Project title:	*Brassica* and lettuce propagation: Scaling-up means of controlling clubroot resting spores in trays to avoid infection of transplants.
Project number:	FV 337a
Project leader:	Dr John A Walsh, University of Warwick
Report:	Final Report, November 2013
Previous report:	Annual Report, November 2012
Key workers:	Dr John A Walsh, University of Warwick Isabel Saunders, University of Warwick Joy Gaze, Campden BRI Dr Taruna Sharma, University of Warwick
Location of project:	University of Warwick, Wellesbourne, Campden BRI
Industry Representative:	James Bean, Crystal Heart Salad Company, Crystal Heart Salad Co Ltd Eastrington Road Brough Humberside HU15 2XS
Date project commenced:	01 September 2011
Date project completed:	30 November 2013

The results and conclusions in this report are based on an investigation conducted over a twenty seven month period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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.

Dr John Walsh
Senior Research Scientist
University of Warwick

Signature .....Dr John Walsh ..... Date 27.11.13……..

Isabel Saunders
Senior Research Technician
University of Warwick

Signature .....Isabel Saunders..... Date 27.11.13……

Taruna Sharma
Research Technician
University of Warwick

Signature .....Dr Taruna Sharma.....Date 27.11.13.....

Report authorised by:

[Name]
[Position]
[Organisation]

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

CONTENTS

GROWER SUMMARY

Headline

This project describes the strategies to control clubroot in trays to avoid infection of transplants

Background and expected deliverables

Why the work was undertaken

Clubroot is one of the most important diseases affecting brassicas in the UK and is virtually impossible to eradicate once established in fields. Plant propagation trays returned from growers to propagators may be contaminated with clubroot resting spores. Reliable methods of controlling clubroot contamination on trays are required, in order to avoid infection of transplants by clubroot.

Overall aim of the project

The overall aim of the project is to evaluate both heat and disinfectant treatments for their ability to control clubroot resting spores and translate this data into guidelines for propagators who wish to sterilise propagation trays.

Expected deliverables

1. Knowledge of the exposure times needed to control clubroot spores at temperatures between 70ºC - 90ºC.
2. Knowledge of the efficacy of disinfectants Jet 5 and Aquaform in controlling the resting spores.
3. Improve the Assured Plant Propagation Scheme by providing data that will lead to improved propagation practices and standards.

Summary of the project and main conclusions

- The exposure times needed to control clubroot spores at temperatures between 70ºC and 90ºC and in different dilutions of the disinfectants Jet 5 and Aquaform were determined.

- Treating clubroot resting spores at temperatures of 70ºC and higher controlled clubroot resting spores, provided treatment duration was long enough.

- The disinfectant Aquaform controlled clubroot resting spores when used at 1%, 5%, 10% and 80%, provided treatment durations were long enough. The disinfectant Jet 5 at 0.5% and 40% also controlled clubroot resting spores, provided exposure times were long enough.

- Previous research (FV 337) has shown that the resting spores of *Olpidium brassicae* (vector of lettuce big-vein disease) are more susceptible to heat and disinfectants than clubroot spores. Hence, any of the treatments reported here that controlled clubroot will control *Olpidium brassicae* resting spores.

A robust, reliable and reproducible bioassay incorporating a molecular test for assessing the efficacy of physical and chemical treatments in controlling clubroot resting spores was developed, evaluated and successfully deployed in this project.

To control clubroot spores within a reasonable time requires high temperatures, or high concentrations of disinfectant.

Temperature

Minimum exposure times required to control clubroot resting spores at 70ºC, 80ºC and 90ºC.

	70ºC	**80ºC**	**90ºC**
Minimum exposure (minutes)	105	45	17.5

- At 90ºC it took 17.5 minutes to control clubroot spores.
- At 80ºC, treatment for 45 minutes was necessary to control clubroot spores.
- At 70ºC, 104 minutes treatment was necessary to control clubroot spores.

Aquaform

Minimum exposure times required to control clubroot resting spores in different concentrations of Aquaform.

	1%	**5%**	**10%**	**80%**
Minimum exposure (minutes)	9 hours	2 hours	100 mins	21 mins

- Aquaform at a concentration of 80% controlled clubroot spores after treatment for 21 minutes.
- Aquaform at a concentration of 10% controlled clubroot spores after treatment for 100 minutes.
- Aquaform at a concentration of 5% took 120 minutes to control clubroot spores.

- Aquaform at a concentration of 1% took 9 hours to control clubroot spores.

Jet 5

Minimum exposure times required to control clubroot resting spores in different concentrations of Jet 5

	0.5%	**2.5%**	**5%**	**40%**
Minimum exposure (minutes)	15 hours	Not killed after 4 hours	Not killed after 3 hours	25 mins

- Jet 5 at a concentration of 40% controlled clubroot spores after exposure for 25 minutes.

- Jet 5 at a concentration of 5% did not control clubroot spores after exposure for 3 hours.

- Jet 5 at a concentration of 2.5% did not control clubroot spores after exposure for 4 hours.

- Jet 5 at a concentration of 0.5% took 15 hours to control spores.

Propagators will now be able utilise the results to treat trays at a range of temperatures and in a range of concentrations of Jet 5 and Aquaform for the appropriate exposure times and thereby improve propagation practices and the Assured Plant Propagation Scheme.

Financial Benefits

- The times needed to control clubroot spores at different temperatures and in different concentrations of two disinfectants provides propagators with a range of options to decontaminate propagation trays of clubroot.

- Development of robust and reliable decontamination options means that time and money will be saved, by eliminating ineffective treatments. This will improve confidence in propagation practices.

- Improved clubroot spore control will further guarantee clean transplants and avoid contamination of fields, thereby sustaining growers' crop yields and providing financial benefits.

Action Points

- The data obtained indicates that clubroot resting spores will need to be treated for a minimum of 105 minutes at 70ºC, 45 minutes at 80ºC, or 17.5 minutes at 90ºC in order to obtain control in the presence of soil. Shorter times at these temperatures are very unlikely to give complete spore control.

