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Assessment of the response of *Plasmodiophora brassicae* in contaminated horticultural land, using lime-based fertilizer concentrations

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[Correction added on 15 November 2019, after first online publication: Mary Hanson and Geoff Petch have been added to the authorship and to reflect this change, the author order and affiliations have been updated in this version.]

Abstract

Infection of brassica crops with the clubroot pathogen, *Plasmodiophora brassicae*, can result in stunted plant growth and wilting, which can severely affect crop yield. Determining *P. brassicae* infection within a field prior to crop planting has long posed a problem for choosing appropriate control treatments. The options for control of this pathogen are limited and in the UK and are based on adjusting pH with soil amendments. In this study quantitative polymerase chain reaction (qPCR) was investigated for measurement of this pathogen in different control treatments. The qPCR was capable of reliably quantifying *P. brassicae* at levels greater than and including 10^3 resting spores/g soil. The assay was used to study the effect of lime-based products (LimeX) on the incidence of the clubroot pathogen in field trials with broccoli crops grown on contaminated land. The results showed that variation occurred in clubroot resting spore levels in treated and untreated plots during the crop growing period. In year one there was a 96% decrease in spore load during the growth of the crop. Treatment with LimeX resulted in a greater marketable head weight of broccoli in 2 years of the field trials, and significantly reduced gall numbers on the roots in 1 year. The rate of lime (calcium carbonate) application was not found to have a significant effect in this study, however a greater reduction in clubroot was observed at higher LimeX concentrations.

KEYWORDS

clubroot, control, lime, *Plasmodiophora brassicae*, qPCR, real-time

1 | INTRODUCTION

Clubroot on brassica crops is caused by the soil-borne protist, *Plasmodiophora brassicae*. It is widely distributed and can result in substantial yield loss in infected crops due to poor nutrient uptake brought about by disruption to the root tissues (Buczacki, 1983; Karling, 1968). Disease recognition depends on the presence of clubbed roots, and mild infections may not have a visible effect on crop growth. Nevertheless, the steady accumulation of *P. brassicae* resting spores in the soil may occur over consecutive years of cropping resulting in severe yield losses.

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The resilience of *P. brassicae* resting spores means they are capable of inducing disease in brassicas years after the initial detection. There are fungicides which control clubroot disease (Cheah, Page, & Koolaard, 1998; Peng et al., 2011), but these are not registered for this purpose worldwide. This is particularly problematic within the UK where no definitive control measures are approved. The half-life of spores has been estimated at 3.6 years (Wallenhammar, 1996). At the present time there are no reliable tests of resting spores viability due to the parasitic nature of the pathogen (Buczacki, 1983). There is no simple method of determining an infestation of *P. brassicae* within crop systems, apart from a traditional plant bait test, which is labor intensive and slow to perform. More recently the quantification of *P. brassicae* in soils has been reported (Jin-Ping, Yan, Xue-wen, A-li, & Bao-ju, 2013; Wallenhammar, Almquist, Söderström, & Jonsson, 2012) and now both qualitative and quantitative assessments of soils are available to growers commercially, based on these approaches.

There are many factors within the soil environment affecting the resting spores, such as temperature, moisture content, soil composition (both nutritionally and structurally). The occurrence of clubroot is particularly influenced by the relationship between calcium and pH (Myers & Campbell, 1985). For maximum severity of disease there is a threshold level of infection and this has been shown to be dependent on environmental conditions such as pH, calcium and boron concentrations (Webster & Dixon, 1991a,b). The incorporation of lime-based compounds within cropping systems is well-established (Van Slyke, 1932); providing a source of calcium for crops, helping the structure, pH and microbial composition of soil, and being particularly useful as a fertilizer application. The application of calcium compounds before crop transplantation also reduces the incidence of clubroot within crops (Donald, Lawrence, & Porter, 2004; Karling, 1968). Comprehensive reviews (Dixon, 2009; Donald & Porter, 2009) discuss the scientific literature relating to the effect of calcium, liming, and pH (among others) on *P. brassicae* development.

However, the individual and interactive effect of calcium and pH on clubroot incidence has yet to be fully determined. Niwa et al. (2007) showed that calcium-rich organic matter suppressed disease. This was due to the effect of increasing pH rather than the presence of calcium. Subsequently they demonstrated that rhizosphere spore germination is inhibited when calcium carbonate (or calcium-rich organic matter) is used to neutralize the soil (Niwa, Nomura, Osaki, & Ezawa, 2008). Myers and Campbell (1985) also studied the effects of pH and calcium, but took into account the effects of magnesium. They found that high pH reduced clubroot incidence, and that calcium and magnesium concentration in the soil also affected disease levels. There is evidence supporting the effect of calcium on *P. brassicae* at more than one time during

its lifecycle. It has its greatest effect on *P. brassicae* when it is present before spore germination and during the period following penetration of root hairs (Dixon, 2009). Calcium can stimulate germination of microbial spores (Sussman & Halvorson, 1966) and this has been shown to be linked to germination in *P. brassicae* (Kageyama & Asano, 2009). It has been suggested that the effect of calcium on *P. brassicae* may be greatest when the organism is in the root hair, rather than in the soil or during the penetration processes (Dixon & Webster, 1988).

