at or $>1x10^7$ resting spores g^{-1} soil (very high clubroot risk) (Figure 7). This is at variance to the lateral flow test which identified 23 soils at or above this level. Nevertheless 29 soils were recorded to have resting spore concentrations at circa $1x10^6$ resting spores g^{-1} soil which would place sown Brassica crops at high risk of developing severe and uniform disease expression when grown under environmental conditions conducive to the disease development. A further 20 soils were

identified as moderate to high risk with resting spore concentrations circa $1x10^4$ to $1x10^5$ resting spores g^{-1} soil. A single field soil (28) was identified at low risk to the clubroot disease. Only one of the samples tested was determined to be below the detection threshold of $1x10^3$ spores g^{-1} soil and was rated as free / low risk to the disease (soil 19) .

When the distribution of measured sample qPCR spores g^{-1} soil was plotted against the confidence limits of the predicted qPCR spores g^{-1} (generated from regression of the LFD OD values), 17 (36.6%) fell outside the confidence intervals while the remaining 30 were inside (64%) (Figure 8).

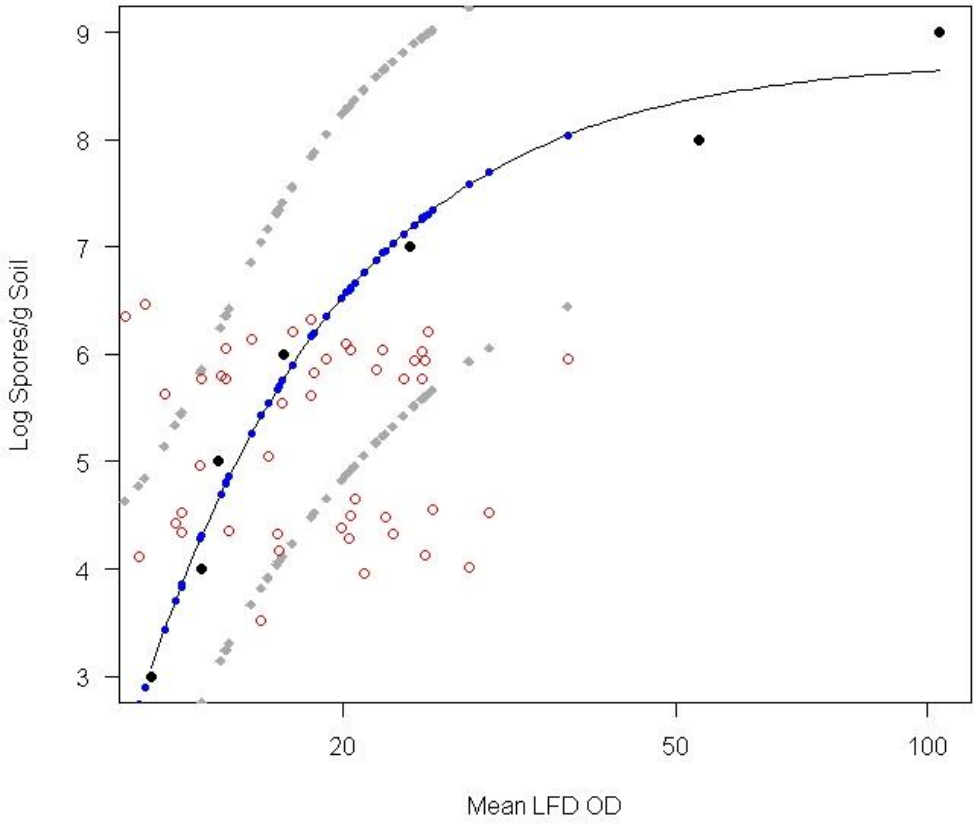

Figure 8: Predicted and actual log spores g^{-1} soil from LFD and qPCR. Black points and line = 4 parameter logistic regression of LFD and qPCR standards, blue points = predicted output based on LFD OD, open red circles = actual LFD against qPCR results, grey points = confidence intervals for predicted outputs.

4.1.6. **Lateral flow shelf life: Antibody and antigen stability**

With the exception of the conjugate pads which received a sucrose concentration of 10% or greater, the immune-reactivity of each was compromised over time when compared to a conjugate pad which had been prepared within 1hr of production (*i.e.* fresh / no storage) (Figure 9). This confers with other studies which report improved immune-stability when samples were stored at a 2% sucrose concentration.

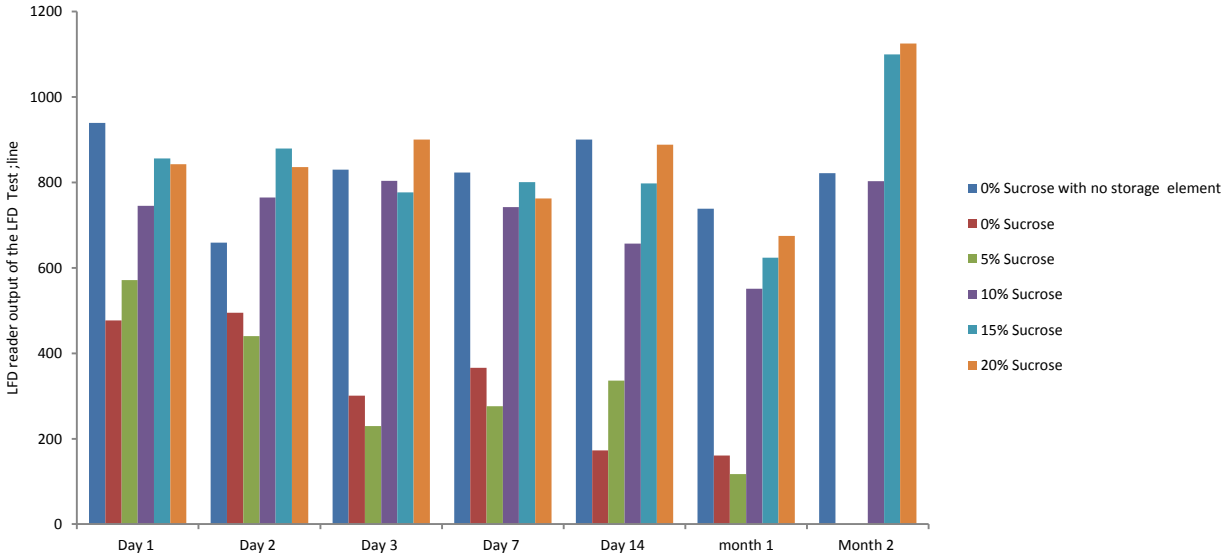

Figure 9: Time course study to determine the immune-reactivity of the air-dried conjugate pads over time

Over an eight month period the addition of both Trehalose and Sucrose to the standard test line application enabled both visual and OD test stability to be retained and at a sensitivity similar to that recorded at the initial time of application (Figure 10). In contrast the standard test line application gave rise to a 50 % reduction in test line signal after a two month storage period. This equated to near or no test line visability when viewed by visual eye recognition. An OD reading however could still be generated using an ESE reader but a reduced signal. After two months the OD reading was at 0 for both the standard test line application (no sucrose applied) and at a 5% sucrose application.

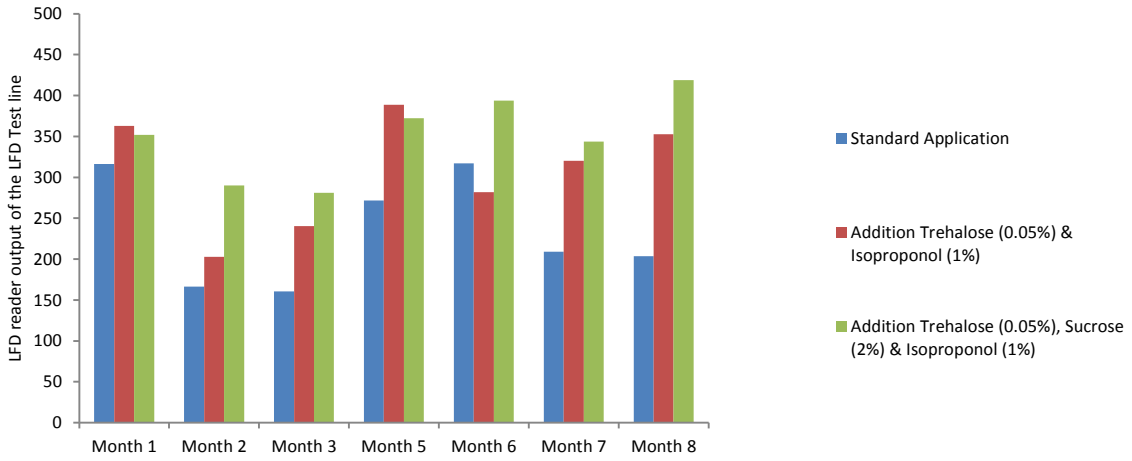

Figure 10: The effect of Trehalose, Sucrose and Isopropanol on the stability of *P. brassicae* resting spores when applied as a test line to an LFD membrane and monitored over time

The results in Figure 11 show that the disruption of P*. brassicae* resting spores to release soluble antigen prior to test line application has no effect on LFD test line stability when applied in a 10% sucrose application buffer.

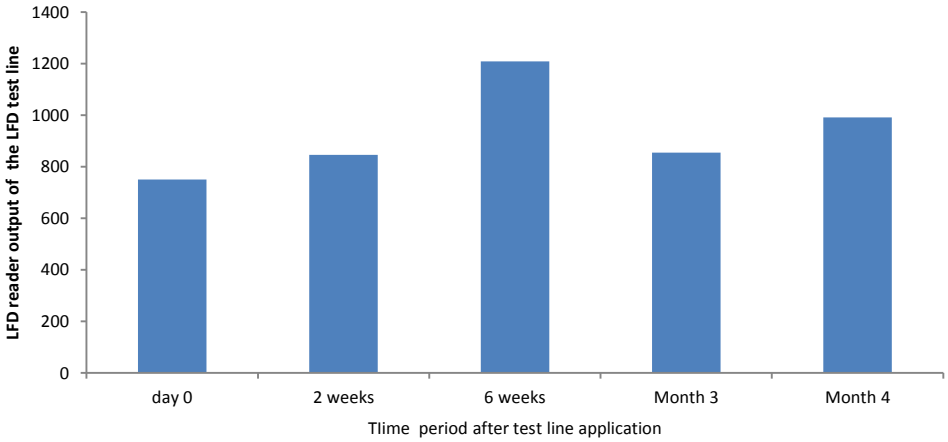

Figure 11: Stability of disrupted *P. brassicae* resting spores in a 10% Sucrose, 0.05% trehalose, 1% isopropanol and 0.05% sodium azide application buffer when applied to a LFD membrane and the test line measured over time.

