Project title:	Methods for determining environmental factors affecting spore viability of the club root pathogen of vegetable Brassicas *Plasmodiophora brassicae* (Woronin) (HDC Studentship)
Project number:	CP 45
Project leader:	Dr Roy Kennedy
Year of project:	Final
Date of report:	1 November 2010
Locations:	National Pollen and Aerobiology Research Unit, University of Worcester. Worcester
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Project commenced:	1 October 2007
Completion:	30 September 2010
Key words:	Clubroot, resting spores, spore viability

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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.
The project leaders as defined in the Research Funding agreement need to sign the report.

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Signature ..... Date .....

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GROWER SUMMARY

Headline

Magnesium and ammonium adversely affect clubroot development. Control of clubroot could be achieved using nutrient amendments to the soil but would this would be dependent on soil type.

Background and expected deliverables

Clubroot caused by the protist *Plasmodiophora brassicae* Woronin is one of the most intractable plant pathological problems of cultivated cruciferous crops world-wide. Infection leads to wilting, death and total crop loss especially when young plants are infected. Traditional control measures include improving drainage, liming to raise soil pH and rotation of susceptible crops with non-cruciferous crops. Previous studies have indicated that symptom expression is dependent on the presence of a threshold level of infection. This means that the concentration of clubroot spores plays an important part in the incidence and severity of clubroot development within crops. In reality these thresholds are determined by environmental and control measures operating within the soil.

Clubroot resting spores are capable of inducing disease in vegetable brassicas years after initial detection of disease. There are no reliable tests to measure resting spores viability; as an obligate parasite it can only develop in its host. Molecular detection of clubroot DNA has been used to aid identification. Detection and quantification is central to investigating the viability of clubroot resting spores. Examples of other methods used to investigate *P. brassicae* include serological tests to detect *P. brassicae* in soil samples but these cannot be used to determine spore viability.

Environmental factors can have an effect on the incidence of clubroot disease. It is widely accepted that high and low temperatures can reduce the incidence of clubroot disease, with optimum temperature for disease occurrence being between 20 and 24^{o}C. Soil temperature has also been measured in relation to severity of clubs on plants in field plots as well as in oven trials to determine the relationship between temperature, time and disease prevalence. High soil moisture content has been shown to increase the incidence of clubroot by providing good spore germination conditions and subsequent infection. The effects of pH on clubroot disease have also been extensively studied as it is well known that alkaline soils show an

inhibitory effect. Many of the environmental studies on clubroot focus on disease incidence rather than actual spore levels and viability.

The expected deliverables from this project are:

- Reliable methods for measuring clubroot resting spores viability.
- Information (as models) on environmental and host factors which affect clubroot resting spore viability.

Summary of the results and main conclusions

Molecular detection of clubroot DNA

Molecular tests were used to determine the limit of clubroot detection in soil that was artificially infested with clubroot resting spores at a range of concentrations. These tests could not detect low numbers of spores in the soil reliably below 1000 resting spores per gram of soil. A commercially available kit was used to extract the clubroot DNA from the soil. Using a molecular test (PCR – polymerase chain reaction) showed that using a 1:10 dilution of the DNA extraction was necessary to give a good level of detection. If the DNA extraction is not diluted or diluted to a level of 1:100, there is little or no detection at all.

Staining viable and non viable clubroot spores

The viability of *P. brassicae* spores can be identified using staining techniques. Viable spores show up as a solid colour where the nucleus is present. However, the spores containing a nucleus cannot be definitively considered to be viable as it is possible they will never germinate even under optimal conditions.

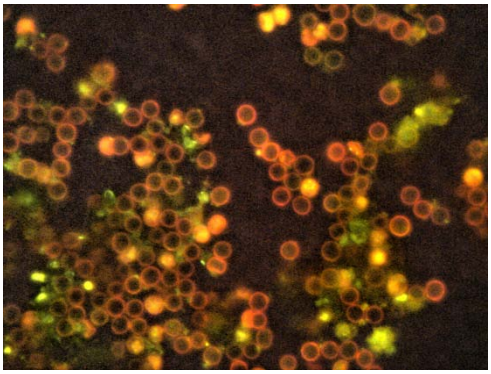

Figure 1. Stained spores: the viable spores are a solid colour.

The method used here did have limitations as clear images of the spores were difficult to obtain and this depended on camera set up and steadiness. It was found that image adjustment did allow for easier identification of viable and non-viable spores. It was also found that capturing images using a digital camera was easier than obtaining microscope counts, because the red staining of the empty spores could be difficult to distinguish from the black background by eye. Clumping of spores on the slide also meant that a slightly lower dilution of spore suspension was easier to count than neat spore suspensions. The different methods of applying the stains and their concentration did appear to greatly affect the end result.

A different combination of stains and microscope filters may provide a greater ease of distinction between full and empty spores, but the cost and time required to test all of the different combinations available could be restrictive.

Seedling assay investigating effect of environmental factors on clubroot spore viability

A seedling assay was used as a method of assessing the germination potential of *P. brassicae* resting spores. The results suggest the number of ungerminated clubroot spores is related to pH levels. The percentage infection level also rises as pH increases.

The effect of calcium, magnesium and ammonium on resting spore germination and infection was studied using DNA detection. Germination and infection were considered as measureable outcomes of resting spore viability. The germination and infection of the clubroot resting spores was regarded as the difference between the number of spores germinating and the number of spores present in the root tissue during the assay as assessed by clubroot DNA measurement. Statistical treatment of results showed that there was no significant effect of calcium on ungerminated clubroot spore levels. There was a strong statistical relationship between Mg levels and ungerminated spores remaining in suspension. The results of the effect of NH_4 on *P.brassicae* DNA levels showed a similar pattern to that of Magnesium levels when analysed. One-way ANOVA showed a highly significant relationship between ungerminated spores in suspension and NH_4 mEq/l levels. There was also a significant relationship between Ammonium concentration and infection of the roots by clubroot.

Viability of resting spores from different geographic areas

The different gall populations run through the seedling assay were assessed by statistical tests to determine the significance of any difference between clubroot populations. There was significance detected between the Northern Ireland clubroot gall samples and the East

Scotland clubroot gall samples, but there was no significance between the East Scotland and Warwickshire clubroot gall samples.

Anticipated practical and financial benefit

- Information detailing the viability of clubroot resting spores will be useful in designating successful clubroot control strategies and crop rotation practices.
- Nutrient amendments to the soil which can successfully control clubroot can be designated from the results in this report.
- Control practices which reduce clubroot resting spore viability will be important in improving crop yields in the presence of this pathogen.

Action points for growers

This project will deliver further information on soil amendments which affect clubroot development in relation to clubroot contamination levels.

SCIENCE SECTION

Introduction

Importance of Clubroot in vegetable Brassicas production

Clubroot is caused by the protist *Plasmodiophora brassicae* Woronin. Symptoms are characteristically galls or clubs formed on the roots which reduce plant vigour and yield. Infection leads to wilting, death and total crop loss especially when young plants are invaded. Older plants may produce limited yields but plant maturity will be erratic and harvesting schedules disrupted. Traditional control measures include improving drainage, liming to raise soil pH and rotation of susceptible crops with non-cruciferous crops. Previous studies have indicated that symptom expression is dependent on the presence of a threshold level of infection. Inoculum concentration plays an important part in the incidence and severity of clubroot development within crops. In reality these thresholds are determined by environmental and control measures operating within the crop. Cruciferous crops are of high economic importance globally, and in the United Kingdom. One of the main diseases affecting cruciferous crops is Clubroot, caused by the soil borne organism *Plasmodiophora brassicae.* This disease results in stunted plant growth, wilting of leaves and poor nutrient uptake due to disruption of the root system. Infection is recognisable by swelling of root tissue causing galls and club shaped structures (Bulman *et al*., 2001).

The life cycle of *P. brassicae* consists (in part) of a single motile zoospore infecting a root hair cell, followed by replication within host cells before transposition to the root cortical cells where spore formation occurs. (Ingram and Tommerup, 1972). Spore structure, mechanisms of germination and impact of environmental factors have been suggested by several authors (Aist and Williams, 1971; Macfarlane, 1970; Naiki *et al.,* 1987; Buczacki, 1978; Buczacki, 1983).

P. brassicae resting spores are capable of inducing disease in vegetable Brassicas years after initial detection of disease. There are no reliable tests of resting spores viability and, as an obligate parasite, it is impossible to culture. (Buczacki, 1983). There is no easy method of determining *P. brassicae* prevalence within a field prior to crop planting, apart from a traditional plant bait test, which is labour intensive and slow to perform.

Molecular Detection of P. brassicae

PCR amplification of DNA has been used to aid detection and study the diversity of *P. brassicae* (Buhariwalla and Mithen, 1995, Buhariwalla *et al*., 1995). Initial work using a single copy DNA sequence unique to *P. brassicae* led to the development of three primers; PBTZS-2, PBTZS-3 and PBTZS-4 that were used in a species specific single-tube nested polymerase chain reaction (STN-PCR) to detect *P. brassicae* DNA (Ito *et al.,* 1999; Ito *et al.,* 1997).

Similar methods of *P. brassicae* PCR detection are based on ribosomal RNA (rRNA) gene internal transcribed spacer regions (ITS) (Faggian *et al.,* 1999) as, in fungal-type organisms, these regions are variable at species level allowing distinction from other species. This method has been applied for investigating inter- and intraspecific variation and detection assays in several organisms such as *Phytophthora infestans* (Hussain *et al*., 2005*), P. capsici* (Silvar *et al.,* 2005), *Colletotrichum coccodes* (Cullen *et al.,* 2002) and other plasmodiophorids such as *Spongospora subterranea* f. sp *subterranea*(Bell *et al.,*1999).

Detection and quantification is central to investigating the viability of *P. brassicae* resting spores. Examples of other methods used to investigate *P. brassicae* include serological tests to detect *P. brassicae* in soil samples (Wakeham and White, 1996) (Pers. Comm. Dr. R. Kennedy University of Warwick), and there have also been molecular genetic studies within *P. brassicae* to aid understanding of host pathogen interactions (Klewer *et al*., 2001, Bulman *et al*., 2006).

Wallenhammer and Arwidsson (2001) developed a nested PCR protocol using several different primers. They investigated several different field samples, and took into account the different soil types and physiochemical properties of the soils. They found that Swedish isolates differed in a few bases from UK isolates and when they tested PCR on infected root tissue, it was found that nested PCR was more sensitive than single round PCR.

P. brassicae resting spores and the environment

P. brassicae produces large numbers of resting spores, which reside within soils. An estimate of the half life of resting spores has been put at 3.6 years (Wallenhammer, 2001). During this time spent in soil, the interaction of resting spores with their environment is 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, soil composition (both nutritionally and structurally).

Field trial data relating to the persistence of *P. brassicae* resting spores within soil environments is fairly limited, having been restricted in the past to looking at disease occurrence rather than pathogen numbers, and the majority of the work investigating factors such as moisture, temperature, pH and light intensity were performed several years ago. However, it has become widely accepted that high and low temperatures can reduce the incidence of clubroot disease, with optimum temperature for disease occurrence being between 20 and 24^{o}C (Buczacki, 1978; Myers *et al.,* 1983). Soil temperature has also been measured in relation to severity of clubs on plants in field plots as well as in oven trials to determine the relationship between temperature, time and disease prevalence, (White and Buczacki,1979). Mattusch (1977) found that high soil moisture content increased the incidence of disease by providing good germination conditions and subsequent infection.

The effects of pH on clubroot disease have also been extensively studied as it is well known that alkaline soils show an inhibitory effect (Tremblay *et al.,* 2005) and liming fields before transplanting reduces the incidence of disease. In order for maximum severity of disease to be present a threshold level of infection exists and this is dependent on environmental conditions such as pH Calcium and Boron concentrations (Webster and Dixon, 1991). Many of the environmental studies on clubroot disease focus on disease incidence as opposed to actual spore levels and viability.

Materials and Methods

Soil Extraction

Samples of soil with no previous record of *Brassica* planting (therefore presumed to be clubroot free) were inoculated with known amounts of *P. brassicae* resting spores (10through to 1000 million spores) obtained from galls by maceration in a Waring blender and filtration to give spore suspensions. DNA was extracted from the soils using the MOBIO UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc.) following the manufacturers protocol. A Polyvinylpolypyrrolidone (PVP) DNAclean-up was then performed (Klemsdal *et al.,* 2008) and the DNA diluted by 1:10 and 1:100 in reverse osmosis (RO) water.

Optimisation and comparison of primers for the amplification of *P. brassicae* DNA

Several primers were tested in the PCR detection of *P. brassicae* from soil. The primers tested were PbITS1 and PbITS2, PbITS6 and PbITS7, TC1F and TC1R, TC2F and TC2R, PBTZS3

and PBTZS4, PBAW-10 and PBAW- 11, and PBAW- 12 with PBAW- 13. All primers were supplied by Invitrogen™ and are as described in Table 2.

The PCR conditions used were 94^{o}C for 2 mins, followed by 40 cycles of; 94^{o}C for 30 secs, 55^{o}C for 30 secs, 72^{o}C for 1 min, then 10 mins at 72^{o}C. Primer sets PbITS6 and PbITS7 used an annealing temperature of 58^{o}C, while TC1F and TC1R and TC2F and TC2R used an annealing temperature of 65^{o}C (all other conditions were kept uniform). The PCR reaction mixture total volume was 25µl and consisted of 10 x PCR Buffer, 50mM MgCl2, 25mM dNTPs, ROH_2O, 0.4µM each primer, 5µl each soil DNA extract and Platinum Taq Polymerase (Invitrogen™). 5µl PCR amplification product was run on a 1.5% agarose gel incorporating 0.005% (v/v) GelRed (Biotium, Inc.) as a stain. The gel was electrophoresed at 150V for 1 hour before visualization under UV light.