Conventionally the detection of *P. brassicae* was carried out by gall observations and bait tests. With the advent of molecular techniques, PCR amplification of DNA has been used to aid detection and study the diversity of *P. brassicae* (Buhariwalla, Greaves, Magrath, & Mithen, 1995; Buhariwalla & Mithen, 1995; Cao, Tewari, & Strelkov, 2007; Faggian, Bulman, Lawrie, & Porter, 1999; Faggian & Strelkov, 2009; Ito et al., 1997, 1999; Wallenhammar & Ardwisson, 2001). Methods of *P. brassicae* PCR detection are often based on ribosomal RNA (rRNA) gene internal transcribed spacer regions (ITS) (Ito et al., 1999). Detection and quantification is central to investigating the control of *P. brassicae*. The objectives of this study were to quantify *P. brassicae* spores using real-time PCR (qPCR) and investigate the effect of Limex (LimeX70) and conventional lime-based products on clubroot control. Limex is a form of CaCO3 produced as a by product of the sugar beet refining process (Harling, 2007). Limex contains P_2O_5, MgO, and SO_3. The addition of calcium carbonate to clubroot contaminated land has been shown to improve control. However large quantities of calcium carbonate are required to produce consistent results. This can raise the pH of the soil to levels which are inconsistent with the other crop production in the rotation (potatoes). The number of *P. brassicae* resting spores in soil was monitored in different control treatments and the subsequent effect on crop yield was ascertained.

2 | MATERIALS AND METHODS

2.1 | Real-time quantification

Total DNA was extracted and purified from soil which had tested negative for clubroot by standard PCR and bait planting (data not shown). An aliquot of this soil was inoculated with 10^9 spores/ml *P. brassicae* suspension collected from macerated gall tissue. A 10-fold serial dilution of the 10^9 spores/ml *P. brassicae* DNA extract into the total DNA extracts from the clubroot negative soil provided a set of standards against which unknown samples could be interpolated.

DNA was extracted from all soil samples using the MOBIO UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc. Carlsbad, CA, USA) following the

manufacturer's protocol with amendments. The vortex step was replaced by homogenization in a FastPrep® Instrument (QBiogene, Irvine, CA, USA) at a speed setting of 5.5 for 25 s, with samples resting on ice in between runs for a total of three runs. A Polyvinylpolypyrrolidone (PVPP) DNA clean-up was performed on all DNA extracts using a protocol modified from Klemsdal, Herrero, Wanner, Lund, and Hermansen (2008), where empty Micro Bio-Spin® Chromatography Columns (BioRad Laboratories Ltd., Hertfordshire, UK) were placed in 2 ml Starstedt tubes and filled to the shoulder with PVPP. The PVPP was rehydrated with 500 µl molecular H_2O and left to hydrate at room temperature for 5 min. Columns were centrifuged for 3 min. at 1500 g. Flow through was discarded, and these steps repeated. A further centrifugation of 4500 g for 1 min was performed and columns were placed in a clean 1.5 ml low-bind Eppendorf tube. The 100 µl DNA samples were applied to the surface of the PVPP and incubated at room temperature for five min. A final centrifugation step of 3500 g for 3 min was included and the supernatant retained. Nucleic acid concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Samples were immediately stored at −20°C until use.

Quantitative PCR (qPCR) was set up manually using LightCycler® 480 384 Multiwell plates using a 20 µl reaction mix composed of 10 µl Lightcycler® 480 Sybr Green I Master (Roche Diagnostics, Burgess Hill, UK), 1 µl each primer (desalted, final concentration 200 nM) (Sigma-Aldrich Company Ltd. Dorset, UK), 6 µl ROH_2O, and 2 µl sample DNA. The primers used, PbITS3 and PbITS4 (Faggian & Parsons, 2002), were designed to amplify 86 bp within the internal transcribed spacer (ITS) region of the *P. brassicae* genome, as detailed in GenBank Accession AF231027 and searched against NCBI GenBank database using the nucleotide Basic Local Alignment Search Tool (BLASTN) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm specificity for *P. brassicae*. Cycling was performed on a LightCycler 480 instrument (Roche Diagnostics, Burgess Hill, UK), with preamplification incubation of 95°C for 5 min, followed by 50 cycles of 95°C for 10 s, 62°C for 20 s, 72°C for 25 s. A subsequent melting curve was generated by a cycle of 95°C for 5 s, 40°C for 1 min. and an increase to 95°C of 0.06°C/s, recording fluorescence 10 times/°C. Finally samples were cooled to 40°C and held for 1 min.

Validation of qPCR was performed by assessing the concentration and melt curves of the qPCR assay. Assay performance was assessed on the results of five parameters; the no template control (ntc) samples, the qPCR efficiency, the linear dynamic range, the limit of detection and the repeatability of the assay, all in accordance with MIQE guidelines (Bustin et al., 2009). Run repeatability and individual assay repeatability were tested by running the same set of standards on five different plates at different times by three different operators, with each sample in triplicate on each plate. Intersample repeatability was examined by extracting five replicate DNA samples from three soils as previously detailed and calculating the % CV of the quantified results.

2.2 | Measurement of pH

Soil was air-dried and milled before wetting with water and the pH measured electrometrically. Soil (10 g) was placed into a 50 ml container with 25 ml distilled water. The suspension was shaken immediately and at frequent intervals for 15 min. A pH meter electrode calibrated with standard buffers was lowered into the suspension to a depth of 1 cm and the pH recorded after 30 s.