- Aquaform at 80% for 21 minutes, 10% for 100 minutes, 5% for 120 minutes, or 1% for 9 hours controlled clubroot spores in the presence of soil. Shorter exposures at these concentrations are unlikely to effectively control spores.

- Jet 5 at 40% for 25 minutes and 0.5% for 15 hours controlled clubroot spores in the presence of soil. Shorter exposure at these concentrations is unlikely to effectively control spores.

SCIENCE SECTION

Introduction

The project aims were to test different combinations of temperatures and exposure times for controlling clubroot resting spores and to determine the range of concentrations and contact times of two disinfectants, Jet 5 and Aquaform, needed to control spores.

In 2007/8 vegetable brassicas were grown over approximately 26,000 Kha of land and had a farm gate value of £225 million (DEFRA, 2012).

Clubroot is one of the most important diseases affecting brassica crops in the UK, and is prevalent in all major areas of vegetable brassica production. It is caused by a pathogenic protist called *Plasmodiophora brassicae* which forms characteristic clubs, or galls on the roots of infected plants, resulting in reduced crop yield potential. Mild crop infections result in up to 20% root damage, resulting in slowed growth and delayed harvesting. Severe infections can result in total crop failure. Clubroot resting spores can remain dormant in contaminated soil for up to 18 years, meaning that infected fields often cannot be used for brassica cultivation for considerable periods of time. Measures for ridding land of clubroot spores often involve disease control treatments which are expensive and have associated environmental concerns. It is therefore essential that spores are not introduced into clean soils.

It is very important to avoid introduction of spores into fields via infected transplants. Uncontaminated transplants are therefore an essential component of integrated clubroot control strategies. If effective control procedures can be achieved using heat and disinfectants, risk of disease transfer to soil via propagation trays will be minimised. The sudden appearance of clubroot in a field which had previously produced uninfected crops may not be a direct result of incoming inoculum from contaminated transplants. Fields can appear to be uninfected whilst in fact they may be harbouring low levels of spores that do not affect production until they reach a threshold level.

.

Materials and methods

Techniques are available at University of Warwick for purifying and quantifying clubroot spores. Batches of $5x10^7$ spores were produced using these techniques and subjected to various heat and disinfectant treatments. This number of spores was shown to consistently produce clubs on young broccoli plants in sterilised M2 compost in a previous project (FV 337). Bioassays also established in FV 337 to determine the viability of spores following treatments, were deployed to determine the degree of control obtained. This involved inoculating treated batches of spores on to individual broccoli cv Marathon plants and examining the root systems for signs of infection (root swelling and clubs) 7 weeks later. A sample of bioassay plants showing no signs of infection and any plants suspected to be infected, but not showing clear symptoms, had DNA extracted from their roots. The DNA was tested for the presence of clubroot DNA using the polymerase chain reaction (PCR).

Campden BRI collaborators have the knowledge, qualified staff and practical facilities required to carry out thermal sterilisation treatments, whilst all disinfectant treatments were carried out at the University of Warwick.

Clubroot spores were subjected to treatments at three temperatures, 70^0C, 80^0C and 90^0C, for various time periods. Quantified batches of spores were treated in temperature-controlled water baths prior to being inoculated onto test plants in the bioassay to test spore viability. Appropriate controls of untreated spores, autoclave-killed spores and roots of uninoculated test plants were included in experiments. Experimental data was analysed and 'thermal death points' for spores were calculated.

A system for measuring the efficacy of chemical disinfectants, Jet 5 and Aquaform, in controlling clubroot spores was established in Project FV 337. Batches of spores were aliquoted into Falcon tubes and treated with disinfectants for various periods of time. Disinfectants were then chemically neutralised and the spores recovered for testing in the bioassay. The neutralising solution consisted of a universal neutralisation medium based on BS EN 1672, the Food Hygiene bactericidal suspension test [Lecithin, 3g/L, Polysorbate 80, 30g/L (V/V); Sodium thiosulphate, 5g/L; L-histidine, 1g/L; Saponin, 30g/L; made up in diluent consisting of Tryptone, 1.0g/L and NaCl, 8.5g/L].

Objective 1. Determining the exposure times required to control clubroot resting spores at temperatures of 70 ºC, 80 ºC and 90 ºC.

Quantified aliquots of clubroot spores combined with 0.05g of John Innes No. 2 were supplied to Campden BRI in glass bijou bottles (Fig. 1). These bottles were sealed to ensure that they were watertight and then treated in a temperature-controlled water bath at 70^0C, 80^0C or 90^0C for the appropriate times. A test bottle containing water was also placed in the bath and the temperature of the water monitored to ensure that the liquid within reached and remained at the desired temperature.

Figure 1. Glass bijou bottles used to heat-treat clubroot spores.

The list of treatments tested in Experiments 1 (preliminary), 2, 3 and 4 are shown in Tables 1-4 below. The treatments tested in Experiment 1 were based on the outcomes of Project FV 337 and results used to inform treatments in further experiments.

The maximum number of bioassay plants (and therefore spore batches) that could be handled per experiment was 270. Thirty batches of spores per experimental treatment were tested (batches of 10-30 were used for controls), though due to poor seed germination, this number was not achieved in the preliminary experiment.

Table 1. Treatments and number of spore batches tested in temperature Experiment 1 (preliminary).

Temperature	Treatment duration (mins)	Number of spore batches treated
70ºC	20	20
70ºC	40	20
80ºC	12.5	20
80ºC	25	20
90ºC	7.5	13
90ºC	15	13
Untreated spores		30
Autoclave-killed spores		20
Healthy Root Solution		20

Table 2. Treatments and number of spore batches tested in temperature Experiment 2.

Temperature	Treatment duration (mins)	Number of spore batches treated
70ºC	20	30
70ºC	40	30
80ºC	12.5	30
80ºC	25	30
90ºC	7.5	30
90ºC	15	30
Untreated spores		30
Autoclave-killed spores		30
Healthy Root Solution		30

Table 3. Treatments and number of spore batches tested in temperature Experiment 3.