Table 2. Primer details of those used for the detection of *P. brassicae* DNA from soil extracts

Primers	Sequence (5'-3')	Author(s)
PbITS1	ACT TGC ATC GAT TAC GTC CC	R. Faggian *et al.* 1999
PbITS2	GGC ATT CTC GAG GGT ATC AA	
PbITS6	CAACGA GTC AGCTTG AAT GC	
PbITS7	TGTTTCGGCTAG GAT GGT TC	
PBTZS-3	CCA CGT CGATCA CGT TGC AAT	Ito *et al* 1997
PBTZS-4	GCT GGC GTT GAT GTA CTG AAT T	
TC1F	GTG GTC GAA CTTCAT TAA ATT TGG GCT CTT	Cao *et al.* 2007
TC1R	TTC ACC TAC GGA ACG TAT ATGTGCATG TGA	
TC2F	AAA CAACGA GTC AGCTTG AAT GCT AGT GTG	
TC2R	CTTTAGTTGTGTTTCGGCTAG GAT GGT TCG	
PBAW-10	CCC CGG GGA TCACGA TAA ATA ACA	Wallenhammar and
PBAW-11	GGA AGG CCGCCC AGG ACT ACC	Ardwisson 2001
PBAW-12	GCC GGCCAGCATCTCCAT	
PBAW-13	CCCCAG GGT TCACAG CGT TCA A	

Purification and Sequencing

Ten µl of PCR amplification products were purified using a QIAquick®PCR Purification Kit (Qiagen) following the manufacturer's instructions. Purified PCR amplicons were sequenced directly in both directions using Big Dye v3.1 chemistry according to manufacturer's instructions and an ABI PRISM 3100 genetic analyser (Applied Biosystems). Sequences

were edited and aligned using DNAstar v.7 (Lasergene Ltd.) and Chromas (Technelysium Pty Ltd), and searched against the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST).

Cloning

LB agar was autoclaved at 126^{o}C, 16 mins and stock solutions of carbenicillin (50mg/ml in H_20), tetracycline (5mg/ml in ethanol), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (20mg/ml in dimethylformamide) and isopropylthio-β-galactoside (IPTG) (2g/8ml in ROH_20, adjusted to 10ml with H_20 and filter sterilized) were prepared aseptically. LB agar containing 50µg/ml carbenicillin, 15µg/ml tetracycline, 70µg/ml X-Gal and 80µM IPTG was poured into Petri dishes, allowed to set and stored at 5^{o}C.

PCR amplification products (from soil extracts containing 10^9 spores per gram soil) of TC1F /TC1R as well as TC2F/ TC2R were cloned by transforming NovaBlue Singles™ Competent Cells and selecting for Blue/white phenotype (AccepTor™ Vector Kits) according to the manufacturer's instructions. Plates were incubated at 37°C overnight.

Once the colonies had grown, twenty colonies were selected from each using sterile pipette tips, touched onto and replica LB plate and transferred to 0.2ml microtubes containing 50µl sterile RC water. The samples were vortexed and then placed in a water bath at 99^{o}C for 5 mins then placed on ice. The presence of inserts was confirmed by PCR using the appropriate primers as described. The resulting amplicons were then run on a 1.5% agarose gel stained with 0.005% v/v GelRed (Biotium, Inc.). Those positive for the insert were purified and sequenced as described.

Diagnostic primers

Forward primers designed for the insertion/deletion sequences (as detailed in cloning results) were designated PbITS+In1, PbITS+In2, PbITS+In3, PbITS-In1, PbITS-In2. All were coupled with TC1R, TC2R or PbITS7 as reverse primers. After initial tests using a range of annealing temperatures (61^{o}C to 55^{o}C) touchdown PCR was utilised with conditions of: 94^{o}C for 2 mins, followed by 16 cycles of; 94^{o}C for 30 secs, 68^{o}C (– 0.5^{o}C every cycle) for 30 secs, 72^{o}C for 1 min. Then followed 24 cycles of: 94^{o}C for 30 secs, 60^{o}C for 30 secs, 72^{o}C for 1 min and after the cycling followed by 10 mins at 72^{o}C. A $MgCl_2^+$ titration of varying concentrations was also performed to optimise PCR. The details of the diagnostic primers used are given in Table 3.

Table 3. Details of designed primers for amplification of different *P. brassicae* sequences. Bases highlighted red indicates 5 bp insertion/deletion (indel) region.

Primer	Sequence
PbITS+In1	TGTGGCGCAGCGCGCGCCATG
PbITS+In2	GCAGCGCGCGCCATGCGACA
PbITS+In3	GCTAGTGTGGCGCAGCGCGCG
PbITS-In1	GCTAGTGTGGCGCGCGCCATG
PbITS-In2	GCGCGCGCCATGCGACACT

Sequence Analysis

Sequence analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA) Software version 4.0. Sequences were those of cloned DNA amplified using TC1F/R primers, and eight sequences deposited in GenBank as genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA. Alignment was performed by ClustalW and trees were constructed using the Neighbour Joining method with Jukes-Cantor distances (Saitou and Nei, 1987). Bootstrap analysis was performed on 1000 replicates with Pairwise deletion of missing data or alignment gaps.

Soil Characterisation

Soil samples were obtained from a store of 26 different soil types from various locations within the UK (South Wales, Lancashire, East Scotland and Warwickshire). Sample numbers 1 through to 26 (minus samples 14 and 15) underwent textural analysis (LandLook (Midlands), and samples 22, 18, 9, 23, 11 and 6 were assessed for Organic C and Organic N, Exchangeable Ca, K, Mg, Al and Fe, and also pH (A. Jukes, University of Warwick).

PCR on ten different soil samples containing *P. brassicae*

From the 26 soil samples (as detailed in Table6 – p32) the ten which had been determined to contain the most *P. brassicae* DNA by Quantitative PCR (qPCR) (Pers Comm. Dr. E. Clewes and A. Wakeham, University of Warwick) were sample numbers 18, 19, 25, 14, 23, 24, 15, 26, 3 and 2. DNA extracted from these soil samples was diluted to 1:10 and 1:100 before amplification by PCR using the primers described in Table 1. PCR cycling parameters and reaction mix were as described above. Amplification products from samples 18, 25, 14, 23, 24 and 15 using primers PBTZS3 and PBTZS4 were purified and sequenced as described above.

HPLC to study internal sugar alcohol and trehalose content of *P. brassicae* spores

Frozen galls previously collected from infected *Brassica oleracea* were freeze dried for 24h before being cut up and divided into approximate 50mg samples in 2ml eppendorfs. 1.6 litres of mobile phase (consisting of 60:40 water to acetonitrile) was prepared. Stock solutions of glycerol, erythritol, trehalose, glucose, mannitol and arabitol were prepared (100 mg sugar with 10ml mobile phase to give a final concentration of 10000ppm).

Standards of 100ppm (9.9ml mobile phase/0. 1ml Stock), 200ppm (9.8 mobile phase/0.2ml Stock), 400ppm (9.6 mobile phase/0.4ml Stock, 600ppm (9.4 mobile phase/0.6ml Stock) and 800ppm (9.2 mobile phase/0.8ml Stock) were dispensed into 2ml amber vials with rubber centred lids. Helium gas was then run through the mobile phase for approximately one hour to degas the mobile phase.

A small quantity of liquid nitrogen was poured into a mortar and the freeze dried samples were immersed in it. The samples were then ground using a pestle as the liquid nitrogen evaporated. This was repeated for each of eight samples. Each sample was then dispensed into a 4ml amber vial and 1ml of HPLC grade water was added. The samples were boiled in a water bath for 5 mins and cooled for 10 mins. 665µl acetonitrile was added to each sample and then the samples were centrifuged for 10 mins at 13000rpm. Supernatant was removed and then filtered through 2 micron filter into 2ml amber vials with rubber centered lids. The stock solutions and samples were left overnight at 4^{o}C. The samples were then injected into a Gilson HPLC fitted with a Hamilton HC-75 Ca+ form column and a refraction index detector. Software integrated the peaks to produce a refractive index signal for each sample (Pers Comm. N. Magan, CranfieldUniversity)

The development of assays for assessing the viability of P. brassicae resting spores

Staining optimisation

Spore suspension

Galls (approx. 1-2g) collected from cauliflower plants were stored at -20°C for approximately 6 months. After removal from the freezer samples were thawed and washed in distilled H_2O before homogenisation using a pestle and mortar. The crushed galls were then suspended in 120ml ROH_2O and filtered through three-fold muslin. The resulting filtrate was divided equally between eight 15ml falcon tubes and balanced up to 15g each using ROH_2O.

Sample tubes were centrifuged at 2500rpm for 10 mins at 4°C. The supernatant was then discarded and the pellets transferred to two 2ml eppendorfs (4 pellets per eppendorf). 2ml final volume was achieved using RO (Reverse osmosis) H_2O and the tubes were centrifuged in a microcentrifuge at 2000rpm for 15 mins. The supernatant was discarded and the pellets combined in one 2ml eppendorf and made up to 2ml with ROH_2O. Resulting samples were centrifuged at 3000rpm for 5 mins, and the supernatant discarded. The creamy coloured spore layer of the pellet was removed using a Pasteur pipette and this was re-suspended in 1.8ml ROH_2O.

Optimisation of nucleic acid staining

Four different protocols (sets) for nucleic acid staining were followed using fresh and frozen spore suspensions as described above.

Set 1 - A 10ml aliquot of each vortexed spore suspension was air dried onto microscope slides. 2μl of a 1mM Syto 16 nucleic acid stain (Invitrogen Ltd.) stock solution was combined with 398μl ROH2O and 1 drop of Evans Blue/Eriochrome Black counterstain (3ml 0.5% Evans Blue, 4.5ml Phosphate Buffered Saline (PBS), 0.5g Eriochrome Black (Sigma Diagnostics)). 20μl of the stain mixture was then applied to each slide. Slides were subsequently covered in foil and left to incubate for 30 mins before being rinsed thoroughly with ROH_2O. The slides were then air dried in the dark and a small drop of fluorescent mounting media (Dako) was applied to each slide before the coverslip was applied.

Set 2 – As for Set 1 but with stain mixture amended to 240μl ROH_2O, 160μl 5μM Syto 16 nucleic acid stain stock solution and 1 drop of Evans Blue/Eriochrome Black.

Set 3 - 398μl spore suspension was combined with 2μl 1mM Syto 16 nucleic acid stain stock solution and 1 drop Evans Blue/Eriochrome Black in a 2ml eppendorf. This was incubated in the dark for half an hour and then 10μl aliquots were applied to slides. The slides were then air dried in a darkened fume hood before a small drop of DAKO mounting media and coverslip were applied.

Set 4 – As for set 3 however 240μl spores suspension was combined with 160μl 5μM Syto 16 nucleic acid stain stock solution and a drop of Evans Blue/Eriochrome Black mixture.

Two slides were prepared for each of the fresh and frozen spore suspensions in each set, giving a total of 16 slides.

Microscopy and digital image capture.

A Nikon Fluorescent microscope (Optech Ltd.) fitted with a B-2A filter **(**Exicitation wavelength 450-490nm, Diachromic Mirror 505nm and Barrier filter of 520nm) was used to examine the slides. SYTO 16 nucleic acid stain has an absorbance peak of 486nM and an Emission peak of 516nM. A Nikon Coolpix 990 v1.1 digital camera allowed slide images to be captured using an eyepiece adapter. Images were taken of areas of spore concentration under x100 oil immersion lens (using fluorescent free immersion oil (Fluka Analytical)). Images collected were opened in Image J (Rasband, W.S., Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009.) and the contrast, brightness and colours adapted to enable clearer observation of the nucleic acid stain within the spores.

Seedling Assay

Spore suspension preparation

Galls from Oilseed Rape plants were collected in December 2008 from a field at Warwick HRI, Wellesbourne, Warwickshire, and frozen at -20ºC. 405g galls were thawed and washed in sterile H_2O to remove soil debris then cut into 1cm^3 (approximately) pieces using a sterile scalpel. Pieces were then macerated in a Waring Blender for 2 mins at the highest speed with 250ml ROH_2O. The resulting suspension was filtered through four-fold butter muslin and the filtrate collected. Filtrate was passed incrementally through filter membranes under vacuum down to 10 micron (other filter membranes used were 43, 20, 15 and 11 micron). The final filtrate was split equally between six 50ml centrifuge tubes (approx. 35ml in each) before centrifugation at 2500rpm for 10 mins. The supernatants were discarded and the pellets combined and resuspended in 8ml sterile distilled H_2O. The final spore concentration was counted using a Neubauer haemocytometer and found to contain 5x10^7 spores/ml.

Seedling Production

Approx. 240 Chinese cabbage cv Granaat seeds (*Brassicae campestris* ssp. *Pekinesis*) were provided by the European Clubroot Differential Set (Warwick HRI, Wellesbourne, Warwickshire). Seeds were surface sterilized by immersion in 70% Ethanol for 2 mins and subsequently in a mixture of bleach, sterile H_2O and Sodium Docecyl Sulphate (SDS) following the protocol of Agarwal *et al* (2009) with an amendment of SDS concentration to 0.05% instead of 5%. Seeds were sown on the surface of 6 crystallising dishes each filled with 250ml coarse sand (Leighton Buzzard, Lower Green Sand, washed, aired, dried and screened sand (WBB Minerals) which had been sterilized in a bench top autoclave at 126^oC for 16 mins and which had been dampened with sterile distilled H_2O. Dishes were then sealed in a clear plastic sandwich tray and incubated for 12 days at 22ºC (18h photoperiod).

Seedling Assay

A factorial assay was designed (Rodney Edmondson, Pers Comm. University of Warwick) to study the effects of calcium and pH on the viability of *Plasmodiophora brassicae* resting spores. 25 treatments were allocated to represent 5 different levels of Calcium (0, 2, 4, 6, 8 mEq) and five different pH levels (4, 4.5, 5, 5.5 and 6). Each treatment was applied randomly to a set of four seedlings and the experiment was replicated twice. 500ml modified Hewitt's solution (Hewitt and Smith) was prepared in two 496ml batches, without the addition of calcium, and these were subsequently divided into ten 99.2ml aliquots. Each aliquot was then assigned a treatment number from 1 to 25, and Calcium Chloride was added, followed by pH adjustment (with H_2SO_4) to achieve the factorial treatment structure.

Each aliquot was inoculated with 800µl spore suspension bringing the final spore concentration of each aliquot to $4x10^5$ spores/ml. Seedlings were gently removed from dishes and washed in sterile distilled H_2O, to remove sand particles before being randomly placed in two hundred 7ml bijous in clear sandwich trays. Each seedling containing bijou was then numbered 1 to 25, giving four seedlings per treatment. From each treatment aliquot (adjusted nutrient solution containing spore suspension) 20ml was applied to a set of four seedlings at random, and this was repeated until all treatments had been allocated to the seedling bijous. Sandwich tray were then sealed and incubated for 7 days at 22ºC with an 18h photoperiod.