2.3 | Clubroot control experiments

The effect of LimeX and conventional lime-based products on clubroot control was assessed over 2 years. A site in Fife (Scotland) was selected with a recent history of brassica cropping and previous reports of clubroot occurrence. In both years treatments were allocated randomly, with five replicate plots per treatment. The trial locations for the 2 years were in neighboring fields, avoiding reuse of the same plots and therefore eliminating any follow over effects from the first year treatments. In year one three application rates of LimeX70 (7.5, 10.0, and 12.5 t/ha), and untreated plots were assessed. In year two the same treatments were used and a standard lime treatment (5 t/ha) was also included. Each trial site measured 50 m × 50 m and comprised 25 plots, each 10 m × 10 m. The site was planted with broccoli, *Brassica oleracea* cv. Parthanon at an interval spacing of approx. 45 cm. Treatments were applied by hand, and commercially raised transplants were transplanted in June (year one) and July (year two). Soil was sampled prior to planting and throughout the growing period up to, or just after, the time of harvest. Soil cores were taken at a depth of 20–30 cm air-dried, milled and sieved to <2 mm prior to DNA extraction, purification, and quantification following the real-time quantification methods as detailed. In both years 10 plants were selected at random and assessed in August from each plot, taking care not to damage the root tissue, and the percentage of infected plants (with galls visible after washing) was recorded, along with the number of visible galls.

2.4 | Statistical analysis

Data were analyzed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla, CA, USA), with $\log_{10}$ transformation of the number of spores/g soil. Year one and year two data were analyzed separately due to the variation in cultivar and weather conditions.

3 | RESULTS

3.1 | Real-time quantification

When qPCR was used to quantify the amount of *P. brassicae* DNA present in DNA extracts from soil standards, the PCR efficiency was given by the equation: efficiency = $10^{-1/slope} - 1$. For the initial experiment this equaled 1.046 (to 3 dp) or 104.6% and linear regression gave a line with the equation $y = -3.215x + 39.31$ with $r^2 = .9988$ (Figure 1). A further four replicate runs of these standards all showed similar properties and the linear dynamic range spanned 1×10^3 to 1×10^9. The theoretical limit of detection based on the volume of soil used for extraction and the quantity of DNA in each sample run through qPCR was calculated to be 8.0×10^2 spores/g soil, and DNA was detected in 93.3% of replicates at 1×10^2 spores/g soil, and in 100% of replicates at 1×10^3 spores/g soil, therefore giving an actual limit of detection of 1000 spores/g soil.

Assay repeatability (within run variability) was examined from the percent coefficient of variation (%CV) observed within the triplicate samples that make up each concentration point of the standard curve. When the concentration points from all five standard curves were considered the overall range of $\log_{10}$ spore concentration percent %CV was 0.08–5.33, with a mean of 1.22. Further to this the run reproducibility %CV (between run variability) of all five replicate standard curves was calculated from the mean $\log_{10}$ spore concentration of the five runs, and this ranged from 0.635 to 5.269 with a mean of 2.457.

One-way analysis of variance (ANOVA) showed that there was no significant difference between the $\log_{10}$ concentration means of each run $F(4, 60) = 0.003$ (to 3 dp), $p > .9999$. Within three soil samples the intersample reproducibility %CV ranged from 1.95 and 3.42. The melting peaks occurred at 83°C, and the absence of any other peaks on the melt curve analysis also demonstrated that there were no primer dimer,

or nonspecific products present. The "no template control" samples did not produce amplification on any run.

3.2 | Clubroot control experiments

3.2.1 | Estimation of clubroot resting spores

In year one the amount of *P. brassicae* DNA present in soil samples from plots decreased after transplanting but then increased with time after transplanting (Figure 2). Two-way ANOVA showed that there was no significant difference between the *P. brassicae* level across the four Limex70 treatments, $F(3, 80) = 1.016$ (to 3 dp), $p = .390$, but there was a significant difference in the amount of *P. brassicae* present in the soil at the time of sampling, $F(4, 80) = 33.630$, $p < .0001$. The interactions between time after transplanting and *P. brassicae* levels were not significant $F(12, 80) = 0.365$, $p = .972$). The mean *P. brassicae* level at the time of transplanting was 8.20×10^4 estimated spore number. At the minimum point the mean was 3.31×10^3, suggesting a decrease equivalent to 7.87×10^4 spores/g soil (95.96%). In year two there was no significant difference between the *P. brassicae* level across the five treatments, $F(4, 75) = 0.389$ (to 3 dp), $p = .8543$, or in *P. brassicae* level on the DOY sampled $F(3, 75) = 2.489$, $p = .0513$ or the interaction between them $F(12, 75) = 1.214$, $p = .290$.

Neither year showed a significant difference between spore numbers in the soil at the time of brassica transplantation or the time of harvest, however there had been significant variation occurring in between these two time points. The mean number of spores at the time of transplantation in year one ranged from 9.77×10^4 to 1.91×10^5 across the treatments and subsequently measured 2.0×10^5 to 4.57×10^5 at the time of harvest. In year two the range covered 3.08×10^5 to 5.13×10^6 at the time of transplantation and 8.51×10^5 to 3.89×10^6 at the time of harvest.