Temperature	Treatment duration (mins)	Number of spore batches treated
70ºC	60	30
70ºC	80	30
70ºC	100	30
80ºC	30	30
80ºC	35	30
80ºC	40	30
90ºC	17.5	30
90ºC	20	30
Untreated spores		10
Autoclave-killed spores		10
Healthy Root Solution		10

Table 4. Treatments and number of spore batches tested in temperature Experiment 4.

Temperature	Treatment duration (mins)	Number of spore batches treated
70ºC	105	30
70ºC	110	30
70ºC	120	30
80ºC	45	30
80ºC	50	30
80ºC	55	30
90ºC	15	30
90ºC	17.5	30
Untreated spores		10
Autoclave-killed spores		10
Healthy Root Solution		10

Following treatment, each batch of spores was inoculated on to a 10 day-old broccoli seedlings (cv Marathon) growing in M2 compost and then left to grow in a controlled environment (CE) room (18ºC, 12h day length) for 7 weeks. Bioassay plants were kept separate from one another using individual plastic trays (Figs. 2 & 3) and watered every 2-3 days to ensure that the soil remained moist. Following the 7 week growth period, root systems

were harvested and washed and visually examined for signs of clubroot infection. Root systems that had no clear clubroot symptoms, but had the suggestion of symptoms, were tested for the presence of clubroot using a very sensitive molecular test (polymerase chain reaction [PCR], which detects clubroot DNA) along with samples of roots with no symptoms and further appropriate controls. The PCR protocol and primers (TC1F and TC1R) were as described by Cao *et al*. (2007).

Figure 2. Young broccoli seedling inoculated with spores and placed in individual plastic tray.

Figure 3. Bioassay plants growing in CE room.

After collating the results, the kinetic parameter analogous to the 'z' value was calculated. The 'z' value relates the change in temperature required to increase, or decrease the inactivation of organisms by a factor of 10. The 'z' value can be used to calculate exposure times required to control the spores at different heating temperatures. For example, a 'z' value of 25.7ºC means that for every increase of 25.7ºC, there is a 10-fold change (reduction) in the time required for the inactivation of spores. This information will allow growers to choose combinations of times and temperatures that should control clubroot spores.

Objective 2. Determining the time required to control clubroot resting spores in different concentrations of the disinfectants Jet 5 and Aquaform.

Quantified batches of 5 x 10^7 clubroot spores combined with 0.05g of John Innes No. 2 were aliquoted into Falcon tubes and treated for various times with Jet 5 and Aquaform at different concentrations. In preliminary experiments, the strongest concentrations of

disinfectant solutions to be tested (Jet 5, 40%, or Aquaform, 80%) were added to the spore solutions, shaken and incubated at room temperature for the desired exposure times. Following this, neutralising solution was added, shaken and the contents of the tube left at room temperature for 10 minutes. Falcon tubes were then centrifuged for 10 minutes to pellet the clubroot spores. The liquid was poured off and the spores re-suspended in tap water before bioassay plants were inoculated.

The very high concentrations of Jet 5 (10x recommended rate) and Aquaform (240x recommended rate) were used in this preliminary experiment in order to investigate the efficacy of the neutralising solution and inform treatments for further experiments. Controls designed specifically to test the efficacy of the neutraliser involved adding the neutraliser to each disinfectant solution and mixing, before adding spores (if the neutraliser was effective, the spores would not have been adversely affected by the solution). Another control (spores + neutralising solution only) was included to determine whether spores were harmed by the neutraliser itself.

The combination of treatment times and disinfectant concentrations tested in the preliminary experiment (Table 5) were based on the results from Project FV 337.

Table 5. Treatments and number of spore batches tested in disinfectant Experiment 1 (preliminary).

Disinfectant,	**Concentration (%)**	**Treatment duration (mins)**	**Number of spore batches treated**
Aquaform	80	1	30
Aquaform	80	2	30
Aquaform	80	4	30
Jet 5, 40%	40	5	30
Jet 5, 40%	40	10	30
Jet 5, 40%	40	15	30
Aquaform	80 + Neutraliser	10	30
Jet 5	40 + Neutraliser	10	30
Untreated spores	-	-	10
Killed spore control (killed in Jet 5, 40% for 5 days)			10
Neutraliser only + spores, 10 minutes			10

The results of the preliminary treatments informed further experiments testing lower concentrations of disinfectant solutions for their efficacy in controlling spores. Treatment times and disinfectant concentrations tested in Experiments 2, 3, 4, 5 and 6 are shown in Tables 6-10. As durations of Jet 5 disinfectant treatments in experiments 2 and 3 did not give complete control of clubroot spores, further Experiments 5 and 6 with increased exposure times was performed (Tables 9 and 10).

Table 6. Treatments and number of spore batches tested in disinfectant Experiment 2.

Disinfectant	**Concentration (%)**	**Treatment duration (mins)**	**Number of spore batches treated**
Aquaform	10	40	30
Aquaform	10	60	30
Aquaform	10	80	30
Jet 5	40	40	30
Jet 5	60	60	30
Jet 5	80	80	30
Aquaform	10 + Neutraliser	10	30
Jet 5	5 + Neutraliser	10	30
Untreated spores	-	-	10
Killed spore control (killed in Jet 5, 40% for 5 days)			10
Neutraliser only + spores, 10 minutes			10

Table 7. Treatments and number of spore batches tested in disinfectant Experiment 3.

Disinfectant	Concentration (%)	Treatment duration (mins)	Number of spore batches treated
Aquaform	5	40	30
Aquaform	5	80	30
Aquaform	5	120	30
Jet 5	2.5	40	30
Jet 5	2.5	80	30
Jet 5	2.5	120	30
Aquaform	5 + Neutraliser	10	30
Jet 5	2.5 + Neutraliser	10	30
Untreated spores	-	-	10
Killed spore control (killed in Jet 5, 40% for 5 days)			10
Neutraliser only + spores, 10 minutes			10

Table 8. Treatments and number of spore batches tested in disinfectant Experiment 4.