4.2. Validation of the in-field test in arable soils (2013-2016)

4.2.1. Collection of soils with a history of clubroot disease from arable farms in Scotland and England

The soil samples collected from Scotland and England in 2014 and 2015 contained a wide range of *P. brassicae* resting spore concentrations, as quantified by qPCR (Figures 12 and 13). Fields 1 to 14 were Scottish sites of which fields 1 to 7 were harvested in 2014 and fields 8 to 14 were harvested in 2015 (Figure 12). Fields 15 to 22 were English sites with fields 15 to 18 harvested in 2014 and fields 19 to 22 harvested in 2015 (Figure 13). The maximum number of spores g^{-1} soil in all samples was measured at $2.83x10^6$ by qPCR, while one field tested negative for clubroot disease (field 22). Repeated measures two-way ANOVA showed no significant difference in spores g^{-1} soil at the time of planting and the time of harvest. A more valid comparison may be to test the field from one year to the next at the same points, rather than testing pre-planting and at harvest (as clubroot exists both as resting spores and as galls on the surviving root system at harvest).

The majority of fields tested were planted with resistant cultivars; 16 planted with cv. Cracker and two with cv. Mentor (fields 1 and 8), while two other fields were planted with cv. Temple (fields 6 and 13) and one field was planted with cv. Extrovert (field 20) and one was planted with cv. Picto (field 18). There was no significant difference in planting and harvest spore numbers in the resistant cultivars (cv. Cracker and cv. Mentor), or the susceptible cultivars (cv. Temple, cv. Extrovert and cv. Picto).

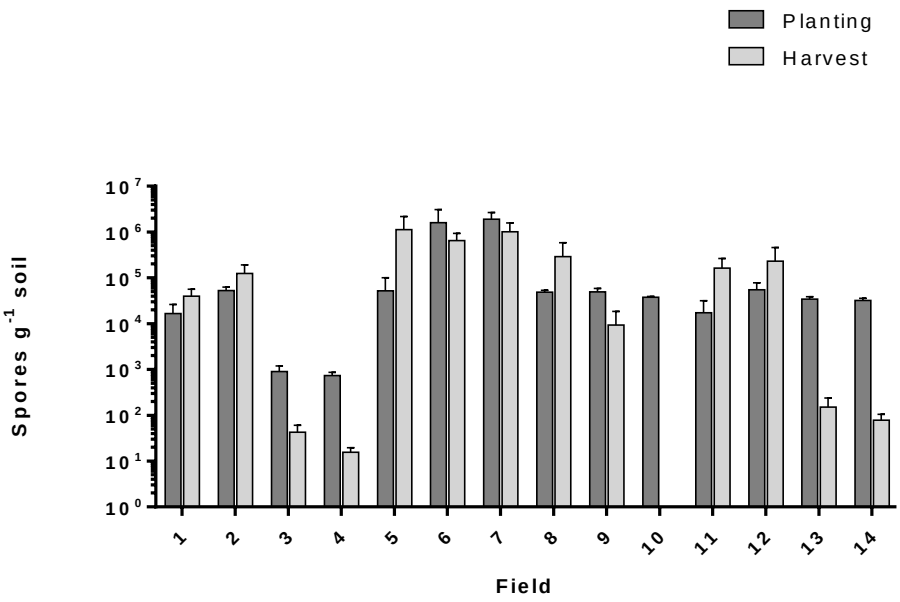

Figure 12. *P. brassicae* levels in Scottish arable soils at the time of planting and time of harvest, as quantified by qPCR.

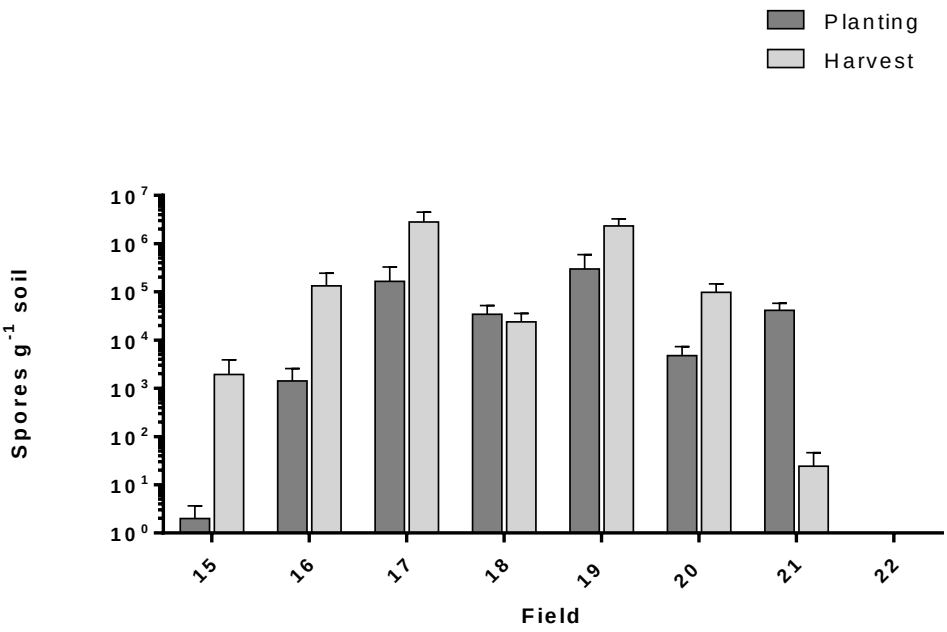

Figure 13. *P. brassicae* levels in English arable soils at the time of planting and time of harvest, as quantified by qPCR.

4.2.2. **Comparison of *P. brassicae* spore numbers in soil by qPCR and LFD**

The number of *P. brassicae* spores g^{-1} soil quantified by qPCR and LFD showed no relationship (R^2=0.02) by linear regression (Figure 14). There were three soils which showed positive results by qPCR but not by LFD, while there were 37 soils which showed positive results by LFD but which were negative by qPCR.

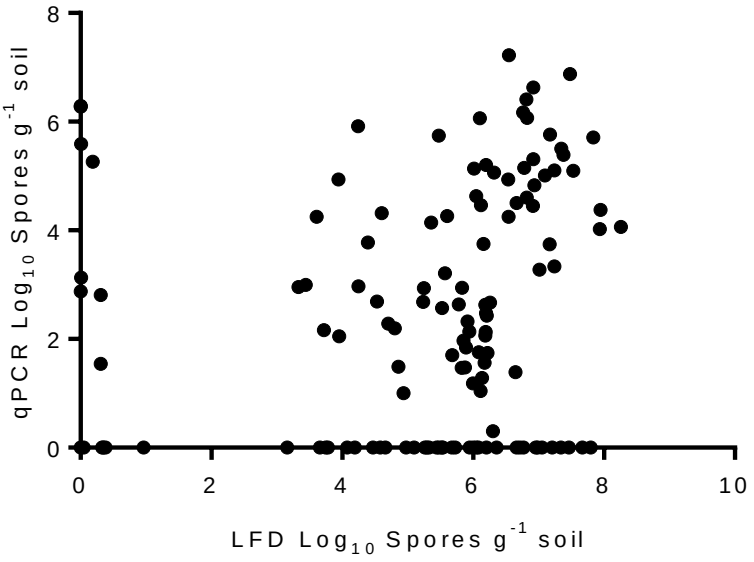

Figure 14. *P. brassicae* spore numbers in soil samples collected from Scotland and England and quantified by qPCR and LFD

4.2.3. The relationship between resting spore concentration and visible symptoms on oilseed rape cultivars (glasshouse)

In both the resistant and susceptible cultivars the largest root damage was observed at the higher *P. brassicae* spore concentrations (Figure 15). However, these experiments experienced some difficulties in applying a measure of clubroot disease, such as the disease index of Dixon and Robinson (1986), due to the symptoms of the damage. Control samples using Chinese cabbage in these pot tests show that the symptomology on vegetable brassicas may be different to those observed in oilseed rape crops (Figure 16). One-way ANOVA did show a significant effect of increasing *P. brassicae* spore number on % root destroyed in both Scottish and English soils for the resistant and susceptible cultivars (Scottish soil, cv. Cracker; $F = 82.87$, $P < 0.0001$, Scottish soil cv. DK Cabernet; and $F = 95.65$, $P < 0.0001$, English soil, cv. Cracker; $F = 37.57$, $P < 0.001$, English soil, cv. DK Cabernet; $F = 48.42$, $P < 0.001$). A visible decrease in galling was observed on cv. DK Cabernet with decreasing spore concentration, as shown on oilseed rape seedlings grown in Warwickshire soil in Figure 17 (a,b). Roots of Cracker seedlings grown in the same soil are also shown for comparison (Figure 17c,d,e,f).