3.2.2 | Assessment of root infection

Infected plants were determined by the presence of galling upon visual inspection of washed roots. In year one the untreated plots had 80% plants with root infection, The 10.0 t/ha LimeX70 plot had the greatest number of infected plants (90%), whereas 7.5 t/ha LimeX70 had the least (60%) and the 12.5 t/ha LimeX70 was intermediate at 70%. In year two the untreated plots displayed the most infection (60%), whereas the 7.5 and 12.5 t/ha displayed the least (10%). The standard lime showed 50% infection, whereas 10 t/ha LimeX70 showed 40% infection. One-way ANOVA showed there was no significant difference between the number of galls on each plant in year one. However in year two there a significant difference was observed; $F(4, 45) = 2.709$, $p \leq .05$. The 7.5 and 12.5 t/ha LimeX70 plots showed the lowest mean number of galls per plant, whereas the untreated plots showed
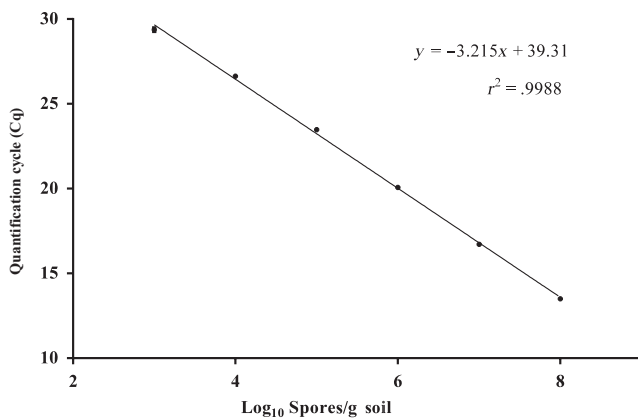

FIGURE 1 Linear regression between Cq and number of *Plasmodiophora brassicae* spores in soils artificially infected with clubroot disease
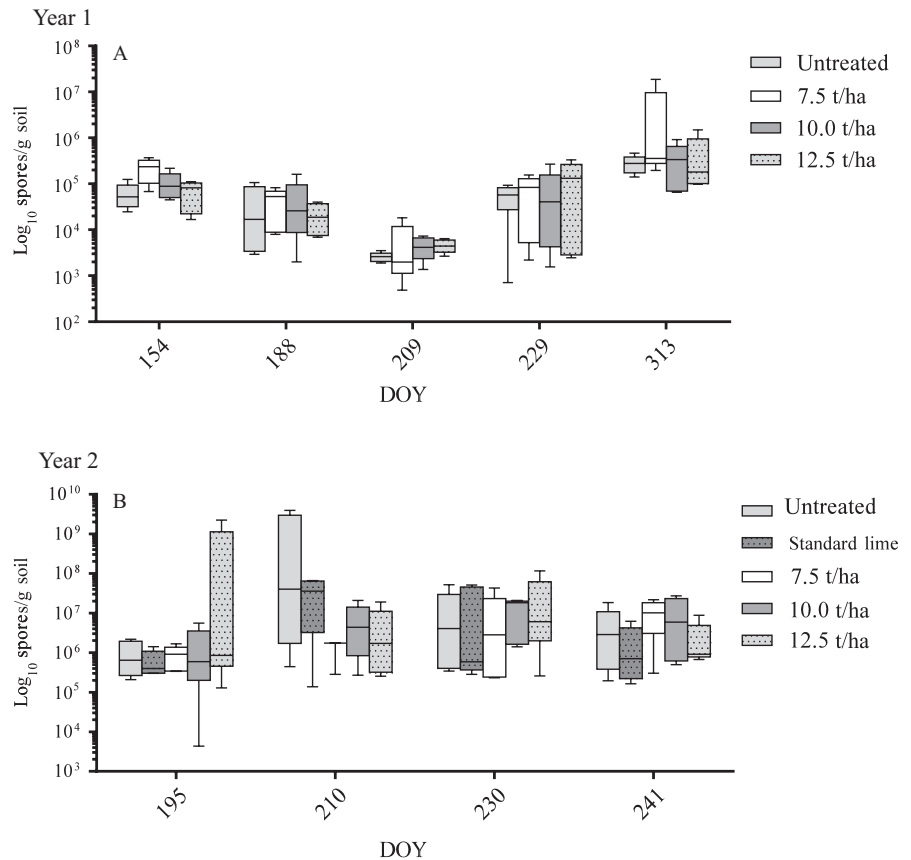
FIGURE 2 Number of *P. brassicae* spores present in soil samples from LimeX70, standard lime and control treatment plots over the course of the growing season for a brassica crop. A) First year data, B) Second year data. Error bars represent SD

the highest (Figure 3). The soil pH results are shown for the treatments in Figure 4.

3.2.3 | Harvest data

Yield (t/ha) was estimated from the total marketable weight of Broccoli heads within the sample plots and the proportion of a hectare covered by the test area. One-way ANOVA of harvest data (Table 1a and b) showed that LimeX70 treatments in both years and the standard lime treatment in year two significantly increased the total marketable head weight and the mean marketable head weight of the Broccoli crop. In year one the number of marketable heads was also significantly increased, but this effect was not observed in year two. No significant difference in marketable head weight between any of the treatments was observed in the year two trials (F (2, 12) = 0.5788, p = .5755). The LimeX70-treated plot yields were greater in year two than year one, and in both years the untreated plots had the lowest yield. The standard lime treatment yield (year two – Table 1b) was higher than in untreated plots, but lower than LimeX70-treated plots.