Germination and subsequent root infection of P. brassicae with different nutrients

Methods of assessing the germination and subsequent infection by *P*. *brassicae* resting spores were used as above (seedling assay system). To investigate the effects of nutrients on the germination and subsequent infection of Brassicas by *P*. *brassicae*, Chinese cabbage cv Granaat seeds (*Brassicae campestris* ssp. *Pekinesis*) (European Clubroot Differential Set, Warwick HRI, Wellesbourne, Warwickshire) were surface sterilised following the protocol of Agarwal et al (2009) with an amendment of SDS concentration to 0.05% instead of 5%. Seeds were sown on the surface of sterile sharp sand (J Arthur Bower) dampened with sterile distilled H_2O in crystallising dishes. Dishes were then sealed in a clear plastic sandwich tray and incubated for 7-12 days at 22ºC (18h photoperiod), while the seeds germinated.

Subsequently seedlings were individually placed in 2ml eppendorf tubes with the lids removed, with four concurrent seedlings being considered as one sample. Three treatment solutions of Calcium Chloride ($CaCl_2.2H_2O$), Magnesium sulphate ($MgSO_4.7H_2O$) and Ammonium Nitrate solutions (NH_4NO_3, K_2SO_4) were then randomly assigned to each sample (group of four seedlings) at one of four different concentrations (25, 50, 70 and 100 mEq/l), with 1ml of each

treatment pipetted into each of the four relevant eppendorfs. The eppendorfs containing seedlings and treatments were placed in clear 81 well trays, of which two were placed inside a larger clear sandwich tray which was then sealed. A spore suspension was made from freshly grown galls produced on *Brassica oleracea* cv. Belot collected at Warwick HRI, Wellesbourne, Warwickshire. 40g galls were washed in sterile H_2O then cut into pieces using a sterile scalpel before maceration in a Waring Blender for 2 minutes at the highest speed with 480ml ROH_2O. The resulting suspension was filtered firstly through muslin and then through nylon membranes (43, 20 and 15 micron) under vacuum. The final spore concentration was found to contain $2.6x10^7$ spores/ml which was estimated by counting using an improved Neubauer haemocytometer. 1ml of the spore suspension was applied to each seedling containing eppendorf.

The eppendorfs were placed within 96 well trays which were then sealed inside clear sandwich trays to prevent evaporation. The seedlings were then incubated again for five days at 15^oC, with a 16h photoperiod after which they seedlings were removed, and the roots from the four concurrent seedlings of each sample were pooled together and the DNA subsequently extracted using DNA was extracted using the FastDNA Spin kit (QBiogene) following the manufacturer's instructions for plant tissue DNA extraction with the vortex step amended to comprise of three runs in the FastPrep instrument at speed 5.5 for 25 s with samples resting on ice in between runs. A subsequent DNA clean-up was performed following the method of Klemsdal et al (2008).

Spore quantification in the seedling assay

Quantitative PCR (qPCR) was performed on the extracted DNA from both experiments using a LightCycler 480 (Roche Diagnostics), using and primer set PbML1F and PbML1R and with a master mix of 10µl Lightcycler® 480 Sybr Green I Master (Roche Diagnostics), 2µl each primer (0.5µM final concentration), 4µl ROH_20 and 2µl DNA. The cycling parameters were pre-incubation at 95^oC for 15 mins, followed by 55 cycles of 95^oC for 10 s, 65^oC for 20 s and 72^oC for 20 s, with acquisition of the fluorescence measurement during the 72^oC step. A subsequent melting curve was generated by 1 cycle of a hold at 95^oC for 5s, 65^oC for 1 min, and an increase to 97^oC of 0.05^oC/s, recording fluorescence 10 times/°C. Finally the Samples were cooled to 40^oC and held for 10 s. Amplification was performed in 384 well plates (Applied Biosystems) and quantification was by the second derivative maximum method against a standard curve generated from *P. brassicae* spore suspension DNA extracts which were diluted in DNA extract solutions of clean Chinese Cabbage (cv. Granaat) root tissue.

Each experiment was replicated three times, and positive and negative controls were included. Each control treatment comprised of one set of four seedlings exposed to either 1ml H_2O instead of spore suspension with 1ml treatment solution (negative), or 1ml H_2O instead of treatment solution with 1ml spore suspension (positive). Statistical analysis on the seedling assay was performed on log transformed data using R: A Language and Environment for Statistical Computing (2010).

Seedling infection assessment

Seedlings were removed from bijous and roots were washed in sterile distilled H_2O. Stems were removed using a sterile scalpel and discarded. The four roots from each treatment were pooled together and stored at -20°C for subsequent DNA extraction.

The remaining nutrient solutions from each seedling were gently vortexed and a 500µl aliquot removed from each one. The four solutions in each treatment were pooled together in 2ml eppendorfs and stored at -20°C for subsequent DNA quantification by qPCR (using primer set MLPb1F/R against spore suspension DNA standards) and nucleic acid staining.

Density Gradient Separation of *P. brassicae* spores from soil and galls

Ludox HS-40 (Colloidal Silica) was diluted with distilled H_2O in 15ml centrifuge tubes to produce solutions of Ludox from 10% to 100% (in 10% increments) with a final volume of 10ml for each solution. Soil samples from the Quarantine Field (Wellesbourne, Warwickshire, UK) were dried and ground using a pestle and mortar then made into a slurry using excess H_2O, then mixed and filtered through four fold butter muslin. Samples of freeze dried galls (obtained from infected *Brassica oleracea*) were crushed with a pestle and mortar and excess H_2O was added. This was mixed and then filtered through four fold butter muslin.

Density gradients were prepared by layering 1ml of each Ludox preparation, in a 15ml centrifuge tube, starting with 100% Ludox at the bottom and ending with 10% Ludox on the top. 1ml of soil slurry was then layered on top of the gradient and then centrifuged at 4000rpm for 30 mins.

Subsequently further Ludox preparations of 15%, 25% and 35% were made as above in 15ml centrifuge tubes. Density gradients of Ludox were then prepared using 50%, 40%,

35%, 30%, 25%, 20%, 15% and 10% Ludox preparations. These were again centrifuged for 30 mins at 4000rpm then studied and centrifuged again for 10 mins at 4000rpm.

Resulting bands were then removed by careful pipetting and dispensed into 2ml microcentrifuge tubes. 1ml distilled H_2O was added and then the tubes were centrifuged for 60 secs at 18000rpm. The resulting supernatant was then discarded and the pellet resuspended in 1ml distilled water. This was again centrifuged at 18000rpm for 60 secs. This was repeated four times to remove the Ludox before aliquots were placed on a heamocytometer to confirm the presence of *P. brassicae* resting spores.

The effect of soil type and environmental factors on resting spore viability

Preliminary field investigation

The preliminary field trial was carried out from April to November 2008, at Warwick HRI, Wellesbourne, Warwickshire (52°12'N 36°00'W) in a quarantined field (0.58 Ha) known to contain significant levels of *P. brassicae* and with a recent history of Brassica growing (Humpherson-Jones, 1993).

A pre-existing pH gradient across the field, running from east (low pH) to west (high pH) was checked by sampling soil at 12 locations in the field at a depth of 15cm using an auger and the pH tested using an elmeco pH meter to gain an indication of existing pH levels.

Each pH area was divided into two 5 m x 7.5 m plots. In order to re-establish the pH gradient to 6, 7 and 8 (6 being referred to as low, 7 as medium and 8 as high), other previously sampled soils from the plots which had been air-dried were measured for pH and a lime titration with Calcium Hydroxide performed following the protocol of Liu et al (2004). It was found that the initial pH level levels of the air dried soil varied considerably from that of the 12 samples taken in April, however the 12 samples had not been air dried or ground and calibration of the pH meter used to take the pH of the air dried samples was more reliable than the elmeco pH calibration (only performed once a year). $CaCO_3$ was applied to the plots in quantities as shown in Table 4, during the week beginning the 16th June 2008.

Table 4. Existing and desired pH levels in experimental field

Soil	Current pH	Desired pH	tonnes $CaCO_3$ required/ha
East (low)	4.45	6	2.39
Middle (medium)	4.62	7	3.81
West (high)	6.78	8	1.72

Plants and Plot Layout

Two Brassica crops, *Brassica oleracea* Cauliflower cv. Belot and *Brassica napus* Oilseed rape (cv. Unknown) were sown in Hassay trays in late April and grown up in a Glasshouse then hardened in a cold frame before transplantation in June 2008. Plots were prepared for planting with herbicide treatment of glyphosphate and harrowing. For each plot 4 rows cauliflower and 4 rows oilseed rape were separated by an unplanted area equivalent in size to 4 rows of plants, planted with 50cm spacing between plants and rows. At the time of transplanting the crops were netted and nitrogen was applied to the plots at a rate of 140Kg, followed by a top dressing of 100Kg N. One set of plots comprised one plot from each pH level, identified as A,B and C and the other set of plots included the remaining plots from each pH; D, E and F.

Weather patterns and soil monitoring

Humidity, air temperature, rainfall, leaf wetness were measured using a centrally situated weather station with an environmental monitoring station (Skye Instruments Ltd.) set to record at half hourly intervals. Soil temperature and soil moisture were measured every half an hour using a 10k thermistor temperature sensor and an SM200 theta probe (Delta-T Devices Ltd.) Data was downloaded from the weather station logger and the GP1 logger at intervals throughout the growing season.

Soil sampling and analysis

For each crop, and unplanted area within the plots 9 samples were taken in between the rows of plants (or equivalent to between rows for the unplanted area), following a W pattern at a depth of 15cm. The samples for each crop in each plot were then pooled. For plots D, E and F samples were taken from directly around the roots. For each crop type five plants were selected at random and uprooted. The five samples taken for each crop type were pooled within each plot. The roots were then removed from the plants and bagged, then stored at -20^{o}C for future use.

Soil samples were then transferred to a glasshouse and air dried for at least 5 days before grinding using a pestle and mortar. DNA was then extracted and cleaned up as described in 2.1.1 and quantified by qPCR using PbITS3 and 4 (M. Proctor. Pers. Comm. University of Warwick).

Samples from plots A, B and C were taken on the 1st August, 18th September and 6th Nov 2008. Samples from plots D, E and F were taken on the 2nd October and 6th November 2008.

The effect of using the seedling assay against the clubroot differential set.
Freshly galls grown on *Brassica oleracea* cv. Belot were collected from a field at Warwick HRI, Wellesbourne, Warwickshire. 50g galls were used to prepare a spore suspension as previously described. The final spore concentration was counted using a Neubauer haemocytometer and found to contain $3x10^7$ spores/ml.

Seeds were provided by the European Clubroot Differential Set (Warwick HRI, Wellesbourne, Warwickshire).

Seeds were surface sterilized by immersion in 70% ethanol for 2 mins and subsequently in a mixture of bleach, sterile H_2O and sodium docecyl sulphate (SDS) following the protocol of Agarwal et al (2009) with an amendment of SDS concentration to 10%. Seeds of each differential were sown on the surface of a small crystallising dish filled with approximately 150ml sharp sand (J. Arthur Bower) which had been sterilized in a bench top autoclave at 126^{o}C for 16 minutes. Sterile distilled H_2O was used to dampen the sand and dishes were then sealed in clear plastic sandwich trays and incubated for 7-12 days at 15ºC (12h photoperiod?).

Following seedling germination 2ml eppendorfs were filled with 2ml spore suspension and placed in a clear 81 well box in groups of four. To each group of four eppendorfs a treatment of one differential was applied at random, resulting in each eppendorf containing one seedling. The clear tray was then sealed inside a clear sandwich box as before and incubated for five days at 15ºC (12h photoperiod).There were fifteen differentials in total and this experiment was replicated three times.

Following incubation the roots from the four seedlings in each treatment were washed in sterile distilled H_2O, removed from the aerial parts of the seedling and pooled together. The suspensions in which each seedling had been incubated were also pooled together in their

treatment groups. A 200µl aliquot of each of the pooled suspensions was removed for DNA extraction using the Fast DNA Spin kit (QBiogene) as previously described (this chapter), DNA from the seedling root tissues were also extracted using the Fast DNA Spin Kit, however using the protocol for plant tissue instead of fungal cells. A PVP DNA clean-up was then performed on both sets of DNA extractions (Klemsdal et al 2008).

qPCR was then performed on the DNA extracts using primer set MLPb1F and MLPb1R and a master mix containing 10µl Lightcycler® 480 Sybr Green I Master (Roche Diagnostics), 2µl each primer (0.5µM final concentration), 4µl ROH_20 and 2µl DNA. The cycling parameters were pre-incubation at 95^oC for 15 mins, followed by 55 cycles of 95^oC for 10 s, 65^oC for 20 s and 72^oC for 20 s. Acquisition of the fluorescence measurement took place during the 72^oC step. A subsequent melting curve was generated by 1 cycle of a hold at 95^oC for 5s, 65^oC for 1 min, and an increase to 97^oC of 0.05^oC/s, recording fluorescence 10 times/°C. Finally the samples were cooled to 40^oC and held for 10 s. Amplification was performed in 384 well plates (Applied Biosystems), as previously described.

Difference in viability of P. brassicae populations from galls

Chinese cabbage seeds (*Brassica rapa* ssp. *Pekinesis* cv Granaat) were sterilized and germinated under the conditions described for the ECD set seeds. Spore suspensions from the East Scotland, Warwickshire and Northern Ireland gall tissues were prepared as previously described using 8g gall tissue. The Charlock spore suspension from East Scotland contained 1.73x107 spores/ml, the Cauliflower spore suspension from Warwickshire contained 3.05x107 spores/ml and the spore suspension from unknown Brassica galls from Northern Ireland contained 7.2x106 spores/ml. Four seedlings were allocated to each different spore suspension treatment. Each seedling within the treatment groups was placed in 2ml spore suspension within an eppendorf and the incubation, sampling and DNA extraction was performed as described for the ECD set above.

Results

Year one Results

Soil Extraction and Optimisation of primers for the detection of *P. brassicae* DNA

P. brassicae molecular detection by PCR from soil extracts was most successful when the DNA was diluted 1:10. DNA only amplified in neat soil extracts containing the lowest

concentrations of *P. brassciae* and while the 1:100 dilution did amplify some of the soil extracts it was not as successful as the 1:10 dilution.

Primers PbITS6/7 gave a weak non-specific banding pattern at both 55^{o}C and 58^{o}C annealing temperatures, whereas TC1F/R and TC2F/R gave a distinct product at 55^{o}C, with little amplification at 65^{o}C (annealing temperature). At 55^{o}C TC1F/R showed amplification from soil extracts containing 10^6 through to 10^9 spores per gram of soil and TC2F/R showed amplification of soil extracts 10^4, 10^7, 10^8, and 10^9 spores per gram of soil. PbITS1/ PbITS2 did amplify some DNA from soil extracts 10^5, 10^8, and 10^9 and PBTZS3/PBTZS4 also showed bands from soil extracts 10^8, and 10^9.