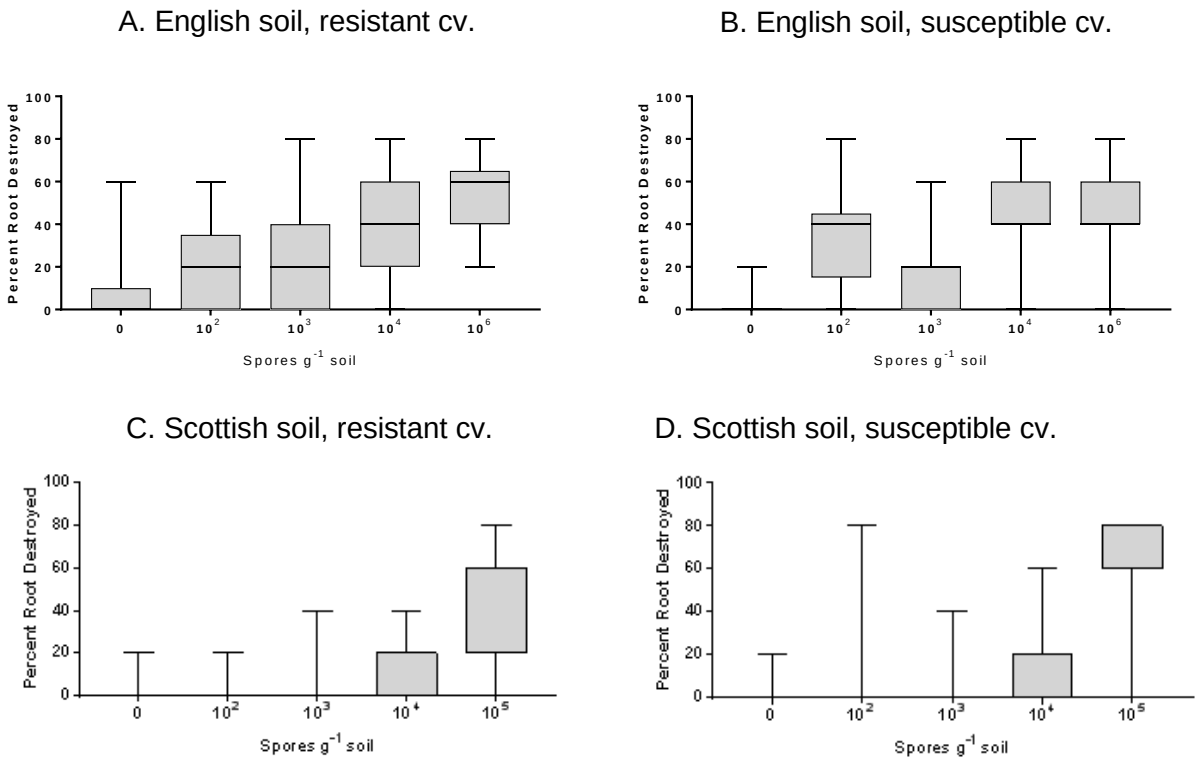

Figure 15. Root damage in seedlings grown in English or Scottish clubroot infected soils at different concentrations of *P. brassicae*, in a resistant and susceptible OSR cultivar.

Figure 16. The roots of Chinese Cabbage cv. Grannat seedlings grown in a soil from Warwick at 10^6 spores g^{-1} soil

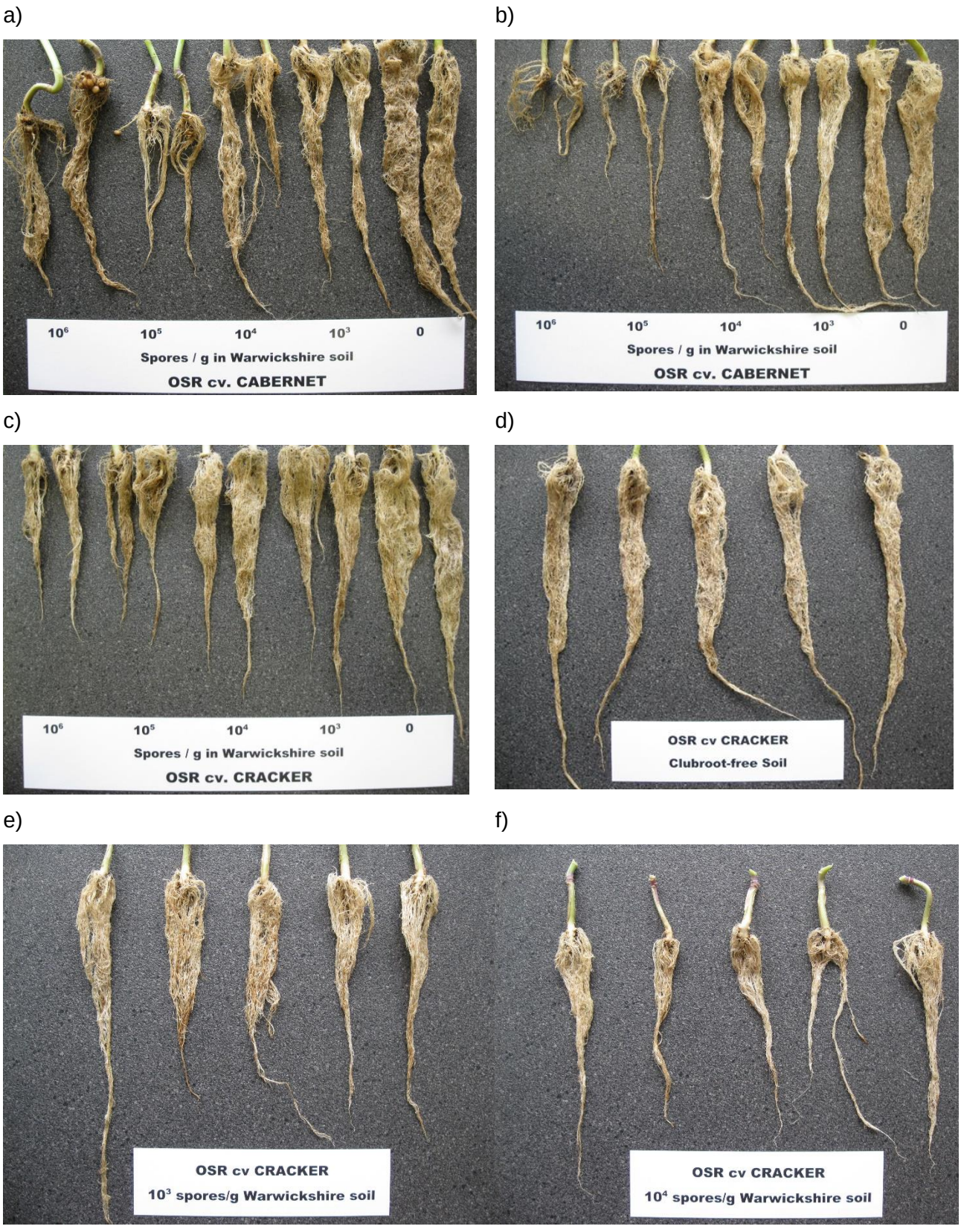

Figure 17. The roots of oilseed rape cvs. DK Cabernet (a,b; susceptible) and Cracker (c- f; resistant) seedlings grown in a soil from Warwick which contained different concentrations of *P. brassicae* resting spores.

4.2.4. Evaluation of the effect of oilseed rape cultivar on clubroot disease development (glasshouse)

In both soils cv. Cracker (resistant) became infected with small amounts of clubroot. In the English soil cv. Temple had the highest numbers of clubroot root galls, while in the Scottish soil cv. PR46W21 showed the greatest degree of disease. For the English soils most cultivars tested were similar in the numbers of root galls produced and in their score (scores 46 – 95) using the Dixon Disease Index scale (Dixon and Robinson, 1986). Only the resistant cultivar (cv. Cracker) had a low score (score 3.7). For the Scottish soils none displayed high levels of clubroot disease (>26% disease index) (Figure 18). Note, however, that the level of *P. brassicae* in the Scottish soil was lower than the level of *P. brassicae* in the English soil which may explain the difference in disease indices as overall the Scottish disease levels were lower than the English. Figure 19 illustrates disease symptoms on a range of cultivars.

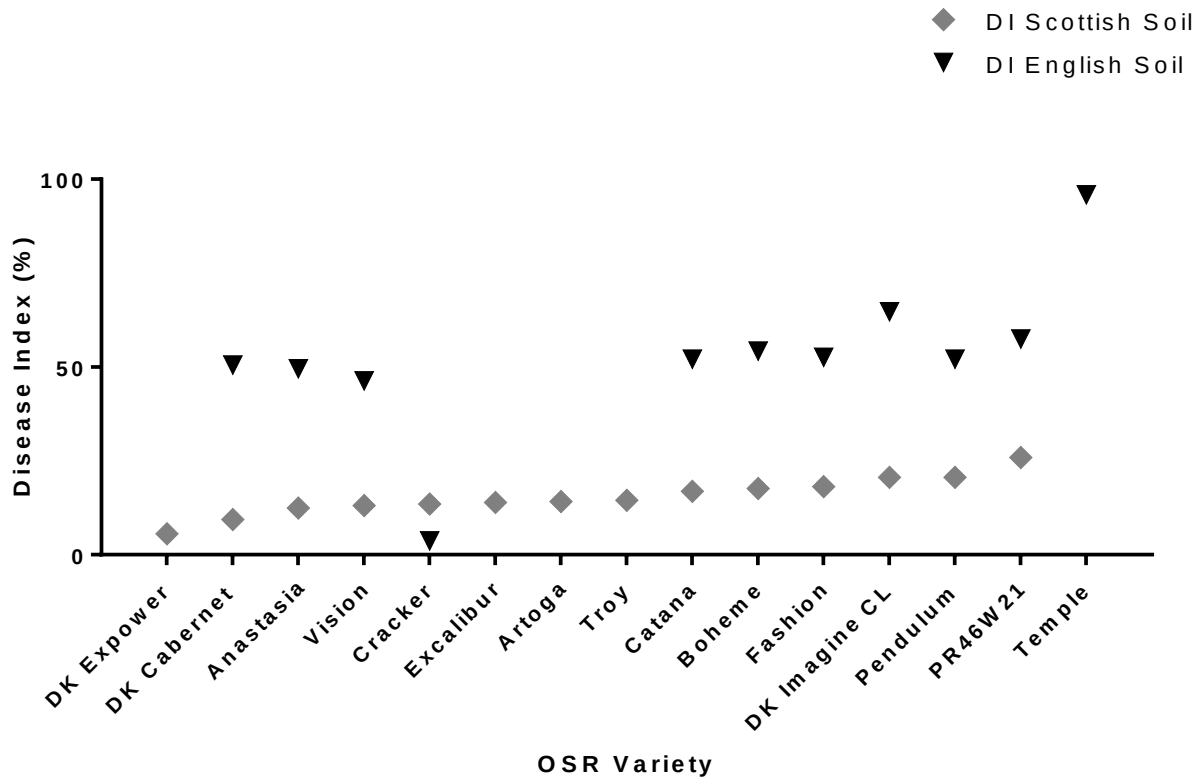

Figure 18. The incidence of clubroot disease (measured disease index; DI) on a variety of oilseed rape cultivars grown in clubroot infected soil. Where there is no disease index the cultivar was only grown in one of the soil types. Note that the Scottish soil contained $1x10^5$ spores g^{-1} and the Warwickshire soil contained $1x10^6$ spores g^{-1} soil.