4 | DISCUSSION

The molecular quantification of *P. brassicae* spores in soil is a relatively recent development (Jin-Ping et al., 2013; Lewis, 2011; Lewis, Wakeham, & Kennedy, 2013; Wallenhammar et al., 2012) but an important step forward in the study of this soil-borne organism. Previous studies have documented detection at 500 spores/g soil (Wallenhammar et al., 2012) and 1000 spores/g soil (Jin-Ping et al., 2013). A detection level of 1000 spores/g soil was achieved in this study, and quantification of *P. brassicae* was performed that demonstrated the robustness of the assay with different operators with a good level of repeatability. Jin-Ping et al., 2013 discussed the benefits of using Sybr Green for quantification instead of a TaqMan probe as used by Wallenhammar et al. (2012) (primarily reproducibility and cost benefits) but acknowledged the slightly lower detection level achieved. One key feature of both previous quantitative PCR examples is the use of plasmid DNA to generate the standard curve. This is a common practice, however, it does not take into account the matrix in which the target amplicon/organism is found, although estimations of inhibition can be performed. Studies have found that using genomic DNA to generate the standard curve helps to account for matrix inhibition of the quantification reaction (Fu et al., 2009; Yun et al., 2006). An alternative approach could be to extract DNA from "*in planta*" cultures but there is also evidence to suggest that this does not provide an accurate representative standard curve either. The DNA used to generate the standard curve should come from the same matrix and have undergone the same procedures as the samples (An, Mainelis, & White, 2006). To this end, the use
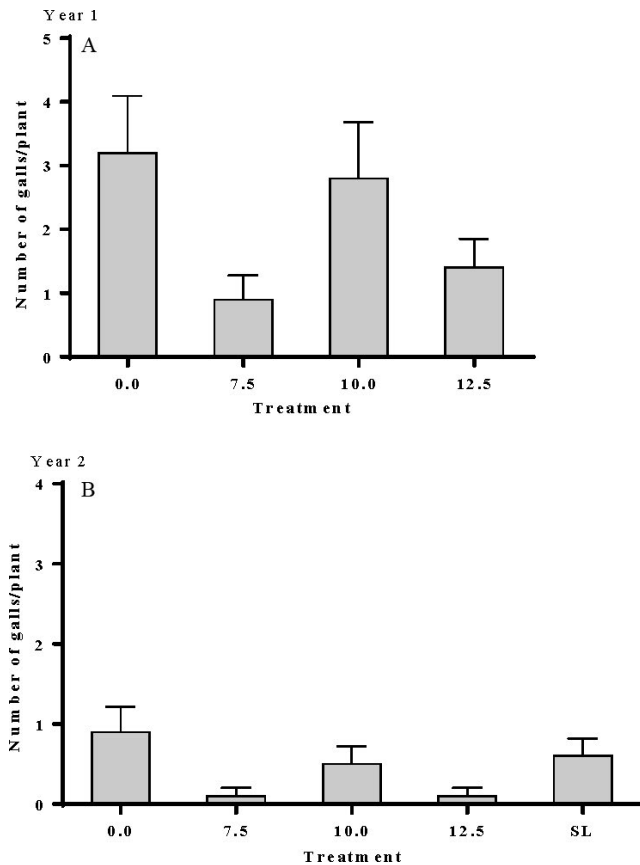
FIGURE 3 Number of galls appearing on washed brassica roots under different treatment rates of LimeX70, standard lime and control plots after growth in *P. brassicae* infected soil. 0 represents the untreated control, 7.5, 10.0 and 12.5 represent rates of LimeX70 application in t ha^{-1} and SL represents standard lime treatment. A) First year data, B) Second year data. Error bars represent SD

of total soil DNA from soils artificially infected with *P. brassicae* diluted into the DNA extracts of uninfected soils was performed to take into account the matrix inhibition.

The usefulness of being able to accurately quantify *P. brassicae* DNA enables studies from a variety of different media (not only soil) to be undertaken, allowing the levels of

P. brassicae to be monitored and control treatments assessed. One of the main findings of this study was the demonstration of variation in *P. brassicae* spore levels in soil throughout the course of the growing season. Despite the lack of difference between spore numbers present at the time of transplantation and at harvest it was clear that significant changes in spore levels occurred during the crop growing period. It has been documented that the lifecycle of *P. brassicae* consists of resting spores within the soil, which germinate to produce primary biflagellate zoospores. These then encyst on root hairs and enter the root hair tissue to form primary plasmodium which differentiate into secondary zoospores. The secondary zoospores are released from the root hairs and root cortical cell infection occurs. This leads to formation of the secondary plasmodium and subsequent production of resting spores which are released as the galls decay (Buczacki, 1983; Ingram & Tommerup, 1972; Kageyama & Asano, 2009).