The remaining primer sets (PBAW10/PBAW 11 and PBAW12/ PBAW13) did not result in any amplification. The most sensitive amplification was obtained using primer sets TC1F/R and TC2F/R.

Sequencing

Sequences of amplification products (obtained with primer sets TC1F/R and TC2F/R) grouped with rRNA ITS sequences deposited to GenBank labelled as *P. brassicae*, indicating the primers were specific for *P. brassicae*. When sequence chromatograms were studied a secondary sequence was present in the background of nearly all products. Other primer sets (PbITS 1/2, PBTZS3/4, PBAW 10/11 and PBAW 12/13) had been tested against the same standards and there had been amplification with primer sets PbITS1/2 and PBTZS3/4.

To confirm the presence of *P. brassicae* DNA as the amplification product using these primer sets, samples were purified and sequenced. The results showed that sequences amplified with PbITS1/2 grouped with rRNA sequences deposited to GenBank as *P. brassicae*, however the sequences obtained from PBTZS3/4 did not (data not shown).

Cloning

It was found that some colonies which were thought to have taken up the insert did not amplify by PCR (using primers TC1F/R and TC2F/R). The resulting gel images are shown in Figure 1.

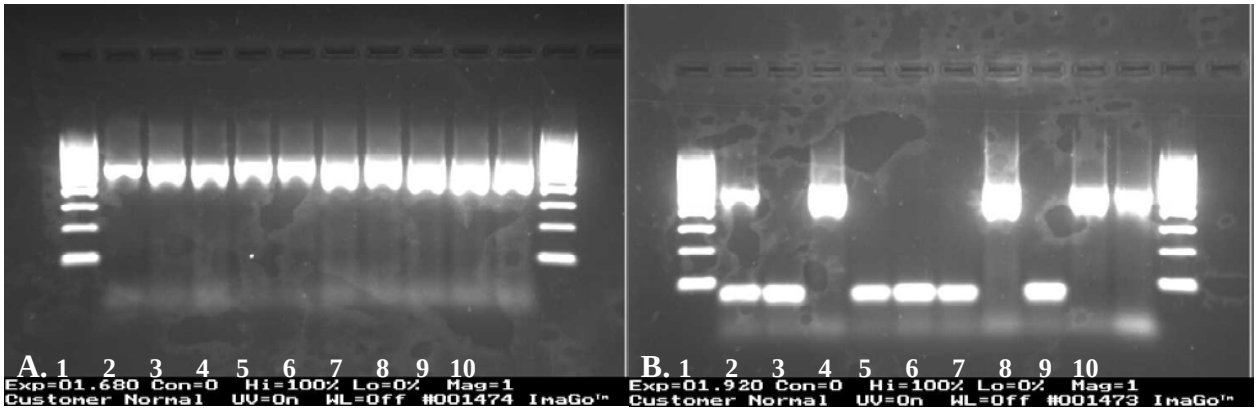

Figure 1. Gel images following PCR amplification on colonies of transformed cells. Image A = amplification with TC1F/R. Image B = amplification of TC2F/R. Each lane numbered 1 to 10 is an individual clone

The products containing the inserts where then purified, sequenced and the resulting sequence chromatograms where studied for secondary sequences with none found. The sequences of the inserts were edited and aligned using DNAstar v.7 (Lasergene Ltd.), and searched against the NCBI GenBank database using BLAST. Sequences grouped with rRNA ITS sequences deposited to GenBank labelled as *P. brassicae*, and alignment indicated two inserts differing by a 5bp insertion/deletion region (indel). For descriptive purposes the sequences containing the indel shall from now on be referred to as PbA+In and the sequences without the indel PbA-In.

Unpublished studies involving quantitative PCR (qPCR) on DNA extractions from 26 soils and standards infected with Clubroot have been performed (Pers Comm. Dr. E.Clewes, University of Warwick). These were amplified using TC1F and TC1R. The resulting amplification curve and melting peak analysis was performed revealed a secondary peak corresponding with a slightly smaller DNA strand than the main product (see Figure 2).

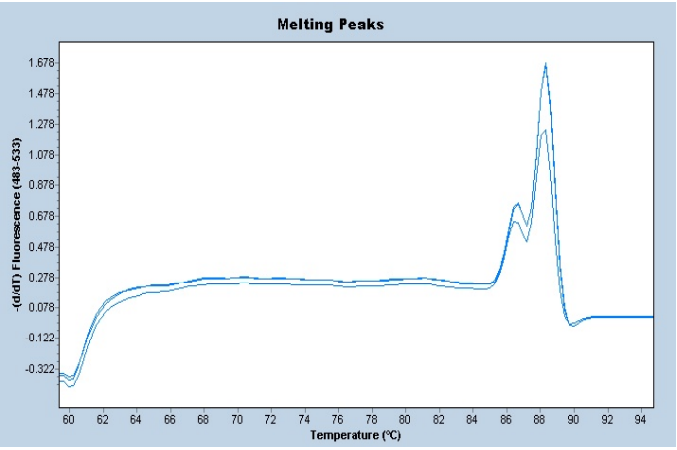

Figure 2. Melting Peak analysis of *P. brassicae* DNA by qPCR, showing the smaller 'secondary' peak

Sequence Analysis

P. brassicae sequences deposited in NCBI GenBank were aligned with PbA+In and PbA-In sequences as detailed in Table 5 (Faggian *et al.,* 1999; Cao *et al.,* 2007). Analysis of aligned sequences shown in Figure 2 shows clearly the indel region, and also highlights other nucleotide variations in the sequences. The bootstrap tree (Figure 3) shows there are four distinct branches showing greater than 50% bootstrap support, demonstrating that there are some significant differences between sequences. The order of pathotypes is generally less precise with low bootstrap values given (<50%). It can be seen that there is a clear distinction between the grouping of sequences with the indel region (PbA2+In, PbA4+In, PbA5+In and PbA9+In) and the sequences without the indel (PbA1-In, PbA3-In, PbA6-In and PbA7-In) which group more closely with sequences deposited on GenBank.

Table 5. Details of isolates deposited in GenBank as *P. brassicae* and used in alignment with sequences PbA1-In, PbA2+In, PbA3-In, PbA4+In, PbA5+In, PbA7-In, PbA9+In and PbA10-In all of which originate from the UK

GenBank Accession Number	Deposited By	Country of Origin	Host	Isolate Name
AB094984	Uchitsu *et al* (2004)	Japan	Chinese Cabbage cultivar Kuukai	Kuk
AB094983	Uchitsu *et al* (2004)	Japan	Chinese Cabbage cultivar Yuuki	Yuk
AB094982	Uchitsu *et al* (2004)	Japan	Chinese Cabbage cultivar Satokaze	Sto
AB094981	Uchitsu *et al* (2004)	Japan	Chinese Cabbage cultivar Sakumidori 3 gou	Sak3
AB094980	Uchitsu *et al* (2004)	Japan	Chinese Cabbage cultivar Sakumidori 3 gou	Sak2
AB094979	Uchitsu *et al* (2004)	Japan	Chinese Cabbage cultivar Sakumidori 3 gou	Sak1
AB094978	Uchitsu *et al* (2004)	Japan	Chinese Cabbage cultivar Daifuku	DaiI
AB094977	Uchitsu *et al* (2004)	Japan	Chinese Cabbage cultivar Daifuku	DaiF
DQ533682	Cao *et al.* (2007)	Canada	-	SACAN03-1
AF231027	Faggian *et al.* (1999)	Australia	-	-

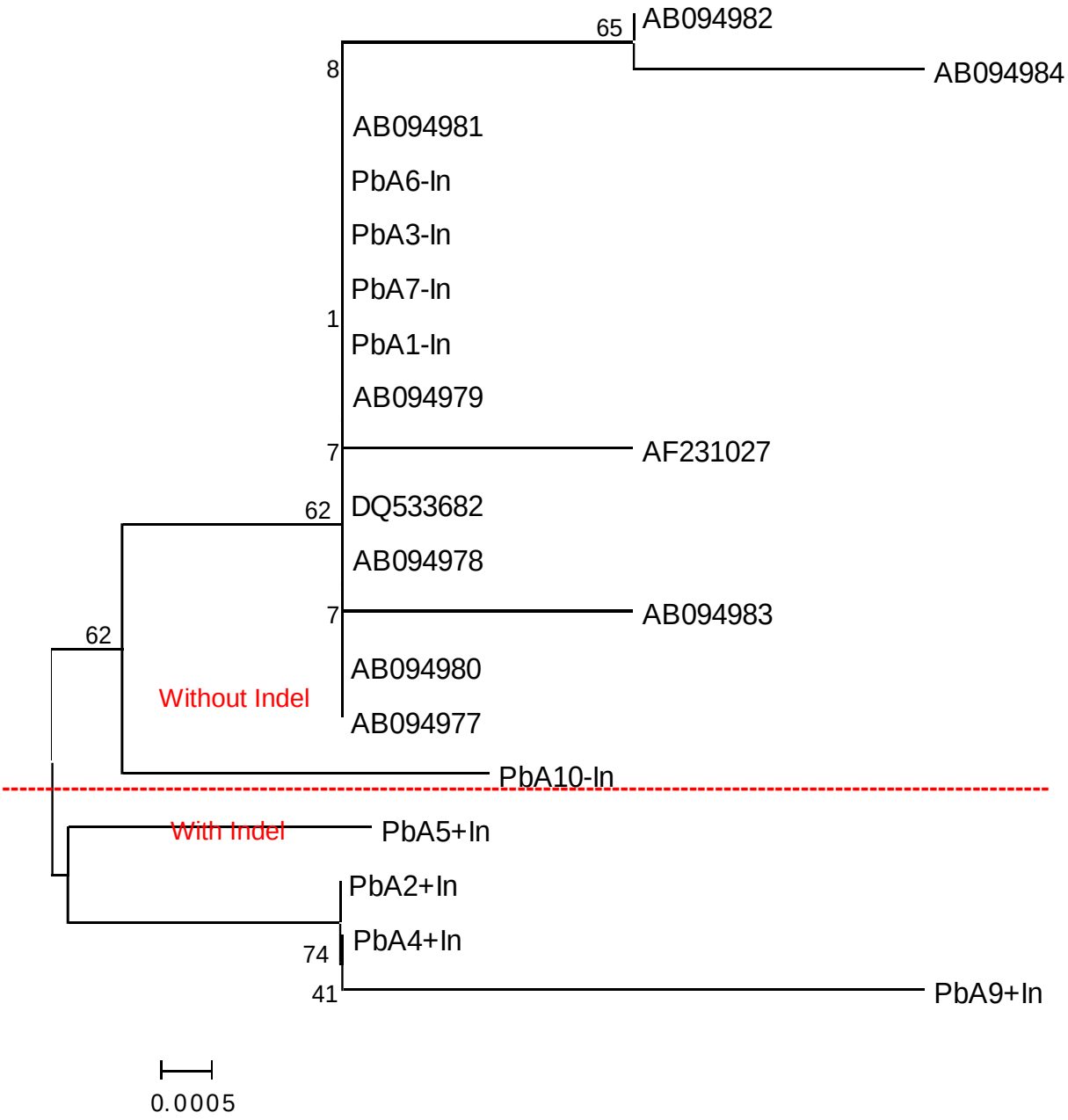

Figure 3. Bootstrap tree of aligned *P. Brassicae* DNA sequences. AB094980, AB094982, AB094984, AB094978, AB094981, AB094983, AB094979, AB094977 are GenBank Acession numbers deposited by Uchitsu *et al* (2004), AF231027 by Faggian *et al*. (1999), and DQ533682 by Cao *et al* (2007). PbA1 – PbA10 are *P. Brassicae* DNA sequences obtained in the course of this study

Diagnostic primers

Optimisation of diagnostic primers to the indel region is an on-going process. When amplified with TC1R as reverse primer under standard PCR conditions there was amplification of both sequences PbA+In and PbA-In. Similar results were obtained using TC2R and PbITS7 as reverse primers under touchdown PCR conditions. However the primer combination of PbITS-In2/PbITS 7 did result in an amplification product from DNA without the indel (PbA-In) and nothing from the DNA containing the indel (PbIA+In) as shown in Figure 4. Despite this, further efforts have also proved largely unsuccessful, but following Magnesium titration some positive results were obtained using PbITS-In1/PbITS 7 under touchdown PCR conditions but these have so far been unrepeatable.

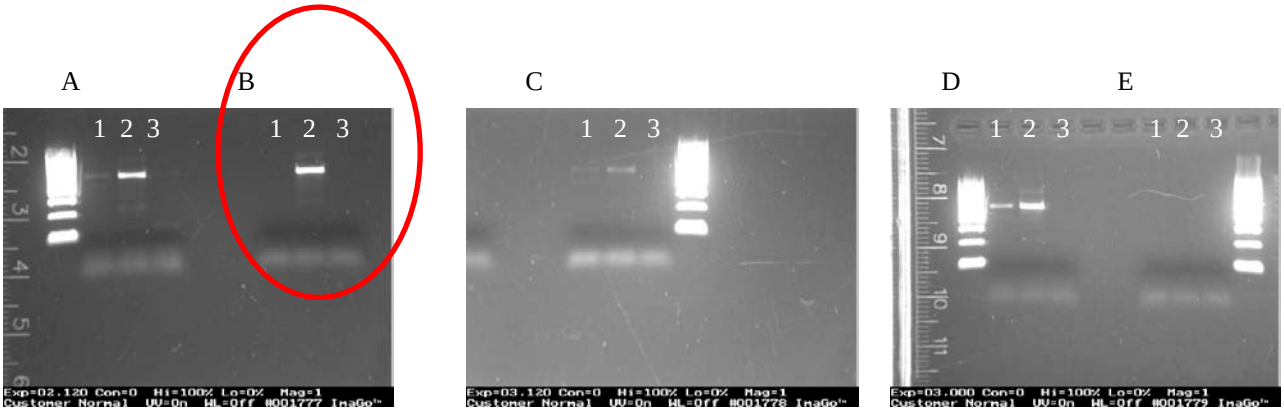

Figure 4. Gel images of sequences with and without the indel and amplification with; A. Forward Primer PBITS–In1, B. Forward Primer PbITS–In2, C. Forward Primer PbITS+In1, D. Forward Primer PbITS+In2, E. Forward Primer PbITS+In3, all combined with reverse primer PbITS7. Lane 1 is PbA+In, Lane 2 is PbA-In and Lane 3 is negative control (H_20)

Soil Characterisation

PCR amplification using primers PbITS 6 and PbITS 7 consistently gave a none specific amplification for all soil samples, as did PBAW10 and PBAW11. PBAW12 and PBAW13 showed either none specific amplification or no amplification. TC1F/R resulted in amplification of six out of the ten soils, while TC2F/R showed seven out of ten amplification products these were all faint bands which could not be easily distinguished. PbITS1 and PbITS2 also amplified seven out of the ten soils, however a secondary band did appear on soil extract 21. Amplification with PBTZS3 and PBTZS4 appeared to produce good amplification of six out of the ten soils, however when these products where purified and

sequenced it was discovered that the sequences did not align with *P. brassicae* sequences on GenBank.