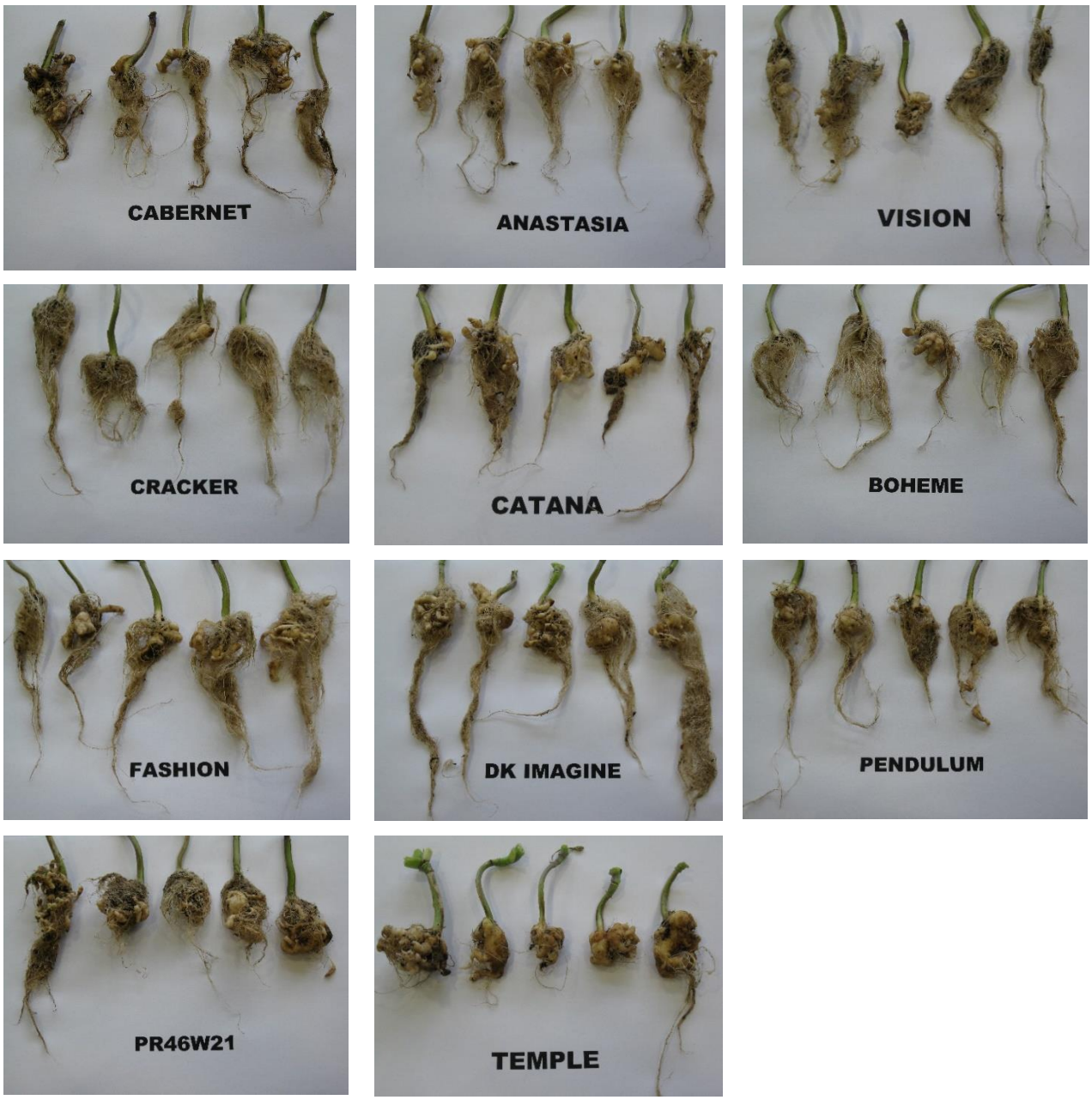

Figure 19: Symptoms of clubroot galling on seedlings of different oilseed rape cultivars grown in soil containing *P. brassicae* at 1 x 10^6 spores g^{-1} soil. Tops of plants were removed prior to photography. Scores on Disease index scale (%): Cabernet 50.5, Anastasia 49.5, Vision 46.3, Cracker 3.7, Catana 52.1, Boheme 54.2, Fashion 52.6, DK Imagine 64.7, Pendulum 52.1, PR46W21 57.4 and Temple, 95.8.

4.2.5. The relationship between clubroot spore number in soil and yield in oilseed crops

A weakly positive relationship existed between cv. Cracker (resistant) seed weight (t Ha^{-1}) and number of *P. brassicae* spores in soil when sampled during the growing season (Pearson's Product Moment Correlation, r = 0.46, n = 38, p = 0.003, two-tailed) (Figure 20), and this association was stronger but not significant at the time of harvest (Pearson's Product Moment Correlation, r = 0.61, n = 8, p = 0.11, 2 tailed). However linear regression was not able to explain the variance of points from the line of best fit with a low R^2 values achieved at both sampling times.

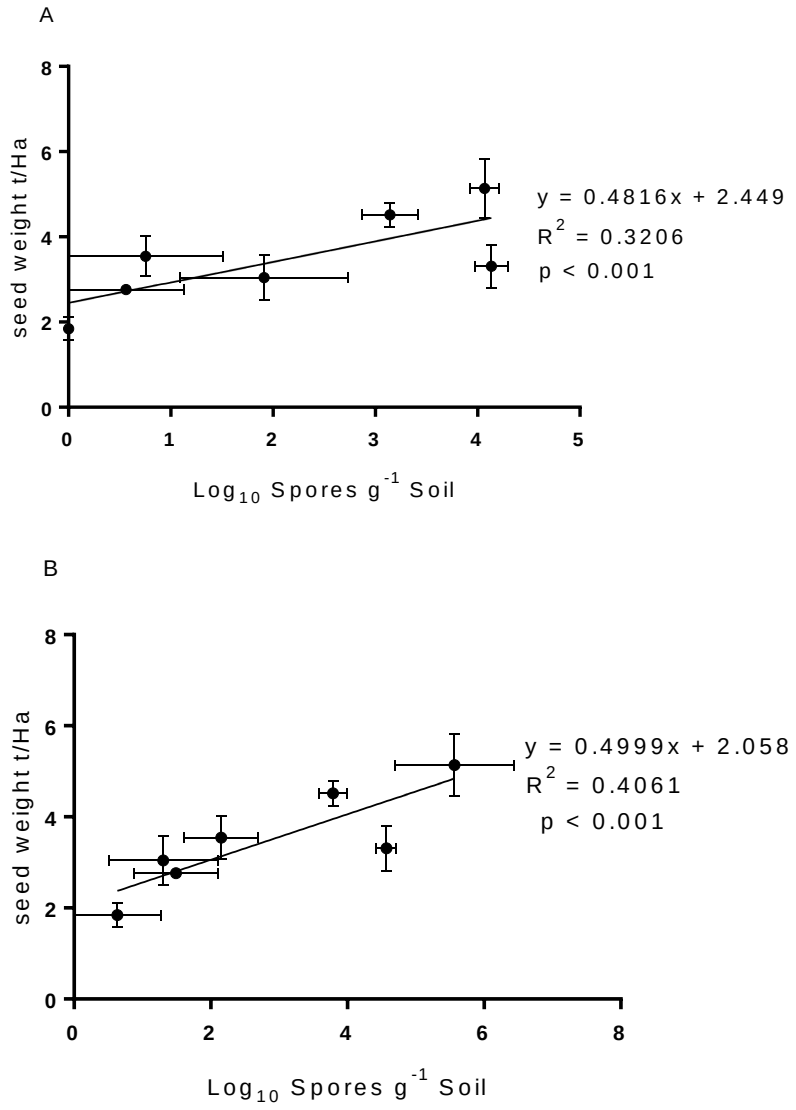

Figure 20. Relationship between OSR seed weight (cv Cracker) and *P. brassicae* spore number A) at the time of planting and B) at the time of harvest

Scottish Agronomy sites

The yield of seven cultivars in the same fields in two regions over three years are shown in Table 2. There was a significant effect of both cultivar and year on yield in the Culross region ($F=5.394$, $P<0.05$, and $F= 8.032$, $P<0.05$ respectively), while only the year was significant in the Borders region ($F = 71.14$, $p<0.0001$. Note that cv. PR46W21 was excluded from two-way ANOVA due to absence of data for 2012). *Plasmodiophora brassicae* had been detected across the fields during 2015 at mean levels of 3.36×10^4 ($\pm 5.3 \times 10^3$ s.d.) spores g^{-1} soil in Culross and 3.68×10^4 ($\pm 1.35 \times 10^4$ s.d.) spores g^{-1} soil in the Borders. These values were obtained as the mean from samples taken within the cv. Cracker plots at the time of planting, based on three soil samples tested by duplicate DNA extraction. At the time of 2015 harvest, two soil samples were taken from seven cultivars, thus giving a total of 14 samples across the field. At this time (harvest) the levels of clubroot per gram of soil had decreased to 0 within the Borders site and to just below the limit of quantification ($<10^3$ spores g^{-1}) at the Culross site.