Disinfectant	Concentration (%)	Treatment duration (hrs)	Number of spore batches treated
Aquaform	1	3	30
Aquaform	1	9	30
Aquaform	1	15	30
Jet 5	0.5	3	30
Jet 5	0.5	9	30
Jet 5	0.5	15	30
Aquaform	1 + Neutraliser	10 mins	30
Jet 5	0.5 + Neutraliser	10 mins	30
Untreated spores	-		10
Killed spore control (killed in Jet 5, 40% for 5 days)			10
Neutraliser only + spores, 10 minutes			10

Table 9. Treatments and number of spore batches tested in disinfectant Experiment 5.

Disinfectant	Concentration (%)	Treatment duration (mins)	Number of spore batches treated
Jet 5	2.5	130	30
Jet 5	2.5	140	30
Jet 5	2.5	160	30
Jet 5	5	90	30
Jet 5	5	100	30
Jet 5	5	120	30
Jet 5	2.5 + Neutraliser	10	30
Jet 5	5 + Neutraliser	10	30
Untreated spores	-	-	10
Killed spore control (killed in Jet 5, 40% for 5 days)			10
Neutraliser only + spores, 10 minutes			10

Table 10. Treatments and number of spore batches tested in disinfectant Experiment 6

Disinfectant	Concentration (%)	Treatment duration (mins)	Number of spore batches treated
Jet 5	2.5	160	30
Jet 5	2.5	200	30
Jet 5	2.5	240	30
Jet 5	5	150	30
Jet 5	5	180	30
Jet 5	2.5 + Neutraliser	10	30
Jet 5	5 + Neutraliser	10	30
Untreated spores	-	-	10
Killed spore control (killed in Jet 5, 100% for 4 hours)			10
Neutraliser only + spores, 10 minutes			10

Following treatment, each batch of spores was inoculated on to a 10 day-old broccoli seedling (cv Marathon) growing in M2 compost and left to grow in a controlled environment (CE) room (18ºC, 12h day length) for 7 weeks. Bioassay plants were kept separate from one another using individual plastic trays (Figs. 2 & 3) and watered every 2-3 days to

ensure that the soil remained moist. Following the 7 week growth period, root systems were harvested, washed and visually examined for signs of clubroot infection. Root systems that had no clear clubroot symptoms, but had the suggestion of symptoms, were tested for the presence of clubroot using a very sensitive molecular test (polymerase chain reaction [PCR], detects clubroot DNA) along with samples of roots with no symptoms and further appropriate controls. The PCR protocol and primers (TC1F and TC1R) were as described by Cao *et al*. (2007).

Results

Objective 1. Exposure times required to control clubroot resting spores at temperatures of 70ºC, 80ºC and 90ºC.

Complete spore control was not achieved at any temperature in Experiment 1 (Table 11). Longer exposure times and higher temperatures gave better spore control. The longest time (15 mins) at the highest temperature (90ºC) was the most effective treatment, with only 1 of the 13 bioassay plants showing visible signs of clubroot infection. The least effective treatment was the shortest exposure (20 mins) at the lowest temperature (70ºC), with almost half of the inoculated plants showing symptoms.

Table 11. Number of bioassay plants infected with clubroot after treatment of spores at 70ºC, 80ºC and 90ºC for a range of exposure times (temperature Experiment 1, preliminary).

Temperature, Treatment duration	Number of bioassay plants infected with clubroot / number of plants in bioassay
70ºC, 20 minutes	9 / 20
70ºC, 40 minutes	5 / 20
80ºC, 12.5 minutes	5 / 20
80ºC, 25 minutes	2 / 20
90ºC, 7.5 minutes	3 / 13
90ºC, 15 minutes	1 / 13
Untreated spores	30 / 30
Autoclave-killed spores	0 / 20
Healthy Root Solution	0 / 20

Complete spore control was not achieved in Experiment 2 (Table 12; a repeat of Experiment 1 with full replication). The same general trend of increased spore control at higher temperatures with longer exposure times was observed in this experiment. There

was almost complete control at 90ºC for 15 minutes exposure (1 of the 30 bioassay plants infected). The least effective treatment was 12.5 minutes exposure at 80ºC, with 11 out of 30 bioassay plants infected by clubroot. These results showed that longer exposure times needed to be tested.

Table 12. Number of bioassay plants infected with clubroot after treatment of spores at 70ºC, 80ºC and 90ºC for a range of exposure times (temperature Experiment 2).

Temperature, Treatment duration	**Number of bioassay plants infected with clubroot / number of plants in bioassay**
70ºC, 20 minutes	9 / 30
70ºC, 40 minutes	6 / 30
80ºC, 12.5 minutes	11 / 30
80ºC, 25 minutes	5 / 30
90ºC, 7.5 minutes	2 / 30
90ºC, 15 minutes	1 / 30
Untreated spores	30 / 30
Autoclave-killed spores	0 / 30
Healthy Root Solution	0 / 30

Complete spore control was achieved in Experiment 3 when spores were subjected to 90ºC for both 17.5 and 20 minutes (Table 13). Full control was not obtained following treatments at 70ºC and 80ºC (2/30 bioassay plants infected by spores treated for 60 minutes at 70ºC, all other exposures at these temperatures resulted in only 1/30 bioassay plants infected with clubroot).

Table 13. Number of bioassay plants infected with clubroot after treatment of spores at 70ºC, 80ºC and 90ºC for a range of exposure times (temperature Experiment 3).

Temperature, Treatment duration	**Number of bioassay plants infected with clubroot / number of plants in bioassay**
70ºC, 60 minutes	2 / 30
70ºC, 80 minutes	1 / 30
70ºC, 100 minutes	1 / 30
80ºC, 30 minutes	1 / 30
80ºC, 35 minutes	1 / 30
80ºC, 40 minutes	1 / 30
90ºC, 17.5 minutes	0 / 30

90ºC, 20 minutes	0 / 30
Untreated spores	10 / 10
Autoclave-killed spores	0 / 10
Healthy root preparation	0 / 10

With increased temperature and longer exposure combinations, the number of bioassay plants infected with clubroot decreased. For example after exposure to 70°C for 20 minutes 9/30 plants were infected (Experiment 2) and increasing the exposure time to 80 minutes resulted in infection of only 1/30 plants (Experiment 3). Similarly, after 12.5 minutes at 80°C, 11/30 plants were infected (Experiment 2) and increasing exposure to 40 minutes resulted in infection of only 1/30 plants (Experiment 3).