Observations regarding the characterisation of different soil types are shown in Table 6. A range of soil types were identified; 4 sandy silt loams, 6 clay loams, 2 clays, 2 sandy loams, 1 peat, 1 sandy clay loam, 1 silty clay loam and 1 loamy sand. The remaining samples were combinations of the above

Sample Number	Sample Ref	Textural Class
1	South Wales	Sandy silt loam
2	South Wales	Sandy silt loam
3	South Wales	Clay loam
4	South Wales	Clay loam
5	South Wales	Fine sandy loam to sandy silt loam
6	South Wales	Clay loam
7	South Wales	Sandy silt loam
8	South Wales	Clay loam to Silty clay loam
9	South Wales	Clay Loam
10	South Wales	Fine sandy silt loam
11	South Wales	Clay
12	South Wales	Clay?
13	South Wales	Clay loam
16	Lancashire	Sandy Loam (probably loamy sand)
17	Lancashire	Loamy sand
18	Ellis Luckhurst	Silty Clay loam?
19	Lancashire	Clay loam
20	Lancashire	Fine sandy loam
21	Lancashire	Clay loam
22	Lancashire	Peat
23	East Scotland	Sandy Loam (probably loamy sand)
24	East Scotland	Sandy silt loam/ Clay loam
25	East Scotland	Sandy Clay loam
26	Warwickshire (Quarantine Field Wellesbourne)	Sandy Loam

Table 6. Textural analysis of 24 different soil samples from across the UK. Textural analysis performed by LandLook (Midlands).PCR on ten different soil samples containing *P*. *brassicae*

The soil DNA extracts which consistently showed good amplification where numbers 18, 23, 24 and 25. Soil extracts 3 and 26 were amplified best by TC1F/R and PbITS1 and PbITS2 respectively. Further work in this area will allow distinction of any soil types which are inhibitory to PCR.

HPLC to study internal sugar alcohol and trehalose content of *P. brassicae* spores

The results show that there are quantities of sugars obtained from galls of (cauliflower) roots infected with *P. brassicae*. The first significant peak on the outputs obtained was Trehalose, followed by Glucose, Glycerol, Erythritol, Arabitol and Mannitol. From the gall samples studied there are distinct peaks correlating to trehalose appearing at approximately 9.5 mins on samples 4b, 4c, 4d, 4g, 4h and 4i.

On the remaining samples (4e and 4f) a peak appeared at approximately 8.8 mins, this also appears on the other samples and therefore may not correlate to trehalose. It is thought that the peak at approximately 15 mins on all samples was erythritol, and it appears that there are sugars that peak prior to trehalose between 4 and 9 mins.

Ludox Density Gradient separation of spores from soils and freeze dried galls

The freeze dried galls produced a clear band approximately 1ml below the meniscus, while the soil samples showed a level of separation but no clear bands that were easily distinguishable were produced.

Year Two Results

The development of assays for assessing the viability of P. brassicae resting spores

Staining Optimisation

The cell counts recorded from the different sets of slide treatments are shown in Table7. Resting spores which showed positive internal constituents (in particular nucleic acids) were designated as ‘spores with nucleus’, and those which did not show any internal components were designated as ‘spores without nucleus’. The percentage of spores with against spores without a nucleus varied with different treatment set. Set 3 spores from frozen galls suspension had 100% containing a nucleus, whereas Set 2 fresh spore suspension had only 4.88% containing a nucleus. The mean percentage of spores with a nucleus across the different treatment sets was 52.47% for the fresh spore suspension and 44.16% for the

frozen gall spore suspension. The total number of spores counted in each set varied according to how many spores were present in one image frame.

Table 7.Results of staining assay investigating viable vs. none viable spores

Slide Set	Number of spores with nucleus	Number of spores without nucleus	Total number of spores	% with nucleus (2dp)	% without nucleus (2dp)
Set 1 Fresh	16	11	27	59.26	40.74
Set 1 Old	24	123	147	16.33	83.67
Set 2 Fresh	2	39	41	4.88	95.12
Set 2 Old	34	43	77	44.16	55.84
Set 3 Fresh	43	11	54	79.63	20.37
Set 3 Old	70	0	70	100.00	0.00
Set 4 Fresh	41	21	62	66.13	33.87
Set 4 Old	26	135	161	16.15	83.85

Of the images captured and adjusted, Sets 1 and 4 proved easiest to distinguish individual spores and the presence/absence of a nucleus. The spores identifiable in unaltered images of all sets were more undefined than was to be hoped, thus meaning that image adjustment was needed to provide sufficient definition for reliable counting (Figure 5). It was noted that in some cases individual spores display variability in stain uptake, and appeared partially full. There was little difference between images captured of the frozen gall spore suspension and the fresh spore suspension, but the 1 in 10 dilution of the spore suspensions was easier to count and capture images as there was less clumping of spores and the overall spore density was reduced. In some instances it appeared spores were clumping because they may be embedded in root tissue.

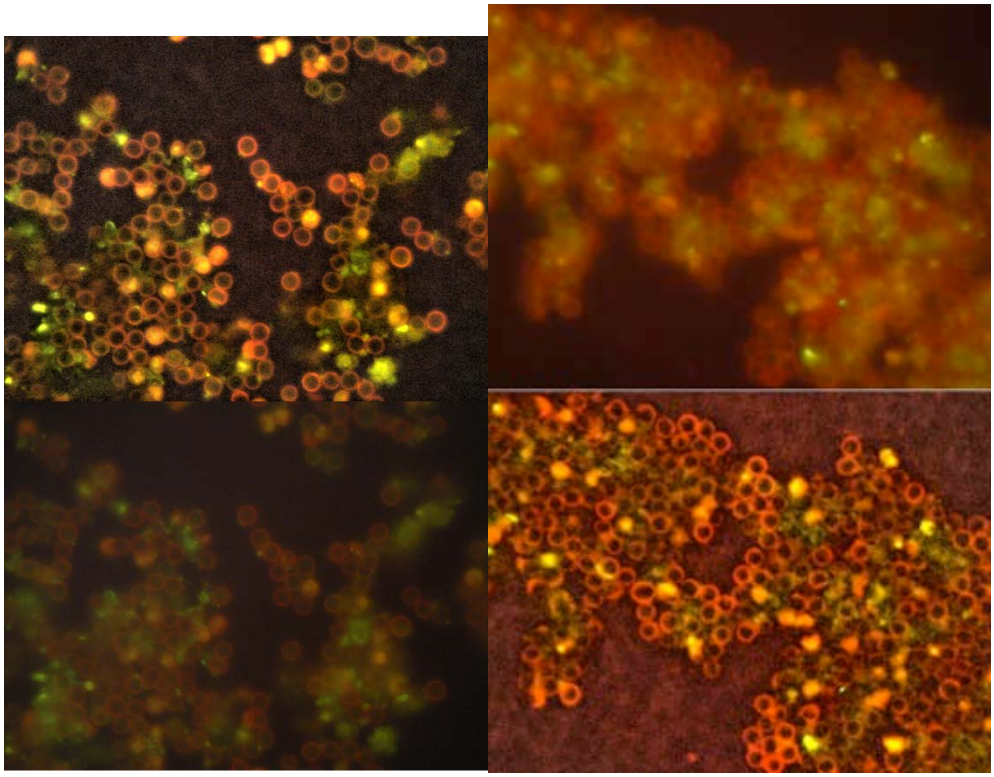

Figure 5. Fluoresence microscopy images of *P. brasssicae* resting spores at x100 magnification. Clockwise from top left: Set 1 10^{-1} 'Old' spore suspension adjusted image, Set 4 10^{-1} 'Old' spore suspension raw image, Set 1 10^{-1} 'Old' spore suspension raw image, Set 4 10^{-1} 'Old' spore suspension adjusted image.

Seedling Assay

Germination levels of spores

Initial inoculation level of nutrient solution was 4 x 10^5 spores/ml for all treatments. The number of ungerminated spores was determined from the qPCR results of the spore suspension after inoculation and incubation; the presence of DNA showing that zoospores had not emerged from the resting spores and entered the roots. The difference between initial inoculum level of nutrient solution and the quantity of ungerminated spores was taken to be the number of germinated spores.

The percentage of spores germinating was calculated from the difference in spore levels prior to and after incubation with seedlings. The mean number of germinated spores was 3.72x10^5 spores/ml, (92.92%), while the minimum was 1.04x10^5 spores/ml (26.0%) and the maximum was 4x10^5 spores/ml (100%). The minimum spore germination occurred at 6mEq Calcium, pH 6, while the maximum occurred several times in a variety of different treatments.

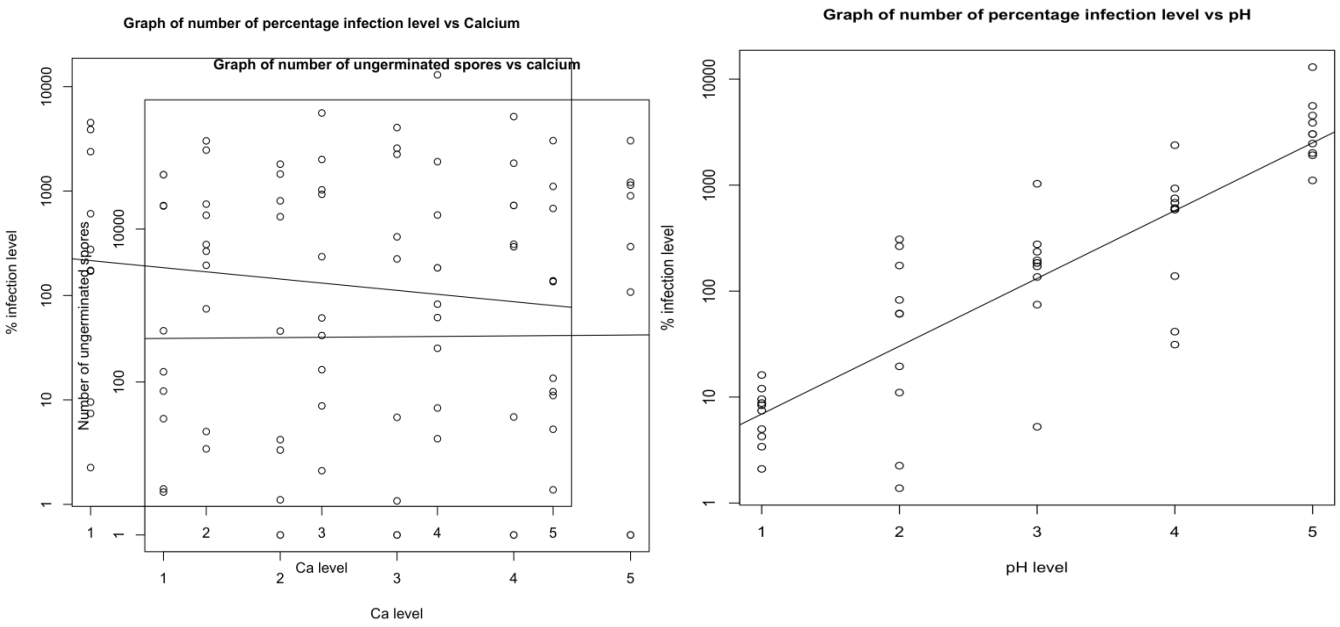

Figure 6. Linear regression to show relationships between pH and Calcium levels and the number of ungerminated spores remaining in solution following the seedling assay

Linear regression on counts of spores remaining in suspension (ungerminated) against pH levels provided a least squares regression line with R^2 = 0.212. In order to plot the regression line on a graph the data had to be transformed by taking the log of spore suspension figures plus one (to remove zero counts) (Figure 6) Pearson's product moment correlation figure was 0.41 for pH against ungerminated spores, however there was very little correlation between calcium levels and ungerminated spores, with a product moment correlation coefficient of 0.12. Two way ANOVA showed that pH did have a significant effect on the number of spores germinating, at the 95% confidence level, but neither calcium or any interactions between calcium and pH were found to be significant (Table 8).

Table 8. Two-way ANOVA to show interactions and effects of Calcium levels and pH levels on the number of ungerminated spores following the seedling assay

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
pH.level	1	2.7416e+10	2.7416e+10	10.0267	0.002738
Ca.level	1	2.3450e+09	2.3450e+09	0.8576	0.359238
pH.level:Ca.level	1	4.0824e+09	4.0824e+09	1.4930	0.227976
Residuals	46	1.2578e+11	2.7343e+09		

Infection levels of spores

The infection level of spores was taken to be the percentage change of germinated spores that were subsequently present in the root tissue as detected by qPCR (Table 9). The mean infection level was a 916.70% increase in spore numbers whilst the minimum was a 98.62%

(2dp.) decrease in spore numbers, and the maximum increase was 12880.00%. The minimum infection level occurred at8mEq Calcium, pH 4.5 while the maximum occurred at 4mEq Calcium, pH 6.