When the years were classified as *P. brassica* 'not detected' or 'detected at low levels' (based on the detection of *P. brassicae* spores in the soil at planting) a t-test showed no significant difference between yield ($t=1.771$ df $=39$, $p = 0.0843$). However, care must be taken with the interpretation of these results. Unlike the Syngenta sites used (see below), where clubroot infection was observed as galls on plants at some sites, no clubbing in any year was observed at the Scottish Agronomy site. Although the qPCR test detected *P. brassicae* there was no visible disease development at the site. Most of the cultivars used in the trial were highly susceptible to clubroot (see pot trial results) so observable clubbing would be expected to some extent. However, it may be that the root sampling frequency of two plants per cultivar was insufficient to find clubs at this level of spores.

Table 2. Yield (t Ha^{-1}) of seven OSR cultivars in three years, where *P. brassicae* was either not detected in soil or detected at 'low' levels (~$3x10^4$ spores g^{-1} soil) at planting. In each year, all cultivars were grown in the same field (within each region).

Site	Cultivar	Year	Yield	*P. brassicae* detected using qPCR
Culross	Vision	2015	6.07	Y
		2014	5.09	N
		2013	5.35	N
	PR46W21	2015	5.31	Y
		2014	4.57	N
		2013	5.35	N
	DK Cabernet	2015	6.32	Y
		2014	5.05	N
		2013	5.09	N
	Cracker	2015	4.67	Y
		2014	4.22	N
		2013	5.03	N
	Boheme	2015	5.56	Y
		2014	5.63	N
		2013	5.87	N
	Troy	2015	5.58	Y
		2014	4.42	N
		2013	5.40	N
	Anastasia	2015	6.31	Y
		2014	5.82	N
		2013	5.97	N
Borders	Vision	2015	5.13	Y
		2014	5.50	N
		2013	3.80	N
	PR46W21	2015	4.90	Y
		2014	5.13	N
		2013	Not Present	N
	DK Cabernet	2015	5.18	Y
		2014	5.35	N
		2013	4.14	N
	Cracker	2015	4.92	Y
		2014	5.38	N
		2013	3.34	N
	Boheme	2015	5.16	Y
		2014	6.14	N
		2013	3.72	N
	Troy	2015	5.17	Y
		2014	5.52	N
		2013	4.26	N
	Anastasia	2015	5.72	Y
		2014	6.28	N
		2013	3.95	N

Syngenta-funded trials

The result of the yield trial, using a resistant and susceptible variety, are shown in Figures 21-26. Clubroot was not detected at all sites used in the trial and only sites where clubroot was detected at planting were used in this analysis. There were nine locations where the Syngenta yield trials were conducted. At each location there were two separate yield comparisons. Yield comparisons were conducted on areas where there was high and low clubroot concentrations at each location. At some sites clubroot was detected as hotspots within the field. There was little or no difference between sites when *P. brassicae* spores per g^{-1} soil at the beginning of the trial were compared to plant infection levels observed at the end of the trial for the susceptible variety used in the trial (Figure 21 and 22). High yields were observed even in the presence of moderate to high clubroot levels. This may have resulted from the uneven distribution of clubroot in the soil at the sites used for the trials. While clubroot was detected in the plots some areas of the plot were less affected by the disease. Some variation in plant infection levels within the plot were observed but in the absence of larger sample volumes the level of clubroot could not be accurately represented across the plot.

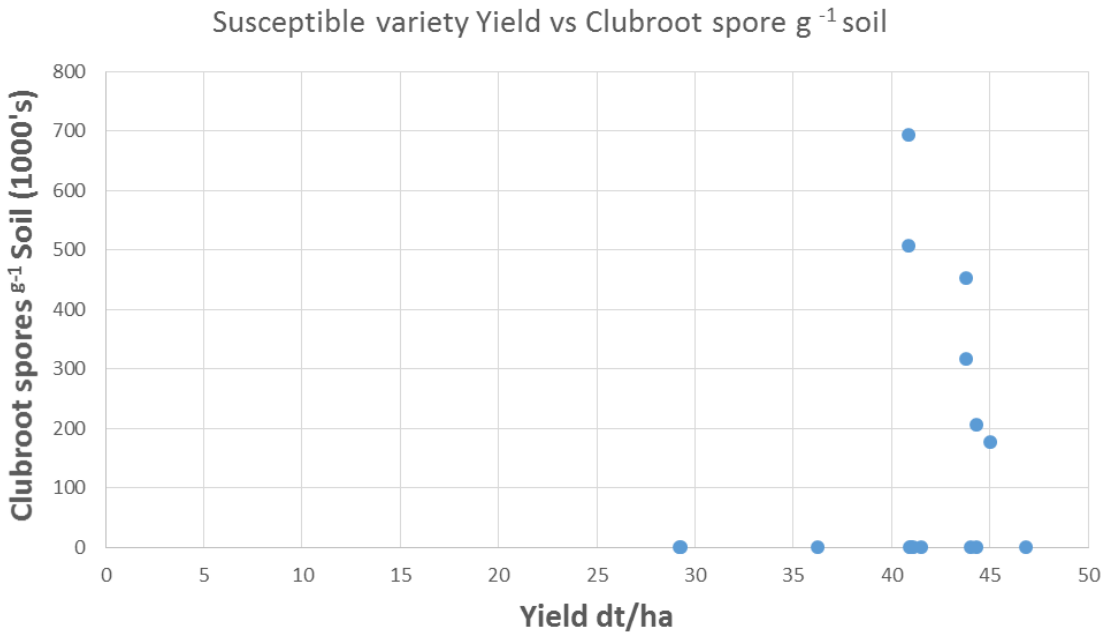

Figure 21. Yield of a susceptible variety (dt/ha) in comparison to clubroot concentration at planting (qPCR test). Each point represents one yield comparison. Sites which appear to have zero clubroot have low levels of detectable clubroot, due to the scale being in 1000's

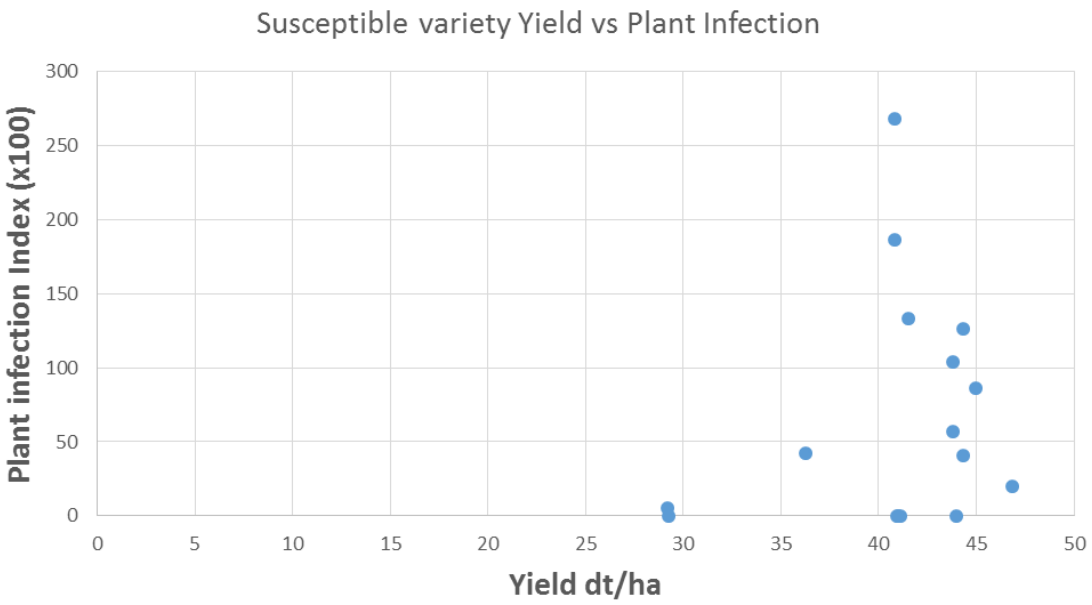

Figure 22. Yield of a susceptible variety (dt/ha) in comparison to clubroot infection at harvest as observed on the susceptible variety.

The effect of clubroot concentration on yield of resistant oilseed rape varieties is shown in Figure 23. Higher yields of oilseed rape were observed in the presence of heavy clubroot infestion g $^{-1}$ soil (Figure 23). A similar result was observed where plant infection at harvest was used to determine clubroot soil infection level (Figure 24).

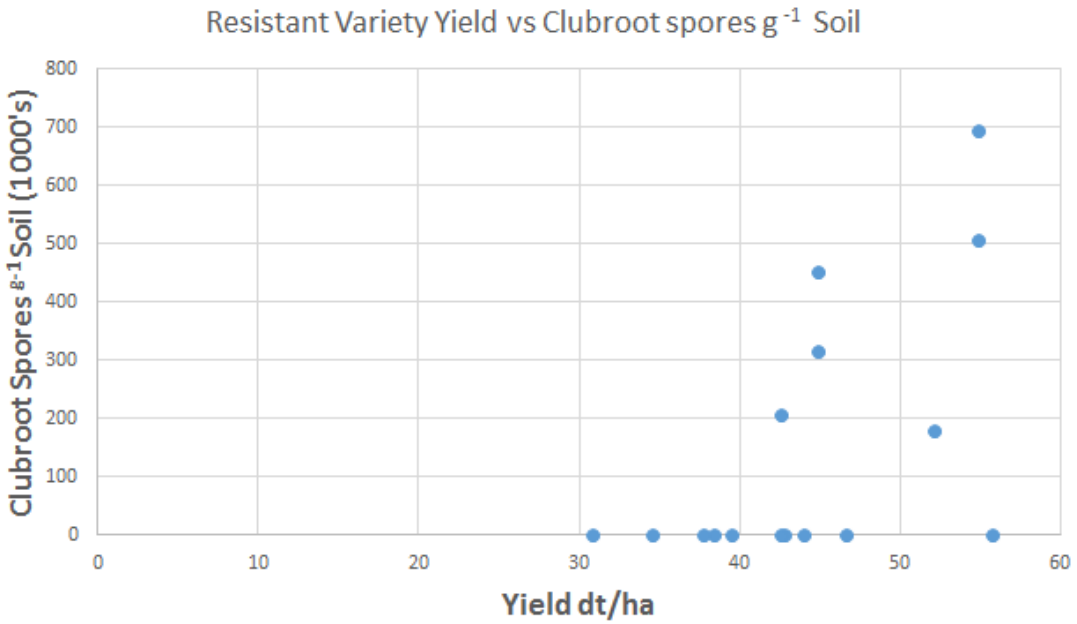

Figure 23. Yield of a resistant variety (dt/ha) in comparison to clubroot concentration at planting (qPCR test)