Using the above data, it was possible to predict that complete control (100% reduction of infection) would be obtained from a 'z' value of 25.7°C. From this, it is possible to predict that it would take at least 110 minutes at 70ºC and at least 45 minutes at 80°C to achieve complete control. Temperature Experiment 4 was designed to test these predictions.

Complete spore control was achieved at all temperatures tested in temperature Experiment 4 at the shortest exposures (Table 14). The exposure times required to control clubroot spores at 70ºC, 80ºC and 90ºC were 105 minutes, 45 minutes and 15 minutes respectively. Exposure for 5 minutes less than predicted by the 'z' value was sufficient to give complete control at 70ºC. Also, as one spore sample tested at 90ºC for 15 minutes in Experiment 2 caused infection, an exposure time of 17.5 minutes must be used at 90ºC in order to ensure complete spore control.

Table 14. Number of bioassay plants infected with clubroot after treatment of spores at 70ºC, 80ºC and 90ºC for a range of exposure times (temperature Experiment 4).

Temperature, duration	**Treatment**	**Number of bioassay plants infected with clubroot / number of plants in bioassay**
70ºC, 105 minutes		0 / 30
70ºC, 110 minutes		0 / 30
70ºC, 120 minutes		0 / 30
80ºC, 45 minutes		0 / 30
80ºC, 50 minutes		0 / 30
80ºC, 55 minutes		0 / 30
90ºC, 15 minutes		0 / 30

90ºC, 17.5 minutes	0 / 30
Untreated spores	10 / 10
Autoclave-killed spores	0 / 10
Healthy root preparation	0 / 10

The ‘z’ value for clubroot was calculated by extrapolating from the above results. This value was then used to create a graph, providing combinations of temperature and exposure that should result in 100% spore control (Fig. 4).

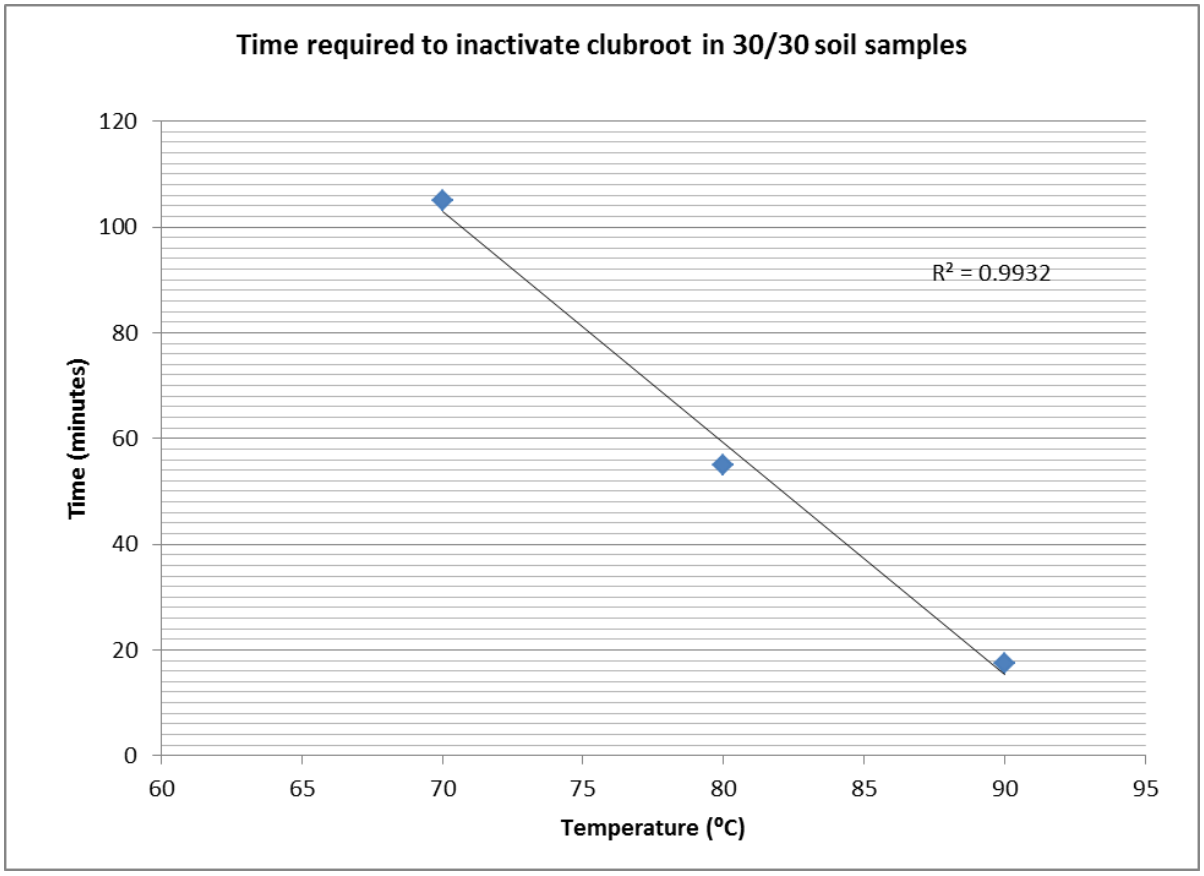

Figure 4. Graph showing the possible combinations of exposure time (minutes) and temperature that should result in complete clubroot spore control.