Table 9. Summary of the seedling assay count data

Ca level (mEq)	pH level	Spore susp. count	Root count	Germinated spores	% ungerminated	% germinated	% infection level
0	4	1.35E+02	2.97E+04	3999865	0.003375	99.996625	-92.57249
2	4	4.62E+02	1.99E+04	3999538	0.01155	99.98845	-95.01925
4	4	3.35E+01	3.51E+04	3999966.5	0.0008375	99.9991625	-91.22427
6	4	0.00E+00	3.35E+04	4000000	0	100	-91.62500
8	4	0.00E+00	4.80E+04	4000000	0	100	-88.00000
0	4.5	4.66E+02	8.99E+03	3999534	0.01165	99.98835	-97.74988
2	4.5	1.19E+01	1.06E+06	3999988.1	0.0002975	99.9997025	165.00788
4	4.5	0.00E+00	7.79E+04	4000000	0	100	-80.52500
6	4.5	0.00E+00	3.30E+05	4000000	0	100	-17.50000
8	4.5	0.00E+00	4.41E+04	4000000	0	100	-88.97500
0	5	3.21E+01	6.84E+05	3999967.9	0.0008025	99.9991975	71.01372
2	5	1.66E+01	2.98E+05	3999983.4	0.000415	99.999585	-25.49691
4	5	0.00E+00	9.41E+05	4000000	0	100	135.25000
6	5	2.04E+04	6.99E+05	3979600	0.51	99.49	84.14120
8	5	1.50E+03	2.09E+04	3998500	0.0375	99.9625	-94.75533
0	5.5	2.99E+00	2.43E+06	3999997.01	0.00007475	99.99992525	507.50454
2	5.5	0.00E+00	3.00E+06	4000000	0	100	650.00000
4	5.5	4.06E+03	1.64E+05	3995940	0.1015	99.8985	-58.57958
6	5.5	0.00E+00	1.25E+05	4000000	0	100	-68.75000
8	5.5	5.88E+03	5.46E+05	3994120	0.147	99.853	38.53649
0	6	2.64E+00	1.55E+07	3999997.36	0.000066	99.999934	3775.02558
2	6	0.00E+00	1.21E+07	4000000	0	100	2925.00000
4	6	1.79E+00	8.03E+06	3999998.21	0.00004475	99.99995525	1907.50898
6	6	5.86E+03	7.54E+06	3994140	0.1465	99.8535	1813.02583
8	6	0.00E+00	4.43E+06	4000000	0	100	1007.50000
0	4	7.50E+01	3.82E+04	3999925	0.001875	99.998125	-90.44821
2	4	1.88E+00	1.36E+04	3999998.12	0.000047	99.999953	-96.59998
4	4	0.00E+00	8.40E+03	4000000	0	100	-97.90000
6	4	3.39E+01	1.70E+04	3999966.1	0.0008475	99.9991525	-95.74964
8	4	0.00E+00	6.46E+04	4000000	0	100	-83.85000
0	4.5	2.03E+04	6.61E+05	3979700	0.5075	99.4925	74.08480
2	4.5	5.24E+04	1.07E+06	3947600	1.31	98.69	207.82509
4	4.5	7.91E+03	2.39E+05	3992090	0.19775	99.80225	-39.04461
6	4.5	6.32E+03	2.42E+05	3993680	0.158	99.842	-38.52875
8	4.5	2.72E+04	5.14E+03	3972800	0.68	99.32	-98.62124
0	5	2.00E+04	1.05E+06	3980000	0.5	99.5	176.31579
2	5	1.45E+04	7.51E+05	3985500	0.3625	99.6375	94.81193
4	5	9.49E+04	3.14E+06	3905100	2.3725	97.6275	929.17076
6	5	2.03E+04	6.99E+05	3979700	0.5075	99.4925	84.09270
8	5	3.75E+04	4.92E+05	3962500	0.9375	99.0625	35.72414
0	5.5	5.14E+04	8.30E+06	3948600	1.285	98.715	2280.95238
2	5.5	2.34E+04	2.21E+06	3976600	0.585	99.415	486.82953
4	5.5	2.12E+05	1.75E+06	3788000	5.3	94.7	830.85106
6	5.5	7.26E+04	1.93E+06	3927400	1.815	98.185	489.49297
8	5.5	4.08E+04	2.45E+06	3959200	1.02	98.98	582.07127
0	6	5.14E+04	1.58E+07	3948600	1.285	98.715	4432.41538
2	6	7.06E+04	8.13E+06	3929400	1.765	98.235	2368.12386
4	6	1.14E+05	1.60E+07	3886000	2.85	97.15	5494.40559
6	6	2.96E+05	1.35E+07	3704000	7.4	92.6	12880.76923
8	6	1.43E+05	7.81E+06	3857000	3.575	96.425	2938.91051

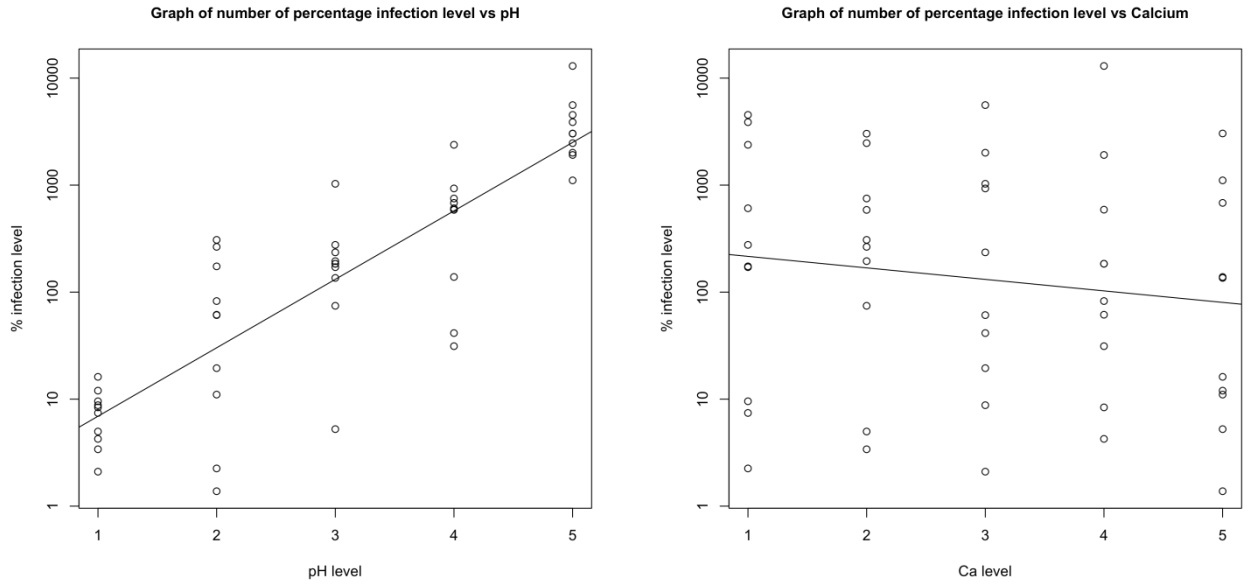

Figure 7. Graphs to show percentage infection vs Calcium and pH levels

Linear regression on the percentage infection level against pH levels provided a least squares regression line with $R^2 = 0.332$. In order to plot the regression line on a graph the data had to be transformed by taking the log of percentage infection level figures plus 100 (to remove zero counts) (Figure 7) Pearson's product moment correlation figure was 0.58 for pH against ungerminated spores, however there was very little correlation between calcium levels and percentage infection level, with a product moment correlation coefficient of -0.03. Two way ANOVA showed that pH did have a significant effect on the number of spores germinating, at the 99.9% confidence level, but neither calcium or any interactions between calcium and pH were found to be significant (Table 10).

Table 10. Two-way ANOVA to show interactions and effects of Calcium levels and pH levels on the infection level of *P. brassicae* spores

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
pH.level	1	75141649	75141649	22.7696	1.883e-05
Ca.level	1	293451	293451	0.0889	0.7669
pH.level:Ca.level	1	33567	33567	0.0102	0.9201
Residuals	46	151804304	3300094		

Field investigations in 2009

Effect of moisture content and pH on mean levels of P. brassicae DNA in the soil over the growing season

Levels of *P. brassicae* DNA obtained from qPCR results were considered against soil parameters over the whole growing season. This was assessed by taking the mean of the monthly recorded levels of *P. brassicae* DNA obtained by qPCR from the three plots. *P. brassicae* levels where found to be greater than $1x10^5$ spores per gram of soil at a water content above $0.3m^3m^{-3}$ (Figure 8).There was a calibration issue with the soil moisture probe due to the high soil water content shown here which will need to be resolved for future experiments. There was no clear relatonship between the mean spore levels for each pH over the course of the growing season, however when pH was examined against spore levels without considering the date of sampling the highest spores numbers were found at pH 6 while the lower and higher pH levels contained lower numbers of spores (Figure 8).

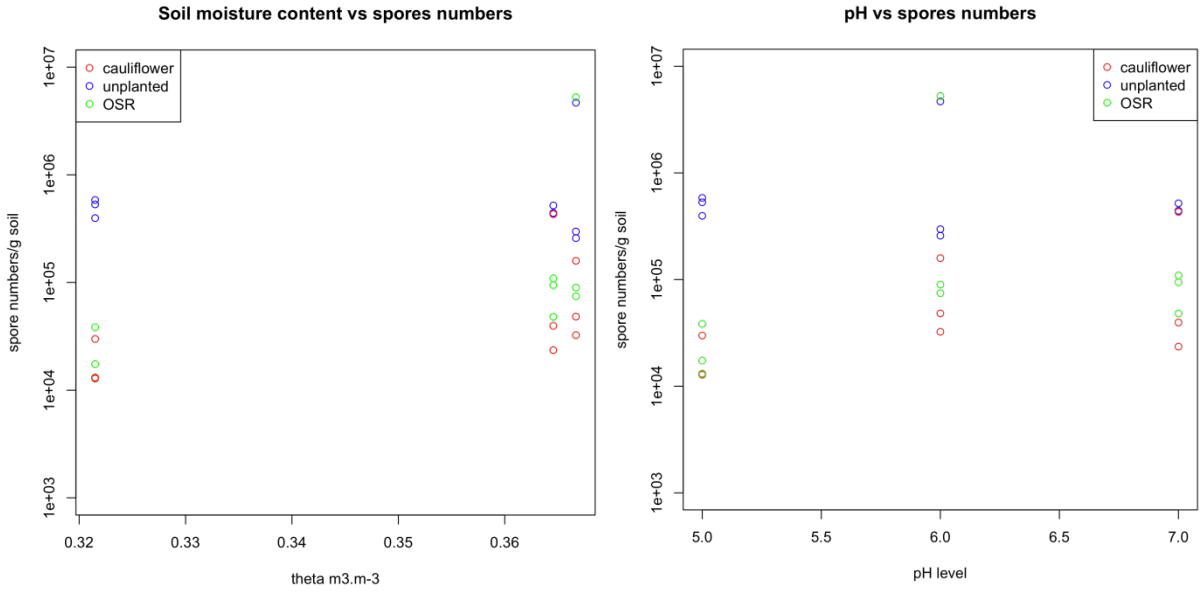

Figure 8. The effects of soil moisture and pH on *P. brassicae* DNA levels detectable in soil*The effect of different crop types on the levels of P. brassicae DNA detectable from soil by qPCR.*

The levels of *P. brassicae* within the plots was consistently between $1x10^4$ and $1x10^7$ spores per gram of soil. In the medium and high pH areas, the levels from the cauliflower planted region followed a very similar pattern starting on roughly the same levels in August then dipping slightly in September before increasing again in November to levels similar to those detected in August. In the low pH area however the August and November levels were similar to those of the other pH plots, but there was a peak up to $4.68x10^6$ clubroot spores (Figure 9).

A similar peak occurred in the low pH area of the OSR cropped area (Figure 9.) but in the OSR there was an increase in DNA levels in the high pH plot between the September and November sampling. In the OSR regions there was no detectable decline in spore numbers in September as shown in the cauliflower regions under any pH conditions, but for the low pH and medium pH areas there was a slight decrease in levels between the September and November samplings. It was not possible to determine whether any of these increases and decreases was statistically important, due to the small sampling size.

The unplanted regions in the QF field showed a similar peak in September as the low pH cauliflower region; however the increase was greater in the high pH plot with an increase of $3.95x10^2$ spores/g soil (Figure9). Soils in the cauliflower planted area displayed the highest levels of average *P. brassicae* DNA levels throughout the growing period while OSR displayed the lowest levels, despite the presence of the unplanted area.

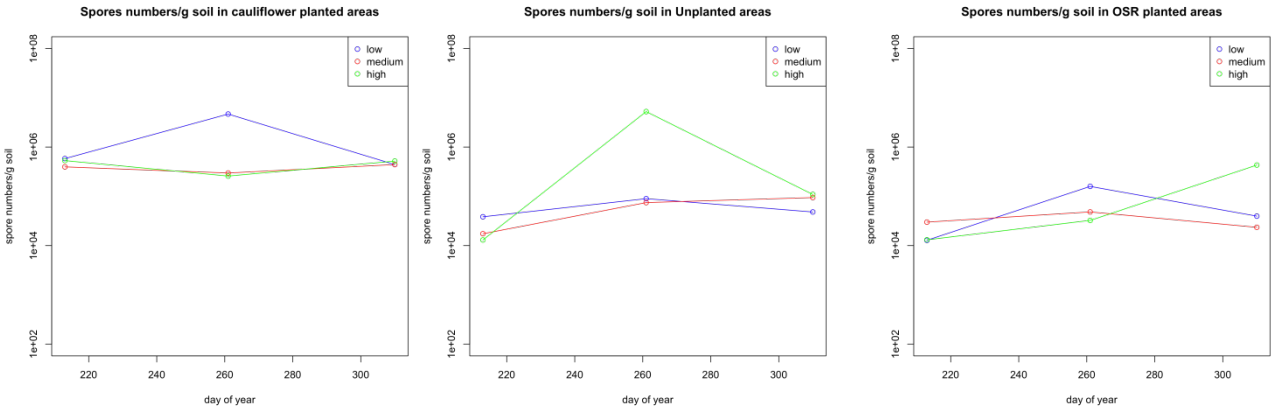

Figure 9. *P. brassicae* DNA levels in soils from the cauliflower, unplanted and OSR planted areas over time

Other meteorological factors

The interactions of various meteorological parameters have been widely documented and so have not been detailed greatly here. Figure 10 shows the relationship between air temperature and soil temperature, as well as soil moisture and rainfall. From these figures it was obvious there was a clear positive correlation between air temperature and soil temperature, (with Pearsons product moment correlation coefficient R^2 = 0.92), and that there was no significant correlation between rainfall and soil moisture (R^2= 0.13).