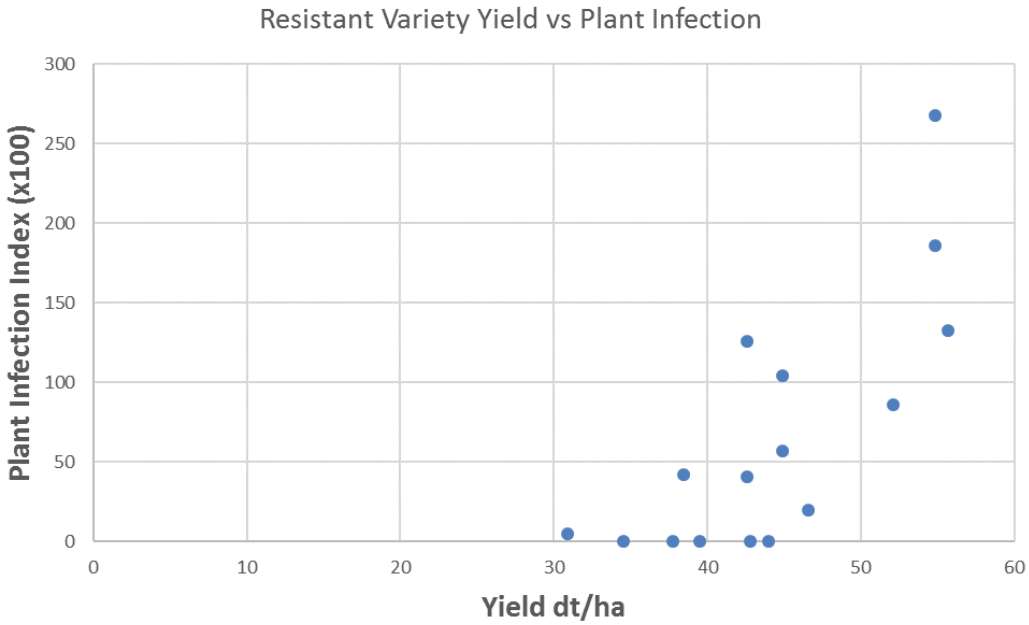

Figure 24. Yield of a resistant variety (dt/ha) in comparison to clubroot infection at harvest as observed on the resistant variety

All sites where clubroot resting spores were present at planting had consistently higher yields where clubroot resistant varieties were used in comparison to clubroot susceptible varieties (Figure 25). Only at one site was there a slightly higher yield for the susceptible variety when compared to the resistant variety. The results indicate that growing clubroot resistant varieties will improve yield in the presence of clubroot in soil. However, there was no effect of clubroot on the increase in yield measured on the susceptible variety in comparison to the resistant variety.

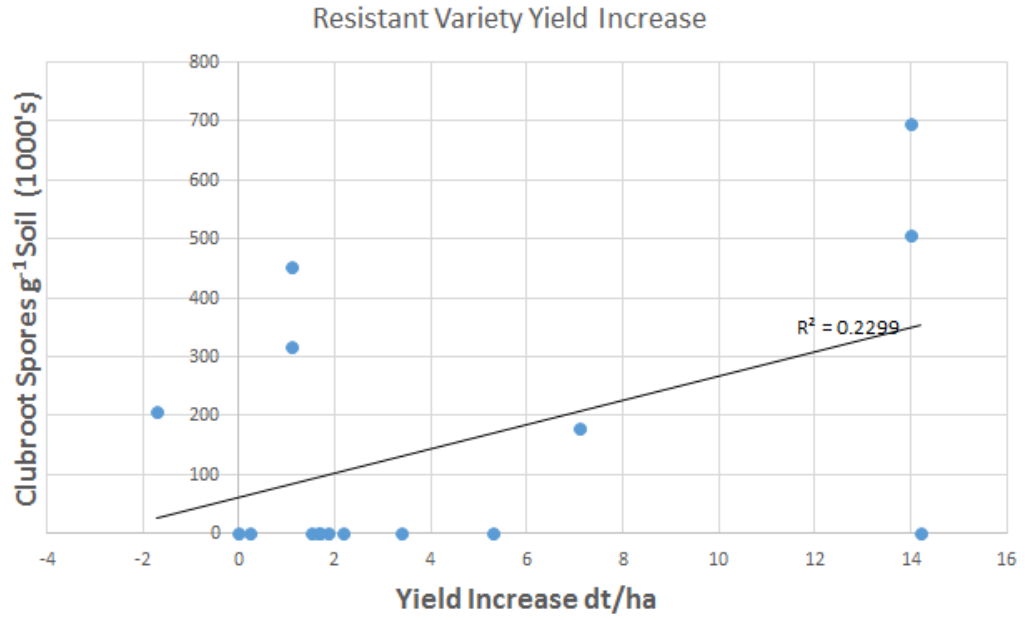

Figure 25. Yield increase of a resistant variety (dt/ha) in relation to clubroot spores present at planting (qPCR test)

The relationship between clubroot spore number at sowing and plant infection at harvest are shown in Figure 26. There was a good relationship between the clubroot levels detected in soil and subsequent infection on plants ($r^2 = 0.726$). This indicates that clubroot quantification at sowing in soil using a diagnostic test is a good indicator of subsequent plant infection. However, this does not necessarily allow for a determination of likely yield loss for oilseed rape. In another study yield loss in oilseed rape crops was correlated with clubroot incidence in the crop. A yield loss of 0.03 t/ha per 1% of plants infected was reported (AHDB Cereals and Oilseeds Project Report 487).

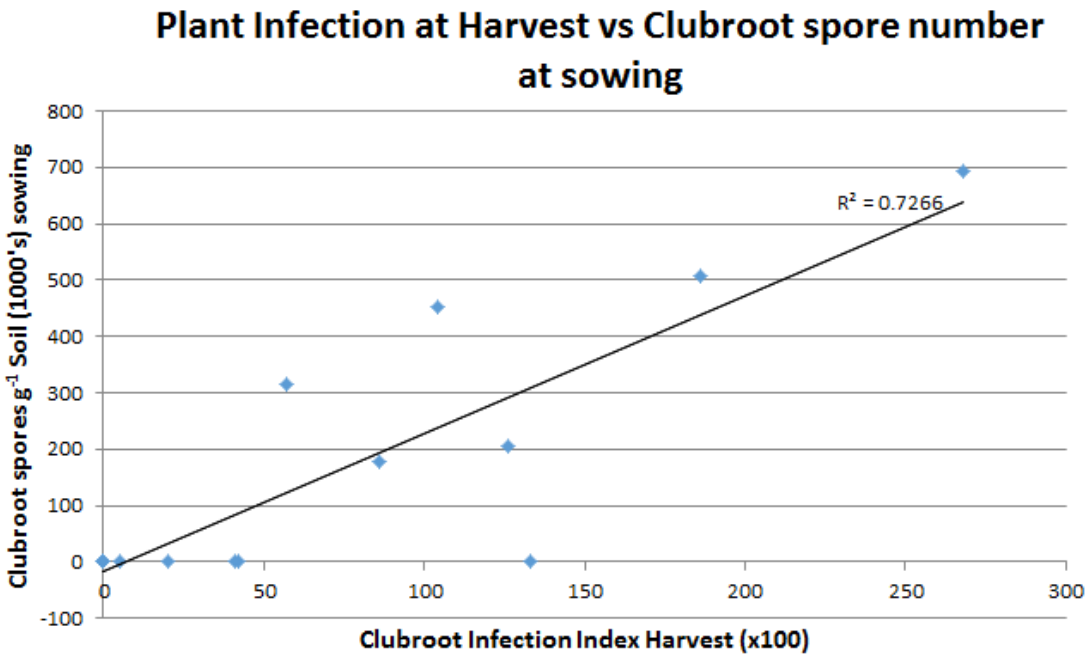

Figure 26. *P. brassicae* concentration at sowing compared to clubroot plant infection index at harvest

5. Discussion

Clubroot is capable of causing economic losses to brassica growers by yield reduction and unmarketable crops. Being able to detect and quantify clubroot resting spores within the soil would enable growers to make informed planting decisions, including whether to sow resistant or susceptible varieties to maximise yields, whether to increase or decrease their crop rotations and whether to incorporate possible control measures such as the application of various forms of lime or other calcium based products. Clubroot has been an important disease in vegetable brassica production for several hundred years. Once soil is contaminated by clubroot it is impossible to eradicate it. Relatively recently arable brassicas have become susceptible to the clubroot pathogen. Oilseed rape is used as a break crop in arable rotations and is grown commonly over wide areas.

The occurrence of clubroot on this crop makes this potentially significant and very damaging for oilseed production in the UK.

The use of detection systems for clubroot is one tool which will help limit the impact of clubroot through identification of sites at risk if oilseed rape is grown. Detection of clubroot will identify areas unsuitable for oilseed rape production or determine the appropriate cultivar which should be used at that site. As a result of this project a new commercial soil test is now offered for clubroot detection in soil. This is based on a molecular test which is offered as a laboratory service. This test can reliably determine the level of clubroot in the soil but it is relatively expensive. The work in this project had the objective of developing a cheaper and easier test for clubroot detection in soil; the aim was to validate an in-field detection test for clubroot based on a lateral flow device format, while also investigating how the quantity of clubroot resting spores is related to the level of plant infection observed and the resulting effect on yield.

One issue which has been addressed in this project has been the shelf life of the LFD test. Work has been carried out on the stability of the clubroot *clfd* test line antigen which has proved to be the main component affecting shelf life. The addition of components to the test line fraction, prior to *clfd* membrane striping, has shown no benefit in attaining a robust test over time. In addition, competition in binding of the clubroot antigen and the incorporated proteins to the membrane was observed and test line optical density values (OD) reduced accordingly. Test line and activity of the dried antibody conjugates in the LFD prototype showed an improved and retained level of stability when sucrose was incorporated as an additive. The concentration of sucrose application proved important in retaining test sensitivity. Sucrose is a known stabiliser and during drying provides a structure around the antibody to assist retention of protein conformation and in doing so maintain the chemical stability, resist denaturation, aggregation and the loss of biological activity of the antibody.