Evidence for aspects of the lifecycle was found in this study. The findings of the first year's field studies support the germination of resting spores and subsequent movement into the host roots, by the reduction in spore levels as detected by qPCR after transplantation of the brassica crop (*P. brassicae* DNA is at reduced levels in the soil as migration of the DNA into the root tissue occurs due to zoospore movement and encystment). The change in levels from the start of sampling to the "dip" in spore numbers suggests that a large proportion (approximately 96%) of the *P. brassicae* population was germinating and infecting the plants in the trials. It has been observed that zoospores can be found in root hairs as early as 10 days after inoculation in pot experiments (MacFarlane & Last, 1959) with root swellings by day 20 and substantial gall formation by day 35. Germination has been observed 12 hr after inoculation in vitro (Ingram & Tommerup, 1972). A study using a model culture system observed primary and secondary phases of infection of *P. brassicae* occurring up to approx. 13–15 days after inoculation, and then subsequent gall formation by 28 days after inoculation (Agarwal, Kaul,
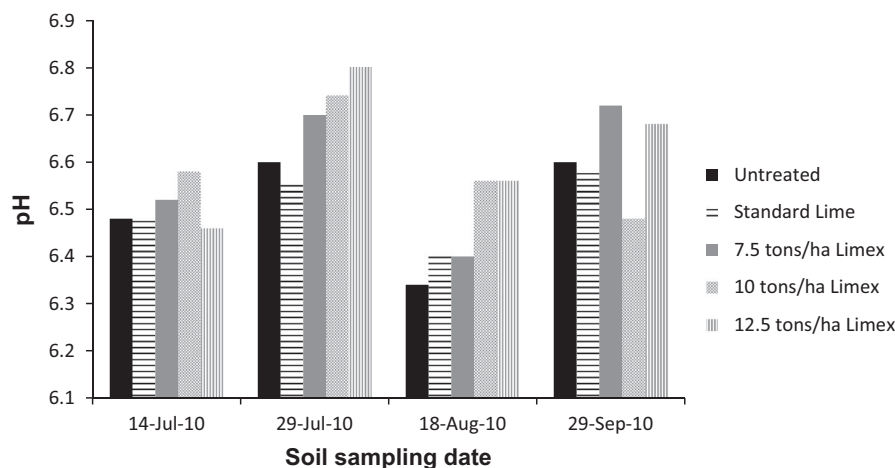

FIGURE 4 Soil pH in different concentrations of LimeX70 and Standard Lime (SL) treatments, in year two of field trials

TABLE 1 ANOVA results showing the effect of different concentrations of LimeX70 on various yield parameters in a brassica crop (a) Year 1 and (b) Year 2 (including a standard lime treatment)

					ANOVA	
Year 1 treatment	0.0 t/ha LimeX	7.5 t/ha LimeX	10.0 t/ha LimeX	12.5 t/ha LimeX	F (df)	p
(a)						
Yield t/ha	0.7	10.8	15.3	12.4	-	-
Number marketable heads	12.2 ± 6.4	23.8 ± 2.7	23.6 ± 2.0	24.4 ± 1.7	12.56 (3,16)	≤.001
Marketable head weight (g)	1979 ± 1011	7196 ± 862	7886 ± 857	8164 ± 415	63.49 (3,16)	≤.0001
Mean marketable head weight (g)	166.0 ± 27.1	304.0 ± 33.9	334.8 ± 36.62	335.8 ± 29.21	32.02 (3,16)	≤.0001

						ANOVA	
Year 2 treatment	0.0 t/ha LimeX	7.5 t/ha LimeX	10.0 t/ha LimeX	12.5 t/ha LimeX	Standard lime	F (df)	p
(b)							
Yield t/ha	2.2	3.9	3.8	4.2	2.7	-	-
Number marketable heads	14.8 ± 3.3	19.8 ± 2.6	17.6 ± 3.9	19.4 ± 4.0	17 ± 2.6	1.825 (4, 20)	= .1636
Marketable head weight (g)	3746 ± 1270	6497 ± 706.0	6302 ± 1300	7061 ± 1355	4690 ± 1204	6.774 (4, 20)	≤.01
Mean marketable head weight (g)	248.7 ± 43.6	329.0 ± 13.8	359.8 ± 21.4	366.3 ± 33.4	273.6 ± 49.7	11.15 (4, 20)	≤.0001

Faggian, & Cahill, 2009). The "dip" in this study was observed at 55 days after transplantation, which would be the period of gall formation, and the point at which most *P. brassicae* could be within the root cortical cells. The subsequent increase in spore levels would support the release of secondary zoospores and resting spores during the later stages of the lifecycle, as the level of *P. brassicae* DNA in the soil increases. The results of the second year did not show the same effect as significant spore number variation was not observed during this year. One reason may be because the field in year two had a higher spore load, and the movement of zoospores was not as apparent due to larger spore numbers. A further reason could be that environmental conditions varied, with wetter conditions present in year two. This would affect the clubroot lifecycle, as a higher soil moisture content is more conducive to clubroot (Mattusch, 1977; Narisawa, Shimura, Usuki, Fukuhara, & Hashiba, 2005). It may also simply be that the sampling frequency was not enough to detect the changes as the site was sampled less frequently than in year one.