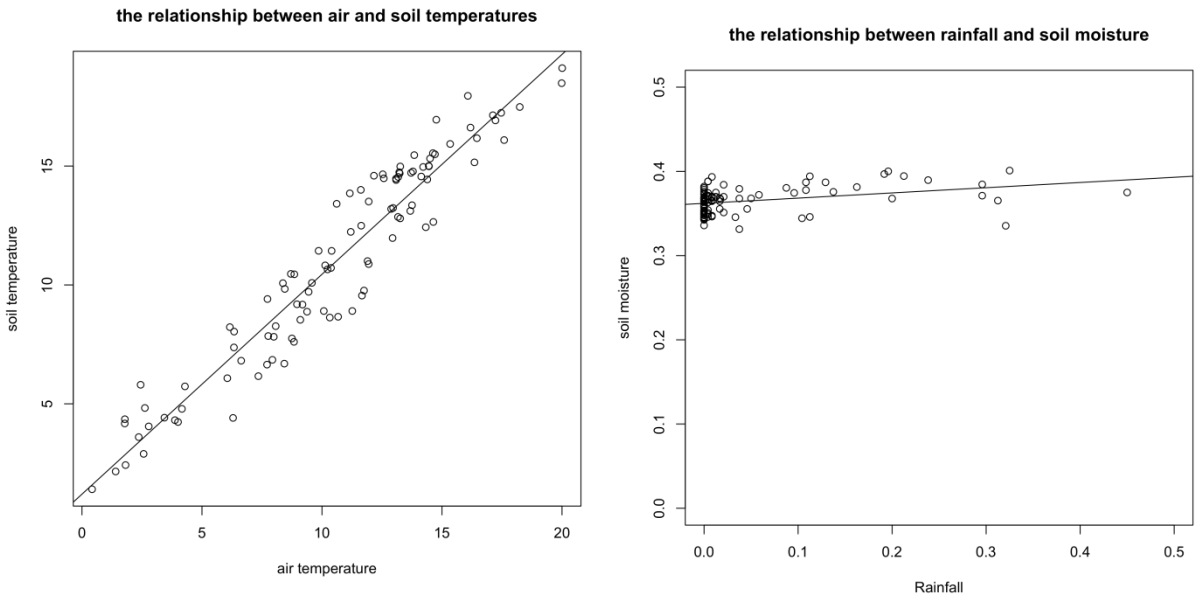

Figure 10. The relationship between air temperature and soil temperature: rainfall and soil moisture content

Year Three Results

Effect of environmental factors on resting spore viability

The effect of $CaCl_2$, $MgSO_4$ and NH_4NO_3 on spore germination and root infection

The number of germinating clubroot resting spores was calculated by subtracting the number of ungerminated spores (those remaining in spore suspension) from the initial inoculum level of $1.3x10^7$. In the positive control the mean number of germinating spores was $8.4x10^6$, in the treatment groups it was $1x10^7$, $9.8x10^6$ and $1.07x10^7$ in Ca, Mg and NH_4 treatments respectively. The germination and infection of the spores was regarded as the difference between the number of spores germinating and the number of spores present in the root tissue. The mean infection level in the positive control roots was $-3.6x10^6$, while in the treatment groups it was $-8.3x10^6$, $-7.7x10^6$ and $-8.7x10^6$ in Ca, Mg and NH_4 treatments respectively.

The results were taken for all the replicates of the qPCR, meaning that each treatment had three data points, and since the experiment had been carried out in triplicate this gave a total of nine data points per treatment. The results were divided so that Ca, Mg and NH_4 could be analysed individually and the data was included for the level of DNA in the remaining spore suspension and the seedling roots. The initial amount of spores from suspension had been diluted when the 1ml spore suspension was added to the 1ml nutrient containing solution /

H_2O, thus the initial starting spores/ml to which each seedling was applied was $2.6x10^7/2$ = 13000000 spores/ml treatment.

T-tests, assuming unequal variance, were performed of each treatment group against the positive control for both the number of spores germinating and the infection level. The summary statistics and t test results are shown in Table 11.

Table 11. The effect of Calcium, Magnesium and Ammonium on clubroot germination and infection

Nutrient	**Mean germinating spores**	**Mean infecting spores**	**T test germinating spores**			**T test infecting spores**		
			d.f.	**t value**	**P**	**d.f.**	**t value**	**P**
Ca^{2+}	$1x10^7$	$-8.3x10^6$	37.85	4.63	$4.2x10^{-5}$	33.45	-9.1383	$1.3x10^{-10}$
Mg^{2+}	$9.8x10^6$	$-7.7x10^6$	33.28	3.08	0.00408	36.994	-6.6135	$9.3x10^{-8}$
NH_4^+	$1x10^7$	$-8.7x10^6$	29.5	4.54	$8.7x10^{-5}$	31.52	-6.41	$3.5x10^{-7}$
positive	$8.4x10^6$	$-3.6x10^6$	-	-	-	-	-	-

When calcium levels (mEq/l) were analysed against log transformed DNA levels in spore suspensions after the incubation period one-way Anova indicated that there was no significant effect of calcium mEq/l on ungerminated spore levels as shown in Table 11. A polynomial model assuming linear trends was used to study the interaction between Ca levels and DNA levels. There was only a very slight negative correlation (R^2= -0.022) observed which suggests that there is no effect of Ca mEq/l on the number of spores remaining in suspension.

There was no statistically significant relationship between Calcium mEq/l and spores present in the seedling roots. A higher (positive) correlation was observed between these than between Calcium mEq/l and the number of ungerminated spores, at R^2 = 0.13 (2dp).

Table 12. The effect of Calcium, Magnesium and Ammonium on clubroot germination and infection

Nutrient	ANOVA germinating spores and nutrient level			ANOVA infecting spores and nutrient level			ANOVA passive spores		
	d.f.	F value	P	d.f.	F value	P	d.f.	F value	P
Ca	1	0.0169	0.8974				1	1.35	0.2532
Mg	1	4.1507	0.05023				1	5.5291	0.02524
NH_4	1	7.389	0.01175	1	22.792	$5.143x10^{-5}$	1	7.8766	0.009562

Magnesium levels (mEq/l) were analysed in the same manner as the Calcium levels, (log transformation of DNA levels detected by qPCR). A polynomial model assuming linear trends was used to examine the relationship between Mg levels and ungerminated spores remaining in suspension. One-way Anova revealed a there was a significant relationship at the 90% confidence level and Pearson's product moment correlation gave a coefficient R^2 value of -0.34 (to 2dp) (Table 12).

There was no significance detected between Magnesium mEq/L and the number of spores in root tissue. However in this instance there was a strong statistically significant relationship between the number of germinated spores (obtained by subtracting from initial spore levels as described in earlier results) and the number of spores in the root tissue, as shown in Table 12.

The results of the effect of NH_4 on *P. brassicae* DNA levels showed a similar pattern to that of Magnesium levels when analysed. One-way ANOVA showed a significant relationship (95% confidence level) between spore remaining in suspension and NH_4 mEq/l levels with a correlation coefficient of -0.48 (to 2 dp) (Table 13).

The effect of NH_4 on the number of spores in the root tissue was found to be highly statistically significant by ANOVA, above 99.9% confidence level. The correlation of the number of spores in the roots to the mEq/L NH_4 was -0.67 (to 2dp). In this instance there

was significance observed between the number of germinated spores and the number of spores in the roots and the correlation was R^2 = -0.49. However when the influence of both the number of ungerminated spores and NH_4 mEq/L on the number of spores in the roots was considered by generating a polynomial model which incorporated interaction it was found that the effect of NH_4 mEq/L and the number of germinated spores were still highly significant, but the interaction between germinated spores and mEq/l NH_4 was not significant (Table 13).

Table 13. The effect of Ammonium on clubroot germination and infection

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
germa	1	1.18038	1.18038	10.6190	0.003457 **
mEq	1	1.16561	1.16561	10.4861	0.003631 **
germa:mEq	1	0.02425	0.02425	0.2182	0.644810
Residuals	23	2.55662	0.11116		

** - Significant statistically significant result.

Viability of resting spores on hosts and from different geographic areas

Infection of European Clubroot Differential Set

The levels of spores within the root tissue varied according to the ECD host in which they were present (as shown in Figure 11) when infected by the isolate of clubroot used in field trials at Warwick HRI. Spores were identified in all hosts, the mean number of spores present in root tissue was $4.2x10^5$ spores/g, while the minimum number of spores ($2.7x10^3$ spores/g) was present in ECD Host 08 (*Brassica napus* L.var. *napus* line DC128) while the maximum clubroot content ($1.7x10^6$) was present in ECD Host 04 (*Brassica rapa*cv fodder turnip line AABBCC).

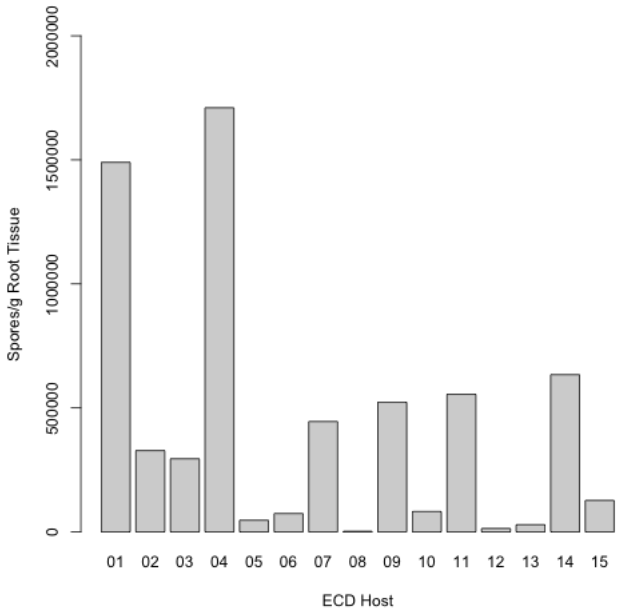

Figure 11. Infection of European differential set by clubroot resting spores.

Viability of clubroot spore populations from different geographic areas

The different gall populations run through the seedling assay were assessed by t tests to determine the significance of any difference between the populations (Figure 12). There was significant differences detected between the Northern Ireland gall samples and the East Scotland gall samples (t = 3.8831, df = 9.39, p-value = 0.003427), but there was no difference between the East Scotland and Warwickshire gall samples (t = 1.8495, df = 15.445, p-value = 0.08362) or the Warwickshire and Northern Ireland gall samples (t = -1.8831, df = 10.022, p-value = 0.089). By looking at the mean values of spores/g root tissue it is possible to determine that a greater number of spores within the root tissue occurred in the East Scotland gall samples, while the least number of spores within the root tissue occurred in the Northern Ireland samples. The Warwickshire samples had a greater range of infection however the mean level fell in between the East Scotland and Northern Ireland samples.

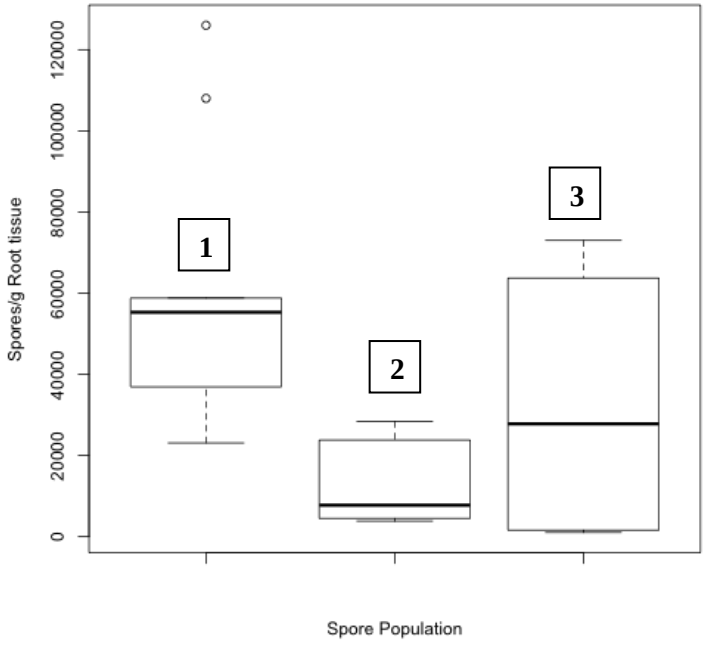

Figure 12. Viability of spore populations from isolates taken from Scotland (1) N. Ireland (2), and Warwick HRI (3).

Discussion

Using molecular tests for clubroot in soils

Molecular tests can confirm the presence of clubroot at low levels in soil samples. In previous work (Fv 259 Final report 2006) the test was successfully validated using clubroot contaminated and uncontaminated soil samples from Lancashire. The use of this test has been successfully extended for use on soil samples from other Brassica growing areas. Molecular tests have been conducted to determine the limit of clubroot detection in soil artificially infested with clubroot resting spores at a range of concentrations. The molecular test was not used to detect clubroot contamination below 1000 resting spores per gram of soil. Detection below this concentration in soil was not tested due to the small numbers of resting spores this equated to per gram of soil. However it is likely that the test could detect clubroot at 100 resting spores per g^{-1} soil. Detection below these levels would be difficult to validate because of the dilution factor in soil. Low numbers of resting spores within dilutions are difficult to replicate and so samples may not be of sufficient accuracy to ensure that they were contaminated at 100 or below. A commercially available kit (MOBIO UltraClean Soil DNA Isolation kit (MoBio Laboratories Inc.) can be used to extract *P. brassicae* DNA from soil, as shown by successful PCR amplification using primers specific for *P. brassicae*

amplification. The results of this PCR show that a 1:10 dilution of the DNA extraction product is necessary to provide a good level of detection, while no dilution or 1:100 dilution results in little or no detection at all at lower spore numbers per gram of soil. This could be due to a reduction in the levels of PCR inhibitory substances by dilution; however this beneficial effect is reduced at more diluted concentrations due to the corresponding reduction in DNA levels. The results of PCR amplification show that detection using either the primers of Faggian *et al* (1999) or Cao *et al* (2007) is repeatable and reliable, with primer set TC1 F/R consistently amplifying as little as 10^2 spores per gram soil, with the potential to amplify 10^5 and 10^4 spores per gram soil using primer sets TC2 F/R and PbITS1/2 respectively. Other primer sets studied did not provide the same consistent results and in some instances amplification of DNA other than that from *P. brassicae* was achieved. The results of sequencing the amplification products from the primer sets of Faggian *et al* (1999) and Cao *et al* (2007) combined with the consistency of detection at levels above 10^6 spores per gram soil suggest it is appropriate to consider these sets of primers as reliable when amplifying DNA extracted from soil using a commercially available kit.