Poly-L-Lysine, a positively charged polymer, routinely used as a binding support for biological material, did not enhance *P. brassicae* binding or retention to the membrane test line application area. Additional studies since have however determined that heat and protease affect the resting spore test line antigen and antibody binding. No effect was observed when a DIG glycan differentiation kit was used indicating that a glycoprotein is not involved in the complex binding between the *P. brassicae* antigen and the monoclonal antibody (MAb). Generally it has been observed that where resting spore fungal surface washings have been used to induce an immune response, the resultant antibodies have bound to glycoprotein fungal antigenic determinants (Macdonald *et al*., 1989, Werres & Steffens, 1994). Analysis of the chemical composition of *P. brassicae* resting spore wall (Buczacki and Moxham, 1983) found no evidence of glycoprotein complexes.

The relationship between quantification by LFD and qPCR is non-linear, and results indicated the LFDs overestimated the number of spores present in samples. Disparity between the two tests may be explained due to a number of reasons, including that the qPCR measures actual DNA (and the expectation would be one genome per resting spore), whereas the LFD measures the amount of antibody binding to the resting spore walls and soluble proteins associated with the resting spore. It is also possible that the antigen is not consistently expressed and concentrations could vary depending on lifecycle stage, age of spore and other environmental factors. For example, it is not known at this stage whether the amount of soluble antigen would be consistent across different *P. brassicae* populations, and there may be an influence of *P. brassicae* race/ pathotype on the level of soluble antigen released. Also it is possible to observe 'empty' resting spores within samples in which the nucleic acids are fluorescently stained (data not shown), and in such a scenario the 'empty' spores would not be detected by qPCR as they do not contain DNA. However, using an antibody based system, the resting spore wall may provide a binding site for the antibody whether or not it contains DNA. Thus the viability of the spores could be important in the comparison of the two assay systems, and again this is something that could be influenced by the predominant race/pathotype of *P. brassicae* present.

In addition, the sampling volume will make a dramatic difference to these measures, and a direct comparison of the qPCR and LFD tests is difficult due to the differences in sample volume used. Both methods of detection were unable to detect low levels of clubroot in soils reliably when small sample volumes of soil were used. The current LFD test could be improved by using larger volumes of soil in the test but in the current format this is not possible. The test could be as accurate as the molecular test if it was based on 10 g of soil (as with the molecular test). This would mean pre-extraction of clubroot from the larger volume of soil before using the extract in the LFD system. AHDB Horticulture project CP099a has investigated methods of extraction of clubroot from larger soil volumes (Wakeham et al., 2015).

Due to the complexity of the soil environment and the longevity of *P. brassicae* within the soil, the interaction of resting spores with their environment is also important to consider. The soil environment does not remain constant throughout different seasons, and there are many factors which could potentially have an effect on the resting spores, such as temperature, moisture content, and soil composition (both nutritionally and structurally). It is well established that soil type can also affect the detection and quantification of organisms by the molecular methods of PCR and qPCR. Immunological based techniques (such as LFD quantification) are also reported to be affected by soils types. Otten et al. (1997) observed antibody retention in some soil types (especially in clay) which proved problematic in quantifying fungal pathogens in soil. Researchers have identified various compounds in soil which are referred to as inhibitory substances (Lloyd-Jones and Hunter 2001, Thakuria et al 2008) and although found in many environmental samples, prove particularly

problematic in a complex soil matrix (Menking et al. 1999). Work in AHDB Horticulture project CP099a (Wakeham *et al.* 2015) supported the importance of diluting DNA extracts for accurate quantification of clubroot by qPCR, which results in lower concentrations of inhibitors within the samples.

Previous field studies in vegetable brassicas (Lewis, 2011) have demonstrated variation in soil *P. brassicae* spore levels throughout the course of the growing season. This was not consistently demonstrated for oilseed rape, and in this work when oilseed crops were sampled at the time of planting and the time of harvest there was no significant difference in the spore levels. During the growing season *P. brassicae* is completing its lifecycle by moving into plant tissue and reproducing. That no increase in soil spore load was observed at the time of harvest suggests that the accumulation of spores in the soil may be a more gradual process than expected (not fast enough to be significant over one growing season), possibly as a result of environmental factors. Alternatively, it may be that the roots (and associated gall tissue) had not decayed at the time of harvest and if sampling had been performed after the subsequent cultivations then the spore load in soil could have been higher. This emphasises that an accurate measurement of spore levels is dependent on the timing of soil sampling. The results suggest that quantifying the levels of spores prior to crop planting is key as this is when the spore levels are at or near to their maximum prior to replication as the lifecycle is completed. The results from this work show that the qPCR test can be used to effectively determine clubroot infestation levels in arable soils prior to planting. This test is now commercially available, following validation in project CP099a (Wakeham et al. 2015), and could be used by growers to decide cultivar selections prior to planting. This might reduce the overall pressure on resistant cultivars by influencing their deployment. Given that clubroot infection levels in arable soils were generally high in the soils tested here, the LFD test could be used as an initial screen to detect those soils which have high clubroot infestation levels. It is clear that the LFD test cannot detect reliably low concentrations of clubroot within a format where 0.25 g of soil is tested. However if 10 g of soil could be pre-filtered or processed before testing with the LFD the accuracy of the test at low clubroot contamination levels would be improved.

Growers require knowledge of clubroot risk before planting to enable them to make cultivar choices. In the FV349 final project report (Kennedy et al 2013) thresholds for clubroot disease risk in vegetable brassicas were proposed: $<10^3$ spores g^{-1} soil as low risk (<33% disease severity), 10^3 and 10^4 spores g^{-1} soil as medium risk (33-66% disease severity) and $>10^5$ spores g^{-1}soil as high risk (>66% disease severity). It remains unclear whether the same risk categories apply to the degree of clubroot damage observed in oilseeds as in vegetable brassicas and the relationship between spore numbers in soil and the effect on yield in oilseed rape is not straightforward. Previous work has demonstrated a relationship between plant infection and OSR yield (Project report 487; Burnett et al., 2013), and here Syngenta trials showed that there was a good relationship between clubroot concentration in

the soil at planting compared to clubroot galling on plants at harvest. In oilseed rape, the degree of root damage occurring over a range of spore levels was comparable to that observed in vegetable brassicas although the symptomology may be different in oilseed rape plants compared to vegetable plants. The symptomology on Chinese cabbage controls in glasshouse bioassays was different to that observed in OSR, when uninfected transplants were planted into heavily infected soils which had been used in pot trials with susceptible and resistant OSR varieties. Symptoms of clubroot infection on OSR appears to include a significant degree of root damage to secondary roots and root hairs. Much of the difference may be related to differences in root production between OSR and vegetable brassicas (rooting habit). This is not surprising given that OSR and vegetable brassicas are two distinct Brassica species. The yield loss mechanism between vegetable brassicas and OSR is very different. Vegetable brassicas are transplanted with a relatively low plant number per hectare (50 cm spacing between plants). The low plant spacing means that total crop failure can result from clubroot infection ie whole marketable plant loss. In contrast, OSR is a directly seeded crop with relatively high plant populations per metre square and overall yield is not as dependent on the survival of an individual plant. From observations taken in the crop and from the pot experiments reported in this project clubroot can have an overall impact of reducing OSR plant populations. This will have an impact on yield but surviving plants may negate this effect through yield compensation. It is clear that smaller OSR plants in the canopy are more likely to be heavily impacted by clubroot compared to larger OSR plants.

In previous studies (Lewis, 2011; Kennedy et al. 2013), the effect of *P. brassicae* inoculum in the soil on disease index was assessed in vegetable brassicas and this has also been examined in published studies such as Cao *et al.* (2007) and Webster and Dixon, (1991). Cao *et al.* (2007) looked at the effect of inoculum level on disease index in oilseed rape and found that at $1x10^5$ spores g^{-1} soil the disease index was 41.8%. This is comparable to the levels of disease reported in vegetable brassicas in Lewis (2011) but lower than the levels reported in chinese cabbage (Webster and Dixon, 1991) thus suggesting there can be varietal differences in the relationship between number of spores and disease severity which fits with existing knowledge of cultivar resistance. As demonstrated in this work, the widespread use of resistant cultivars makes it difficult to observe the relationship between disease severity and spore number in commercial field settings, making accurate glasshouse studies and experimental field trials essential to this work. This has cost implications, is resource intensive and relies on accurate timing of spore sampling methods such as qPCR.

In Scotland our studies found very few growers planting anything other than resistant cultivars. McGrann *et al* (2016) found that all OSR growing areas of the UK were infected with clubroot and control by using resistant varieties is generally effective, although McGrann also observed that varietal control is not as effective in areas that have been reliant on growing resistant cultivars for control. During their studies they documented approximately 0.03t ha^{-1} loss in yield for each 1%

increase in clubroot severity in both a resistant and a susceptible cultivar (McGrann *et al.* 2016). In the cultivar trials, where *P. brassicae* was detected in 2015, but no spores were detected in previous years, the spore levels (10^4 spores g^{-1} soil) would fall into the medium risk category of 33-66% disease observed if disease risk followed the same pattern as in vegetable brassicas. Based on McGrann *et al's* (2016) finding this could result in a yield loss of between 0.99 and 1.98 t ha^{-1}, but no significant difference was found in the yield from fields where clubroot was detected and fields where clubroot was not detected. This is interesting because it may demonstrate that it is difficult to accurately determine risk of clubroot disease based on the number of resting spores in the soil alone. It is likely that other factors, such as weather, seeding rate or establishment rate and soil parameters, must also be considered.