Roots from temperature Experiments 2, 3 and 4 that had no clear clubroot symptoms, but had the suggestion of symptoms were tested for the presence of clubroot along with samples of roots with no symptoms (as controls) and further appropriate controls, using the PCR test. The results of the PCR tests showed that some of the roots with no clear symptoms were infected, but most were not infected. In Experiment 4, all roots with the suggestion of symptoms were negative in the PCR test (Fig. 5).

The combinations of exposure times and temperatures in the range 70-90ºC required to control clubroot resting spores has been calculated (Table 15).

Table 15. The combinations of exposure times and temperatures in the range 70-90ºC required to control clubroot resting spores, based on a 'z' value of 25.7ºC.

Temperature (°C)	Minimum hold time (minutes)
70	105.0
71	96.0
72	87.8
73	80.3
74	73.4
75	67.1
76	61.3
77	56.1
78	51.3
79	46.9
80	42.9
81	39.2
82	35.8
83	32.8
84	30.0
85	27.4
86	25.0
87	22.9
88	20.9
89	19.1
90	17.5

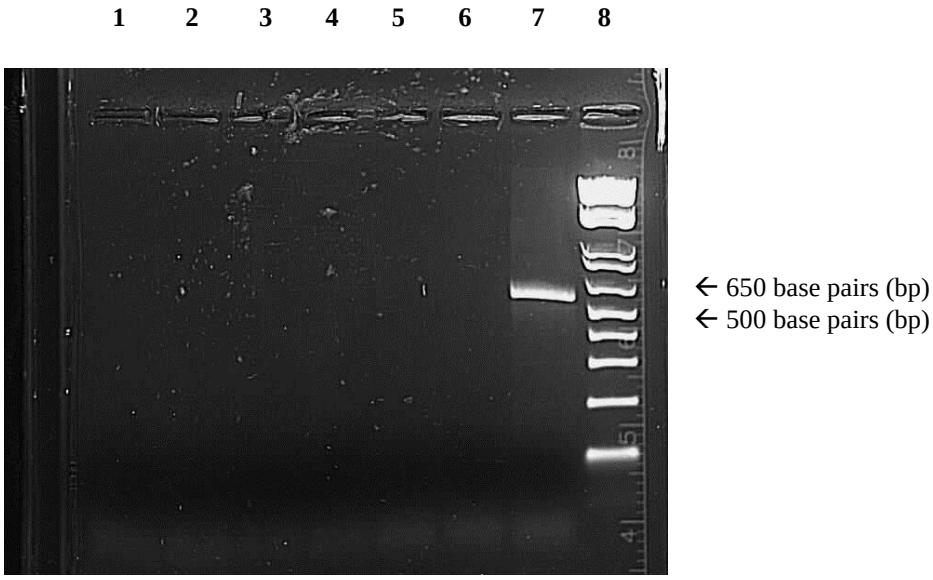

Figure 5. PCR products ran on agarose gel stained with gel red. Lanes 1-3, samples with the suggestion of symptoms. Lanes 4 and 5, root systems with no symptoms. Lane 6, healthy root control sample. Lane 7, clubroot-infected control sample and Lane 8 molecular marker ladder.

There were very clear visual differences between infected control plants and plants inoculated with spores from successful treatments. Plants inoculated with untreated spores, autoclave-killed spores (control) and spores treated at 70ºC for 105 minutes are shown in Figs. 6-8.

Figure 6. Bioassay control plants inoculated with untreated clubroot spores.

Figure 7. Bioassay control plants inoculated with autoclave-killed clubroot spores.

Figure 8. Bioassay plants inoculated with clubroot spores that had been treated at 70^{0}C for 105 minutes .

Objective 2. Determining the time required to control clubroot resting spores in different concentrations of the disinfectants Jet 5 and Aquaform.

In the preliminary experiment, both disinfectants gave 100% spore control at all treatment durations tested (Table 16). However, it was clear that the neutralising solution was not effective against either disinfectant. No infection of any of the 30 bioassay plants was detected after treating spores in disinfectants mixed with neutraliser, showing that the spores had been killed and hence the neutraliser had not worked. As the neutraliser had not been effective, the actual exposure times of spores to disinfectants were significantly (~20 minutes) longer than intended in Table 5. At these prolonged exposures (21, 22 and 24 minutes for Aquaform and 25, 30 and 35 minutes for Jet 5), both disinfectants were very effective against clubroot spores. In the absence of the disinfectant, the neutraliser had no discernible effect on the clubroot resting spores.

Table 16. Number of bioassay plants infected with clubroot after disinfectant treatment of spores for a range of exposure times (disinfectant Experiment 1, preliminary).

Disinfectant	Concentration (%)	Estimated treatment duration (mins)	Number of bioassay plants infected with clubroot / number of plants in bioassay
Aquaform	80	21	0 / 30
Aquaform	80	22	0 / 30
Aquaform	80	24	0 / 30
Jet 5	40	25	0 / 30
Jet 5	40	30	0 / 30
Jet 5	40	35	0 / 30
Aquaform	80 + Neutraliser	10	0 / 30
Jet 5	40 + Neutraliser	10	0 / 30
Untreated spores			10 / 10
Killed spore control (killed in Jet 5, 40% for 5 days)			0 / 10
Neutraliser only + spores, 10 minutes			10 / 10

In the second experiment, precise exposure times could not be guaranteed due to the inefficacy of the neutralising solution, meaning exposure times were again ~20 minutes longer than intended. The control treatments designed to verify the efficacy of the

neutraliser showed it to be relatively ineffective against 10% Aquaform (12/30 plants developed clubroot symptoms; had the neutraliser been effective, all 30 plants would have become infected). Although not completely effective, the neutraliser was more effective against 5% Jet 5 (27/30 plants developed symptoms). Complete spore control was only achieved with 10% Aquaform with an exposure time in excess of 60 minutes, but less than 100 minutes (Table 17). The shortest exposure time at this concentration did not completely control the spores (4/30 plants infected after 60 minutes exposure). Control was not achieved by treating with 5% Jet 5 for up to 100 minutes (2/30 spore batches gave infection following 100 minutes exposure). Infection did decrease with increased duration of exposure.