The lack of significant changes in spore numbers between the time of brassica transplantation and the time of harvest suggests that either the accumulation of spores within the soil is a gradual process occurring over successive brassica crops, or that degradation of the gall tissue had not occurred by the final measurement period. In all plots across both years the estimated number of resting spores had risen (except in

the 12.5 t/ha LimeX70 treatment of year two) so it is possible spore release had started but was incomplete. There was an expected increase in estimated resting spore numbers after transplantation of a brassica crop and therefore it would have been expected that an increase in spore numbers would have been apparent had resting spore release from galls occurred and this should have been particularly visible in the spore numbers observed in the untreated plots. It is possible that migration of resting spores between plots would occur by movement in soil water, and this would be more apparent during wetter years.

From this study it was not possible to determine whether the lime treatments were significantly affecting the germination and infection rate of *P. brassicae*. The interaction been calcium and high pH in reducing disease incidence has been well documented (Dixon, 2009; Donald & Porter, 2009; Myers & Campbell, 1985; Webster & Dixon, 1991a,b) and previous studies have indicated a potential role for calcium in zoospore germination by *P. brassicae* as well as other microbial species, oomycetes being a relevant example (Kageyama & Asano, 2009; Walker & Van West, 2007). Further studies to investigate whether the stimulation of resting spore germination by calcium ions could affect the rate of infection in brassica crops could provide definition to the application rate and timing of lime treatments. Many studies have associated clubroot variation with pH or the level of calcium ions in soil. However both of these parameters vary with soil moisture

and so associations could be artifacts of the spatial and temporal variation in soil moisture. Soil moisture would also be critical in the transmission of clubroot. The results in this trial show that pH could not explain the responses observed. Calcium levels were not recorded.

Further applications of molecular detection and quantification of *P. brassicae* in relation to environmental factors and other chemical control treatments could be conducted. Application of calcium compounds, such as lime-based products, prior to transplanting reduces the incidence of disease (Donald et al., 2004; Karling, 1968). Some significance was found in the application of lime-based products on the number of galls per plant and it was shown that the total weight of marketable heads of broccoli was significantly higher in the lime- or LimeX70-treated plots in association with an increased mean marketable head weight of broccoli, suggesting that the application of LimeX products were having an effect on the incidence and/or severity of disease. On cabbage plants it has been demonstrated that the dry weight of heads and the size of leaves is reduced as spore load increases (MacFarlane & Last, 1959) and it is well-known that one of the key impacts for growers of clubroot infection is the (potentially severe) reduction in yield that can be experienced.

These types of questions can be answered by qPCR, allowing the documentation of the infection process and examination of the impact different factors have on the germination and infection of the resting spores in situ. This information provides scope for targeted treatments and integrated control treatments. The timing of soil sampling for spore quantification appears to be critical to gaining an accurate measurement of clubroot spore levels. The results in combination with an understanding of the lifecycle suggest that quantifying the levels of spores prior to planting brassica crops is key in determining the level of clubroot risk that the crop maybe exposed to. The addition of Limex improved the yield response in the crop which shows that it can be a useful approach in the control of clubroot in vegetable brassica crops. Limex maintains crop yield although Calcium carbonate applied as a standard lime treatment did not, suggesting that Limex has additional advantages in maintaining crop yields in the presence of clubroot.

The results clearly demonstrate that changing the nutrient balance in the root zone had an impact on clubroot infection of roots. These results could not be explained by variation in pH alone as the effect of incorporating standard lime did produce the same results as that observed with the inclusion of Limex. Neither treatment boosted the pH to levels where pH was inhibitory. The results of these field trials indicate that Limex can be used successfully on clubroot contaminated land to improve vegetable brassica yields.

REFERENCES

Agarwal, A., Kaul, V., Faggian, R., & Cahill, D. M. (2009). Development and use of a model system to monitor clubroot disease progression with an Australian field population of *Plasmodiophora brassicae*. *Australasian Plant Pathology*, *38*, 120–127.

An, H. R., Mainelis, G., & White, L. (2006). Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples. *Atmospheric Environment*, *40*, 7924–7939.

Buczacki, S. (1983). *Zoosporic plant pathogens*. London: Academic Press Inc., Ltd.

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

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., … Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, *55*, 611–622.

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

Cheah, L. H., Page, B. B. C., & Koolaard, J. P. (1998). *Soil-incorporation of fungicides for control of clubroot of vegetable brassicas*. Proceedings of the 51st New Zealand Plant Protection Conference, August 11–13, 1998. *Hamilton, New Zealand: New Zealand Plant Protection Society*. p. 130–133.

Dixon, G. (2009). The occurrence and economic impact of *Plasmodiophora brassicae* and Clubroot disease. *Journal of Plant Growth Regulation*, *28*, 194–202.

Dixon, G., & Webster, M. (1988). Antagonistic effect of boron, calcium and pH on pathogenesis caused by *Plasmodiophora brassicae* Woronin (clubroot) — A review of recent work. *Crop Research*, *28*, 84–95.

Donald, C., Lawrence, M., & Porter, I. (2004). Influence of particle size and application method on the efficacy of calcium cyanamide for control of clubroot of vegetable brassicas. *Crop Protection*, *23*, 297–303.

Donald, C., & Porter, I. (2009). Integrated control of clubroot. *Journal of Plant Growth Regulation*, *28*, 289–303.