Low levels of clubroot disease in OSR may be able to act as an indication to growers that there is a clubroot risk on site, despite little evidence of disease symptoms/galling or reduction of yield in that years planting. For the varieties tested, yields were comparable in 2013, 2014 and 2015 despite clubroot only being detected in 2015. Growers would be able to take the 2015 finding as an indication of future risk and this would allow them to decide on future management strategies such as whether to grow resistant or susceptible cultivars, decrease OSR in their rotations or incorporate control measures.

It is possible to hypothesise that; 1) the severity of disease symptoms observed on oilseeds in response to spore load may be similar to vegetable brassicas, 2) the risk of disease symptoms on the roots would therefore follow the same thresholds as in vegetable brassicas, 3) it is unclear whether oilseeds experience the same loss in yield from any given spore number, despite experiencing comparable disease on the roots and, 4) it is possible that environmental conditions and other factors have a greater impact on yield than low to medium spore levels in oilseed crops. The results from Syngenta field trials in Poland and Germany showed that clubroot could have a big impact on yield. However this was observed only at high spore concentrations of 100,000+ clubroot spores per gram of soil. Another aspect of this was the susceptible cultivar against which yield comparisons could be made. There is adequate evidence that clubroot resistant varieties could be generally lower yielding than susceptible varieties. Therefore the impact of clubroot at individual sites can be confounded by the lower yield observed from clubroot resistant varieties.

There is clear evidence that incorporating resistant cultivars and breaks from OSR cropping into rotations can be part of an effective clubroot control strategy (McGrann *et al*, 2016; Peng *et al.* 2015), and observations from controlled studies demonstrate the different disease responses that occur between resistant and susceptible cultivars. However, in the field samples tested here there was no significant difference observed in the number of spores in the soil before and after planting, even in the resistant cultivars. This is in contrast to the results of Peng *et al* (2015), who found a decline in

spore numbers after one year of planting a resistant variety; a key difference between these studies is the size of the areas sampled which may account for the difference in the results observed. Here, the results were gathered from across the UK, so fields would experience variable environmental conditions, while the results of Peng *et al*. (2015) were based on more localised conditions. Other studies have demonstrated the effect of temperature and soil moisture (McDonald and Westerveld, 2008; Nobel and Roberts 2004; Mattusch, 1977) on clubroot disease, and the effects of climate change on clubroot disease have been predicted, raising concerns that a greater proportion of the UK will experience conditions suitable for the development of clubroot disease, particularly in relation to soil moisture content (Burnett *et al*. 2013). It is possible that resistant varieties can be useful in reducing the levels of resting spores in the soil as part of a clubroot control strategy, but consideration should be given to the prevailing environmental conditions.

Therefore, it can be concluded that risk assessment for clubroot disease in oilseeds needs to be multifactorial and should consider the following as a minimum;

1) Levels of resting spores in the soil
2) Cultivar to be grown
3) Rotation history
4) Prevailing soil texture, pH, calcium and magnesium levels
5) Environmental factors
6) Risk of transmission
7) *P. brassicae* pathotype present
8) Soil amendments

Determining whether clubroot disease will occur is easier than predicting how severe yield loss will be. Evidence from this project suggests that even when clubroot spores are present in soil, the yield may be influenced more by factors affecting plant growth. Knowing the number of spores in the soil can act as an indicator of disease risk but offers limited predictive value of yield loss. Controlled glasshouse trials are useful for studying the response of crops to different spore levels, however the abiotic and biotic factors involved in yield loss are complex, and research will continue to inform grower practice to optimise production at a minimal financial and ecological costs.

6. Conclusions

- A reliable test is available (and now offered commercially) which can detect clubroot level in land prior to planting with OSR
- Yield loss results from only high levels of clubroot contamination.
- Cultivar cracker (resistant) shows some level of clubroot gall formation on roots
- An in-field test is available for detection of high levels of clubroot in contaminated fields
- The LFD test could be improved for detection of clubroot reliably at lower levels of soil contamination if a pre-filtering or concentration step was built into the protocol. This would require larger volumes of soil (10 g)

7. References

Buczacki, S. (1983) Zoosporic Plant Pathogens, London: Academic Press Inc. Ltd

Buczacki, S.T. and Moxham, S.E. (1983). Structure of the spore wall of *Plasmodiophora brassicae* revealed by electron microscopy and chemical digestion, Transactions of the British Mycological Society 80, 297-304

Burnett, F., Gladders, P. Smith, J., Theobald, C. (2013) *Management of clubroot (Plasmodiophora brassicae) in winter oilseed rape.* Project Report No. 487, AHDB. Kenilworth.

Cao, T., Tewari, J. and Strelkov, S. E. (2007) Molecular detection of *Plasmodiophora brassicae,* causal agent of clubroot of crucifers, in plant and soil. *Plant Disease,* 91. p. 80-87.

Dixon, G., and Robinson, D. (1986). The susceptibility of *Brassicae oleracea* cultivars to *Plasmodiophora brassicae* (clubroot). *Plant Pathology*, **35**: 101-107.

Dixon, G. (2009). The Occurrence and Economic Impact of *Plasmodiophora brassicae* and Clubroot Disease. *J Plant Growth Regul* **28**:194–202

Faggian R., and Parsons, S. (2002*). A rapid diagnostic test for clubroot.* Final Report Project VG 99008*.* Horticultural Australia Ltd.

Kennedy R., and Wakeham, A. (2007) *Brassicas: Development and validation of detection tests for clubroot*. Project Number FV259. Final Report. AHDB, Kenilworth.

Kennedy, R., and Wakeham, A. J. (2008). Development of detection systems for the sporangia of *Peronospora destructor*. Eur. J. Plant Pathol. 122:147-155.

Kennedy, R., **Wakeham, A., Lewis, M., Keane, G., Petch, G., Proctor, M., and John, S.** (2010) *Brassicas: Further development of in field tests for resting spores of clubroot and the development of clubroot control based on detection.* Project Number FV349. Annual Report. AHDB, Kenilworth.

Kennedy, R., Wakeham, A., Lewis, M., Keane, G., Petch, G., Proctor, M., and John, S. (2012) *Brassicas: Further development of in field tests for resting spores of clubroot and the development of clubroot control based on detection.* Project Number FV349. Annual Report. AHDB, Kenilworth.

Kennedy, R., Wakeham, A., Lewis, M., Keane, G., Petch, G., Proctor, M., and John, S. (2013) *Brassicas: Further development of in field tests for resting spores of clubroot and the development of clubroot control based on detection.* Project Number FV349. Final Report. AHDB, Kenilworth.

Klemsdal S. S., Herrero, M-L., Wanner, La., Lund, G. and Hermansen, A. (2008). PCR based identification of *Pythium* spp. causing cavity spot in carrots and sensitive detection in soils. *Plant Pathol.* **57**, 877-886

Lewis, M (2011). *Methods for determining environmental factors affecting spore viability of the root pathogen of vegetable brassicas Plasmodiophora brassicae (Woronin).* PhD Thesis. University of Warwick.

Lloyd-Jones, G., and Hunter, D. (2001). Comparison of rapid DNA extraction methods applied to contrasting New Zealand soils. *Soil Biology and Biochemistry.* **33**: 2053-2059.

MacDonald, M.M, Dunstan, R.H, Dewey, F.M. (1989) Detection of low-Mr glycoproteins in surface washes of some fungal cultures by gel-filtration HPLC and by monoclonal antibodies. Journal of general microbiology. 135:375-383

McDonald, M., and Westerveld, S. (2008). Temperature Prior to Harvest Influences the Incidence and Severity of Clubroot on Two Asian Brassica Vegetables. *HortScience* **43** (5) 1509-1513.

McGrann G, Gladders P, Smith JA, Burnett FJ, (2016). Control of clubroot (*Plasmodiophora brassicae*) in oilseed rape using varietal resistance and soil amendments. *Field Crops Research,* **186**, 146–156.

Mattusch, P. (1977). *Epidemiology of clubroot of crucifers caused by Plasmodiophora brassicae.* In: Buczacki, S.T. & Williams, P.H. (Eds), Woronin +100 international conference on clubroot.

Menking, D.E., Emanuel, P.A., Valdes, J.J., Kracke, S.K. 1999. Rapid cleanup of bacterial DNA from field samples. *Resources, Conservation and Recycling* **27**: 179-186

Nobel R., and Roberts, S (2004) Eradication of plant pathogens and nematodes during composting: a review. *Plant Pathology.* **53**, 548-568.

Otten, W., Gilligan, CA., Thornton, CR. 1997 Quantification of Fungal Antigens in Soil with a Monoclonal Antibody-Based ELISA: Analysis and Reduction of Soil-Specific Bias *Phytopathology* **87**(7); 730-736

Peng, G., Pageau, D., Strelkov, S.E., Gossen, B.D., Hwang, S.F., and Lahlali, R.L. (2015). A >2-year break from susceptible canola reduces *Plasmodiophora brassicae* resting spores in heavily infested soil and maximizes canola yield. *European Journal of Agronomy,* **70,** 78-84.

Thakuria, D., Schmidt, O., Mac Siúrtáin, M., Egan, D., Dooham, F.M. (2008). Importance of DNA quality in comparative soil microbial community structure analyses. *Soil Biol. Biochem*. **40**. 1390-1403.

Wallenhammar AC, (1996). Prevalence of *Plasmodiophora brassicae* in a spring oilseed rape growing area in Central Sweden and factors influencing soil infestation levels. *J Plant Path*. **45**, 710–719.

Wakeham, A., Lewis., M., Keane, G., Edwards, E., Petch, G., and John, S (2015*). Validation of the clubroot lateral flow in UK commercial Brassica cropping systems*. Project Number CP099a. Final Report. AHDB, Kenilworth.

Webster, M.A and Dixon, G.R. (1991) Calcium, pH and inoculum concentration influencing colonization by *Plasmodiophora brassicae. Mycol. Res.* **95,** 64-73

Webster, M.A and Dixon, G.R. (1991a) Boron, pH and inoculum concentration influencing colonization by *Plasmodiophora brassicae. Mycol. Res.* **95**, 74-79.

Werres S, Steffens C. (1994). Immunological techniques used with fungal plant pathogens: aspects of antigens, antibodies and assays for diagnosis. Annals of Applied Biology 125: 615-643.