Table 17. Number of bioassay plants infected with clubroot after disinfectant treatment of spores for a range of exposure times (disinfectant Experiment 2).

Disinfectant	**Concentration (%)**	**Estimated treatment duration (mins)**	**Number of bioassay plants infected with clubroot / number of plants in bioassay**
Aquaform	10	60	4 / 30
Aquaform	10	80	3 / 30
Aquaform	10	100	0 / 30
Jet 5	5	60	6 / 30
Jet 5	5	80	5 / 30
Jet 5	5	100	2 / 30
Aquaform	10 + Neutraliser	10	12 / 30
Jet 5	5 + Neutraliser	10	27 / 30
Untreated spores			10 / 10
Killed spore control (killed in Jet 5, 40% for 5 days)			0 / 10
Neutraliser only + spores, 10 minutes			10 / 10

The results of the third experiment showed that spore control was achieved using 5% Aquaform for 120 minutes (Table 18). The shorter exposure times of 40 and 80 minutes did not completely control spores (3/30 and 2/30 plants infected respectively). Control was not achieved by treating with 2.5% Jet 5 for up to 120 minutes (2/30 plants infected; 5/30 plants infected following spore treatment for 40 and 80 minutes respectively). The control treatments showed that the neutraliser was effective against these concentrations of

Aquaform and Jet 5, with all 30 spore batches in each treatment giving infection in the bioassay plants.

Table 18. Number of bioassay plants infected with clubroot after disinfectant treatment of spores for a range of exposure times (disinfectant Experiment 3).

Disinfectant	Concentration (%)	Treatment duration (mins)	Number of bioassay plants infected with clubroot / number of plants in bioassay
Aquaform	5	40	3 / 30
Aquaform	5	80	2 / 30
Aquaform	5	120	0 / 30
Jet 5	2.5	40	5 / 30
Jet 5	2.5	80	5 / 30
Jet 5	2.5	120	2 / 30
Aquaform	5 + Neutraliser	10	30 / 30
Jet 5	2.5 + Neutraliser	10	30 / 30
Untreated spores	-	-	10 / 10
Killed spore control (killed in Jet 5, 40% for 5 days)			0 / 10
Neutraliser only + spores, 10 minutes			10 / 10

Spore control was achieved using both 1% Aquaform and 0.5% Jet 5 in Experiment 4 (Table 19). Nine hours exposure time in 1% Aquaform was required (1/30 plants were infected following spore treatment for 3 hours). Fifteen hours was required to achieve control with 0.5% Jet 5 (5/30 plants infected following spore treatment for 3 hours, and 3/30 infected following spore treatment for 9 hours).

Table 19. Number of bioassay plants infected with clubroot after disinfectant treatment of spores for a range of exposure times (disinfectant Experiment 4).

Disinfectant	Concentration (%)	Treatment duration (hrs)	Number of bioassay plants infected with clubroot / number of plants in bioassay
Aquaform	1	3	1 / 30
Aquaform	1	9	0 / 30
Aquaform	1	15	0 / 30
Jet 5	0.5	3	5 / 30
Jet 5	0.5	9	3 / 30
Jet 5	0.5	15	0 / 30
Aquaform	1 + Neutraliser	10 mins	30 / 30
Jet 5	0.5 + Neutraliser	10 mins	30 / 30
Untreated spores	-	-	10 / 10
Killed spore control (killed in Jet 5, 40% for 5 days)			0 / 10
Neutraliser only + spores, 10 minutes			10 / 10

Despite exposure times of 160 minutes in 2.5% Jet 5 and 120 minutes in 5% Jet 5, complete control of clubroot spores was not achieved (Table 20).

Table 20. Number of bioassay plants infected with clubroot after disinfectant treatment of spores for a range of exposure times (disinfectant Experiment 5)

Disinfectant	Concentration (%)	Treatment duration (mins)	Number of bioassay plants infected with clubroot / number of plants in bioassay
Jet 5	2.5	130	27 / 30
Jet 5	2.5	140	28 / 30
Jet 5	2.5	160	19 / 30
Jet 5	5	90	30 / 30
Jet 5	5	100	29 / 30
Jet 5	5	120	9 / 30
Jet 5	2.5 + Neutraliser	10	29 / 30
Jet 5	5 + Neutraliser	10	27 / 30
Untreated spores	-	-	10 / 10
Killed spore control (killed in Jet 5, 40% for 5 days)			0 / 10
Neutraliser only + spores,			10 / 10

10 minutes	

Despite prolonging exposure times further to 240 minutes in 2.5% Jet 5 and 180 minutes in 5% Jet 5, complete control of clubroot spores was still not achieved (Table 21).

Table 21. Number of bioassay plants infected with clubroot after disinfectant treatment of spores for a range of exposure times (disinfectant Experiment 6)

Disinfectant	**Concentration (%)**	**Treatment duration (mins)**	**Number of bioassay plants infected with clubroot / number of plants in bioassay**
Jet 5	2.5	160	22/30
Jet 5	2.5	200	19/30
Jet 5	2.5	240	16/30
Jet 5	5	150	10/30
Jet 5	5	180	7/30
Jet 5	2.5 + Neutraliser	10	30/30
Jet 5	5 + Neutraliser	10	27/30
Untreated spores	-	-	10/10
Killed spore control (killed in Jet 5, 100% for 4 hours)			0/10
Neutraliser only + spores, 10 minutes			10/10

Roots in all disinfectant experiments that had no clear clubroot symptoms, but had the suggestion of symptoms were tested for the presence of clubroot along with samples of roots with no symptoms (as controls) and further appropriate controls, using the PCR test. The results of the PCR tests showed that most of the roots with unclear symptoms were infected. In Experiment 3, six of the eight roots with the suggestion of symptoms were positive in the PCR test (Fig. 9).