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

Faggian, R., & Parsons, S. (2002). A rapid diagnostic test for clubroot. Final Report Project VG 99008. Horticultural Research and Development Corporation

Faggian, R., & Strelkov, S. E. (2009). Detection and measurement of *Plasmodiophora brassicae*. *Journal of Plant Growth Regulation*, *28*, 282–288.

Fu, J., Li, D., Xia, S., Song, H., Dong, Z., Chen, F., … Tang, Z. (2009). Absolute quantification of plasmid DNA by real-time PCR with genomic DNA as external standard and its application to a biodistribution study of an HIV DNA vaccine. *Analytical Sciences*, *25*, 675–680.

Harling, R. (2007). Clubroot control Using Novel and Sustainable methods. Final report DEFRA project HH3227TFV.

Ingram, D. S., & 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., & 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., & Kishi, F. (1997). Cloning of a single-copy DNA sequence unique to *Plasmodiophora brassicae*. *Physiological and Molecular Plant Pathology*, *50*, 289–300.

Jin-Ping, L., Yan, L., Xue-wen, X., A-li, C., & Bao-ju, L. (2013). Development of a real-time PCR assay for *Plasmodiophora brassicae* and its detection in soil samples. *Journal of Integrative Agriculture*, *12*(10), 1799–1806.

Kageyama, K., & Asano, T. (2009). Lifecycle of *Plasmodiophora brassicae*. *Journal of Plant Growth Regulation*, *28*, 203–211.

Karling, J. (1968). *The plasmodiophorales*, 2nd edn. New York: Hafner Pub. Co.

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

Lewis, M. C. (2011). *The development of assays to determine the effect of environmental factors on the viability of Plasmodiophora brassicae resting spores*. PhD Thesis. University of Warwick.

Lewis, M., Wakeham, A., & Kennedy, R. (2013). A molecular based seedling assay system to study the germination and primary infection by *Plasmodiophora brassicae*. *Acta Horticulturae*, *1005*, 583–589.

MacFarlane, I., & Last, F. (1959). Some effects of *Plasmodiophora brassicae* Woron. on the growth of the young cabbage plant. *Annals of Botany*, *23*, 547–570.

Mattusch, P. (1977). Epidemiology of clubroot of crucifers caused by *Plasmodiophora brassicae*. In S. T. Buczacki & P. H. Williams (Eds.), *Woronin +100 international conference on clubroot* (pp. 122–123). Madison: Kluwer Academic Publishers.

Myers, D. F., & Campbell, R. N. (1985). Lime and the control of clubroot of crucifers: Effects of pH, calcium, magnesium and their interactions. *Phytopathology*, *75*(6), 670–674.

Narisawa, K., Shimura, M., Usuki, F., Fukuhara, S., & Hashiba, T. (2005). Effects of pathogen density, soil moisture, and soil pH on biological control of clubroot in Chinese cabbage by *Heteroconium chaetospira*. *Plant Disease*, *89*, 285–290.

Niwa, R., Kumei, T., Nomura, Y., Yoshida, S., Osaki, M., & Ezawa, T. (2007). Increase in soil pH due to Ca-rich organic matter application causes suppression of the clubroot disease of crucifers. *Soil Biology and Biochemistry*, *39*(3), 778–785.

Niwa, R., Nomura, Y., Osaki, M., & Ezawa, T. (2008). Suppression of clubroot disease under neutral pH caused by inhibition of spore germination of *Plasmodiophora brassicae* in the rhizosphere. *Plant Pathology*, *57*(3), 445–452.

Peng, G., McGregor, L., Lahlali, R., Gossen, B. D., Hwang, S. F., Adhikari, K. K., … McDonald, M. R. (2011). Potential biological control of clubroot on canola and crucifer vegetable crops. *Plant Pathology*, *60*(3), 566–574.

Sussman, A., & Halvorson, H. (1966). *Spores – their dormancy and germination*. New York, USA: Harper & Row.

Van Slyke, L. L. (1932). *Fertilizers and crop production* (pp. 1859–1931). New York: Orange Judd Publishing Company, Inc..

Walker, C., & Van West, P. (2007). Zoospore development in the oomycetes. *Fungal Biology Reviews*, *21*, 10–18.

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

Wallenhammar, A. C., Almquist, C., Söderström, M., & Jonsson, A. (2012). In-field distribution of *Plasmodiophora brassicae* measured using quantitative real-time PCR. *Plant Pathology*, *61*(1), 16–28.

Wallenhammar, A. C., & Ardwisson, O. (2001). Detection of *Plasmodiophora brassicae* by PCR in naturally infested soils. *European Journal of Plant Pathology*, *107*, 313–321.

Webster, M. A., & Dixon, G. R. (1991a). Calcium, pH and inoculum concentration influencing colonization by *Plasmodiophora brassicae*. *Mycological Research*, *95*, 64–73.

Webster, M. A., & Dixon, G. R. (1991b). Boron, pH and inoculum concentration influencing colonization by *Plasmodiophora brassicae*. *Mycological Research*, *95*, 74–79.

Yun, J. J., Heisler, L. E., Hwang, I. I., Wilkins, O., Lau, S. K., Hyrcza, M., … Der, S. D. (2006). Genomic DNA functions as a universal external standard in quantitative real-time PCR. *Nucleic Acids Research*, *34*(12), e85.