Quantitative PCR (qPCR)

qPCR is a useful way for quantifying spore numbers of *P. brassicae* in soil, which is less dependent on user subjectiveness than microscopic counts. There are potential limitations to this method with the main limitation being that it cannot distinguish between live and dead spores, but it remains a useful tool nonetheless. It has been shown that three primers sets can be used to quantify *P. brassicae* DNA, but the most effective of these are PBITS3/4 and MLPb1F/R. PBITS3/4 only amplifies an 86bp region of the *P. brassicae* genome (data not shown) which means that it is slightly more difficult to sequence than a longer sequence. The amplicon of MLPb1F/R is longer at 118bp which was sequenced. The different types of DNA extracts for generating a standard curve did not appear to have a great influence on the reaction efficiency, however it may be appropriate to use standard curves generated from the same source as samples when quantifying DNA to eliminate any possible differences that may arise due to the different extraction techniques. Using the optimal parameters for the different primer sets it is possible to confidently quantify levels as low as 100spores/ml or 100 spores/g soil.

The development of assays for assessing the viability of P. brassicae resting spores

Staining optimisation

The nucleic acid staining of *P. brassicae* spores did support the work of Niwa et al (2008), in that spores were identifiable and nucleic acids were stained in some spores and not others of the same samples when using a Syto nucleic acid stain. Spores that do not contain a

nucleus can be considered as non-viable as they will not be able to produce a zoospore and subsequent infection; however it is possible this is due to germination in the past so they may have been viable at some point in their life-cycle. The spores containing a nucleus cannot be definitively considered to be viable as it is possible they will never germinate even under optimal conditions. An assay to distinguish viable and non-viable spores using nucleic acid staining would need to contain a germination stimulant component and the levels of spores containing a nucleus measured before and after exposure to such a stimulus.

The method used here did have limitations as capturing clear images of the spores was difficult but this depended on camera set up and steadiness and it was found that image adjustment did allow for easier identification of viable and non-viable spores. It was also found that capturing images using a digital camera was easier than trying to count down the microscope, as the red staining of the empty spores could be difficult to distinguish from the black background by eye. Clumping of spores on the slide also meant that a slightly lower dilution of spore suspension was easier to count than neat spore suspensions. The different methods of applying the stains did appear to greatly affect the end result, as the clearest results were obtained from sets 1 and 4, which had had the different methods applied. The same could be said about the concentration of Syto 16. The method used for set 4 is slightly easier on the operator as the stains and spore suspension are used together and do not require a rinsing step.

A different combination of stains and microscope filters may provide a greater ease of distinction between full and empty spores, but the cost and time required to test all of the different combinations available could be restrictive.

Seedling Assay

The seedling assay showed much promise as a method of assessing the germination potential of *P. brassicae* resting spores, but there are assumptions involved, such as the assumption that the decline in DNA quantities in the spore suspension during the incubation period are due to germinating spores and not some other means of DNA degradation. It is also difficult to be sure that the roots are washed free of spores when seedlings are removed from the nutrient solution but this effect is counteracted as all roots are exposed in the same manner, meaning any adhering spores should be uniform across different treatments.

The results presented suggest the number of ungerminated spores is positively correlated with increasing pH levels, because although the correlation coefficient is not high it does still show there is some correlation, and this is supported by the ANOVA results. The relationship

between pH and germinating spores found here is not large enough to provide an accurate regression, so at the present time the potential for germinating spore numbers to be predicted if the pH is known is still just out of reach. A larger trial size over a wider range of pH may provide the extra data needed to allow such predictions to be made.

The percentage infection rate of the spores follows a similar pattern to that of spore germination, displaying a positive correlation with pH levels and little or no correlation with calcium levels. The fit of a least squares regression line is better than with ungerminated spores against pH, but is still not a good fit of the data. The importance of the relationship between infection rate and pH is empathised by the ANOVA results where pH is found to be very significant.

From the results it appears that the number of ungerminated spores remaining in spore suspensions after exposure to root tissue increases as pH increases, alternatively stated as the number of spores germinating decreases as pH increases. The percentage infection level also rises as pH increases. These results suggest that pH has a negative effect on spore viability when the spores are not in the plants, but once the zoospores have entered the roots replication within the plant is increased, this could be due to the optimal growing conditions for Brassica crops being around pH 7.5, meaning that closer to this level plants are stronger and have stronger root systems, thus allowing the zoospores to make maximum use of their host plant environment (greater availability of space, nutrients etc. within the roots). This theory remains in line with the commonly held belief that high pH is restrictive to clubroot development, as the number of spores germinating decreases with pH. However this would suggest that there may be a maximum pH level after which no spore germination occurs, and just below this level there is an optimum pH for zoospore replication within host plants, which is higher than the optimum pH for resting spore germination.

Assessing viability of clubroot spores in soil environments using Ludox gradients

In order to be able to assess the viability of *P. brassicae* resting spores in soil. It was first necessary to be able to reliably detect the pathogen and extract it from the soil environment.

While work using density gradients is far from new, the concept of using it to determine the proportions of viable and non-viable spores is not one that has received much attention. It is clear that the use of a Ludox density gradient scan separate spores from plant tissue, producing a clearly defined band of spores within the gradient. It is not clear at this time how successful this will be on soils, as it may be difficult to release the spores from the soil matrix in large enough quantities to achieve a distinct band within the gradient. The hypothesis that viable spores will be more dense than non-viable spores has yet to be elucidated, however it

is reasonable to assume that spores obtained in this manner from freeze dried galls are likely to be viable as they are spores that were produced last season, and as such have not been exposed to any adverse conditions which could affect viability. Thus the separation of the spores from the plant tissue would only result in one band of viable spores. Further work is needed on the release of spores from soils and the distinction between viable and non-viable spores, if this is to be used as a means of assessing clubroot spore viability.

The effect of chemical and root exudate treatments on the viability of P. brassicae resting spores

Endogenous reserves

Preliminary studies show that there is the potential for the identification and quantification of endogenous reserves from clubroot galls using the HPLC technique developed by Hallsworth and Magan (1997). Based on published evidence this may be a useful tool for investigating the viability of *P. brassicae*, however due to the lack of supply of acetonitrile during 2008 and the start of 2009 no further work on this method has been performed and this work may not be fully developed during the course of this project. If this work was furthered it would be necessary to confirm these results presented here are appearing as sugars from the spores and not the plant tissue. This could be achieved by running samples of clean plant roots. Work would also be needed to identify the sugar(s) appearing between 4 and 9 mins.

The effect of soil type and environmental factors on resting spore viability

Preliminary field investigation

The preliminary investigation in a field setting has shown that the spore levels in the soil can be successfully monitored over time and that there are some interesting relationships worthy of further investigation. The results of soil moisture content and DNA levels suggest there could possibly be a positive relationship between soil moisture content and DNA levels detected, with higher soil moisture content relating to slightly higher levels of DNA (Figure 7) which is the result that would be expected. With regards to pH however, it is known that higher pH results in less disease and so the results shown here suggest that this may not be due to an effect on the spores directly. The fact that there were almost the same levels of *P. brassicae* DNA in both the low and high pH plots suggests that the actual levels of spores does not influence disease, and that the higher pH may be more inhibitory to spores entering the plants, rather than a direct effect on the viability of the resting spores.

Some interesting issues are raised by the different results of the levels of DNA associated with different crop types. The main point of interest is why soils surrounding OSR crops should display less *P. brassicae* DNA than unplanted and Cauliflower planted areas. It may

be due to some factor of *P. brassicae* replication inside the host plant, or possibly that OSR stimulated a greater level of germination of resting spores, resulting in less spores being present in the soil, and therefore less DNA. Or another factor could be that the roots did not rot off as quickly as those of cauliflower plants, and therefore there was less release of spores into the soil at the time of sampling. The OSR crop did appear slightly more diseased, with greater numbers of removable clubs, which had not rotted as quickly as those on cauliflower plants.

Key areas of note include the relationship between soil moisture levels and *P. brassicae* DNA levels detectable in the soil. If there is a positive correlation between these two factors then this could be important information to help predict the pattern of disease incidence and whether disease levels can be predicted from monitoring the soil moisture levels.

Secondly, the fact that the high pH area had similar DNA levels to the low pH area but less disease suggests that the preventive measure of lime application to soil may act indirectly on the viability of spores but in some other way, possibly to prevent germination of resting spores into zoospores, thus reducing the level of disease. This is an area which could benefit from further investigation, whereby spore/DNA levels are monitored more closely over time and the levels of disease more stringently recorded in terms of the development of galls on plants.

Finally it will be interesting to determine whether there are any correlations between *P. brassicae* DNA levels and other measurable parameters such as soil temperature, air temperature, humdity, rainfall and leaf wetness. If no relationships are discovered, regular recordings of these would provide a great insight into the quarantine field environment, which may prove useful for future studies.

Conclusions

- Primers developed by Faggian *et al* (1999) and Cao *et al* (2007) are reliable when detecting *P. brassicae* from soil DNA extracted using a commercially available kit.
- These primers are also suitable for detection of DNA from a range of soil types using quantitative PCR.
- Staining clubroot spores with Syto 16 nucleic acid stain can indicate those clubroot spores with the potential to germinate.
- Seedling assays have shown that infection by clubroot spores is affected by pH whereas calcium concentration has little effect.

- Trehalose and Erythritol from freeze dried Clubroot galls can be detected and quantified.
- Spores can be extracted from galls using Ludox density gradient separation.

References

Aist, J.R. andWilliams, P.H. (1971). Cytology and kinetics of cabbage root hair penetration by *Plasmodiophora brassicae*. Canadian Journal of Botany 49:2023-2033.

Bell, K.S., Roberts, J., Verrall, S., Cullen, D.W., Williams, N.A., Harrison, J.G., Toth, I.K., Cooke, D.E.L., Duncan, J.M. and Claxton, J.R. (1999). Detection and quantification of *Spongospora subterranea* f. sp subterranea in soils and on tubers using specific PCR primers. European Journal of Plant Pathology 105:905-915.

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

Buczacki, S., Ockendon, J.G. and Freeman, G.H. (1978). An analysis of some effects of light and soil temperature on clubroot disease. Annals of Applied Biology 88:9.

Buhariwalla, H., Greaves, S., Magrath, R. and Mithen, R. (1995). Development of specific PCR primers for the amplification of polymorphic DNA from the obligate root pathogen *Plasmodiophora brassicae*. Physiological and Molecular Plant Pathology 47:83-94.

Buhariwalla, H. and Mithen, R. (1995). Cloning of a Brassica repetitive DNA element from resting spores of *Plasmodiophora brassicae*. Physiological and Molecular Plant Pathology 47:95-101.

Bulman, S., Siemens, J., Ridgway, H.J., Eady, C. and Conner, A.J. (2006). Identification of genes from the obligate intracellular plant pathogen, *Plasmodiophora brassicae.*FEMS Microbiology Letters 264:198-204.

Bulman, S.R., Kuhn, S.F., Marshall, J.W. & Schnepf, E. (2001). A Phylogenetic Analysis of the SSU rRNA from Members of the Plasmodiophorida and Phagomyxida.Protist 152:43-51.

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: 80-87.

Cullen, D.W., Lees, A.K., Toth, I.K. and Duncan, J.M. (2002). Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. Plant Pathology 51:281-292.

Faggian, R., Bulman, S.R., Lawrie, A.C. and Porter, I.J. (1999). Specific polymerase chain reaction primers for the detection of *Plasmodiophora brassicae* in soil and water. Phytopatholog*y* 89:392-397.

Hallsworth, J.E. and Magan, N. (1997) A rapid HPLC protocol for detection of polyols and trehalose. Journal of Microbiological Methods 29:7-13.

Hussain, S., Lees, A.K., Duncan, J.M. and Cooke, D.E.L. (2005). Development of a species-specific and sensitive detection assay for *Phytophthora infestans* and its application for monitoring of inoculum in tubers and soil. Plant Pathology 54:373-382.

Ingram, D.S. and Tommerup, I.C. (1972). Life History of *Plasmodiophora brassicae* Woronin. Proceedings of the Royal Society of London Series B-Biological Sciences 180:103-112.

Ito, S., Maehara, T., Maruno, E., Tanaka, S., Kameya-Iwaki, M. and Kishi, F. (1999). Development of a PCR-based assay for the detection of *Plasmodiophora brassicae* in soil.Journal of Phytopathology 147:83-88.

Ito, S., Maehara, T., Tanaka, S., Kameya-Iwaki, M., Yano, S. and Kishi, F. (1997). Cloning of a single-copy DNA sequence unique *to Plasmodiophora brassicae.*Physiological and Molecular Plant Pathology 50:289-300.

Klemsdal, S.S., Herrero, M-L., Wanner, L.A., Lund, G. and Hermansen, A. (2008). PCR based identification of *Pythium* spp. causing cavity spot in carrots and sensitive detection in soils. Plant Pathology (on-line early) doi: 10.1111/j.1365-3059.2008.01839.x.

Klewer, A., Luerben, H., Graf, H. and Siemens, J. (2001). Restriction fragment length polymorphism markers to characterize *Plasmodiophora brassicae* single-spore isolates with different virulence patterns. Journal of Phytopathology-Phytopathologische Zeitschrift 149:121-127.

MacFarlane, I. (1970). Germination of resting spores of *Plasmodiophora brassicae.*Transactions of the British Mycological Society 55:97-112.

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, Madison, Wisconsin. p. 24–28a.

Myers, D.F., Campbell, R.N. and Greathead, A.S. (1983). Thermal inactivation of *Plasmodiophora brassicae* Woron. and its attempted control by solarization in the Salinas Valley of California. Crop Protection 2(3):325-333.

Naiki, T., Dixon, G.R. and Ikegami, H. (1987). Quantitative estimation of spore germination of *Plasmodiophora brassicae.*Transactions of the British Mycological Society 89:569-572.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution4:406-425.

Silvar, C., Duncan, J.M., Cooke, D.E.L., Williams, N.A., Diaz, J. and Merino, F. (2005). Development of specific PCR primers for identification and detection of *Phytophthora capsici*Leon.European Journal of Plant Pathology 112:43-52.

Tremblay, N., Belec, C., Coulombe, J. and Godin, C. (2005). Evaluation of calcium cyanamide and liming for control of clubroot disease in cauliflower. Crop Protection 24(9):798-803.

Wakeham, A. and White, J. (1996). Serological detection in soil of *Plasmodiophora brassicae* resting spores. Physiological and Molecular Plant Pathology 48:14.

Wallwnhammar, A.C. and Ardwisson, O. (2001). Detection of *Plasmodiophora brassicae* by PCR in naturally infested soils. European Journal of Plant Pathology107:313–321.

Webster M.A.,and Dixon G. R. (1991) Calcium, pH and inoculum concentration influencing colonisation by *Plasmodiophora brassicae*. Mycological Research 95: 64-73.

White, J.G. and Buczacki, S.T. (1979). Observations on suppression of Clubroot by artificial or natural heating of soil. Transactions of the British Mycological Society 73:4.