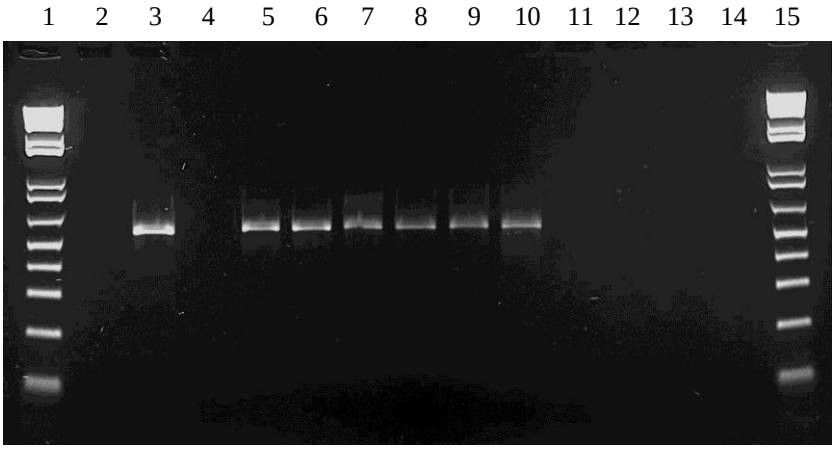

Figure 9. PCR products on agarose gel stained with gel red. Lanes 1 and 15, molecular marker ladder. Lanes 2-9, samples with the suggestion of symptoms (positive samples in lanes 3, 5, 6, 7, 8 and 9). Lane 10, clubroot-infected control sample. Lane 11, healthy root control sample. Lanes 12 and 13, root systems with no symptoms. Lane 14, disinfectant-killed control sample.

Discussion

The results of the heat treatment experiments have provided thermal death point data, giving propagators a range of treatment times at 70ºC (105 mins), 80ºC (45 mins) and 90ºC (17.5 mins) that will control clubroot spores in the presence of soil. All the data obtained has been used to establish a relationship between temperature and treatment duration that will allow propagators to predict combinations that will control clubroot spores. Previous work (FV 337) showed that *Olpidium brassicae* (vector of big-vein disease of lettuce) resting spores were more susceptible to heat treatment than clubroot spores. Control of the *Olpidium* spores was achieved at 65ºC after exposure for just 5 minutes.

The results of the preliminary disinfectant Experiments 1 and 2 were problematic in that the neutraliser failed to inactivate both disinfectants at these high concentrations (10% and 80% Aquaform; 5% and 40% Jet 5) and hence actual exposure times were longer than planned. At 10%, Aquaform controlled spores after treatment for 100 minutes. At 80% it controlled spores in 21 minutes. At 5% Jet 5 failed to control spores after treatment for 100 minutes. At 40% it controlled spores after treatment for 25 minutes. Again, previous work (FV 337) showed that Olpidium brassicae resting spores were more susceptible to disinfectants than clubroot spores. Control of the Olpidium spores was achieved in 20% Jet 5 after treatment for just 5 minutes.

In most of the subsequent experiments with lower concentrations of the disinfectants, the neutraliser did inactivate both disinfectants, providing results for precise exposure times. As the concentrations of the disinfectants decreased, the exposure time required for spore

control increased. At 5% and 1% Aquaform it took 120 minutes and 9 hours respectively to achieve control. Exposure at 5% and 1% Aquaform for 80 minutes and three hours respectively did not give complete control. At 0.5% Jet 5, it took 15 hours to achieve control. Exposure at 5% and 2.5% Jet 5 for three hours and four hours respectively, did not give complete control. Aquaform appears to be more effective than Jet 5 at controlling spores at the concentrations tested.

A range of treatments have been identified that gave complete clubroot spore control. This will provide propagators with a range of options for controlling clubroot spores and avoid wasting time and money on ineffective treatments. The results confirm the resilience of the spores and the necessity for high temperatures, high concentrations of disinfectants, or prolonged treatment times to ensure control.

Conclusions

- Robust, reliable and reproducible bioassays for determining the efficacy of heat and disinfectant treatments in controlling clubroot resting spores were improved and successfully deployed in this project.
- A PCR test to accurately confirm the presence, or absence of clubroot DNA in root systems was developed and successfully deployed in this project.
- Clubroot spores were controlled at 70ºC in the presence of soil following 105 minutes exposure, at 80ºC following 45 minutes exposure and at 90ºC following 17.5 minutes exposure.
- A graph has been produced to allow propagators to work out a range of combinations of temperature and exposure times that will control clubroot spores, over and above those determined in this project.
- Clubroot spores were controlled in Aquaform at concentrations of 80%, 10%, 5% and 1% in the presence of soil following exposure for 21 minutes, 100 minutes, 120 minutes and 9 hours respectively.
- Clubroot spores were controlled in Jet 5 at concentrations of 40% and 0.5% in the presence of soil following exposure for 25 minutes and 15 hours respectively.
- A range of options of disinfectants, concentrations thereof and exposure times that will control clubroot spores are now available for propagators.
- The provision of treatments which effectively control clubroot spores means that propagators can be confident they are producing transplants free of infection and avoid wasteful ineffective sterilisation techniques, thereby improving the Assured Plant Propagation Scheme.

- As spores of *Olpidium brassicae* are more susceptible to heat and disinfectants than clubroot spores, all of the treatments that have been identified in this study as effective in controlling clubroot, will also be capable of controlling *Olpidium* spores.

Knowledge and Technology Transfer

John Walsh gave a presentation on progress of the project to Plant Propagators Ltd. in October 2012 at the NFU headquarters, Stoneleigh Park. A further presentation will be given at the Brassica Technical Seminar in Edinburgh on 28 January, 2014. A Factsheet is in preparation as is an article for HDC News.

References

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

DEFRA (2012). Leafy vegetable genetic improvement network (VeGIN): pre-breeding research to support sustainable farming of leafy vegetables and salads.
Accessed via:
http://randd.defra.gov.uk/Default.aspx?Menu=Menu&Module=More&Location=None&Completed=2&ProjectID=16328

Walsh, J. (2010). Brassica and lettuce propagation: identifying means of killing clubroot and *Olpidium* resting spores in trays to avoid infection of transplants. Horticultural Development Company. Final Report for project FV 337.