Project title:	*Brassica* and lettuce propagation: Scaling-up means of controlling clubroot resting spores in trays to avoid infection of transplants.
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Project leader:	Dr John A Walsh, University of Warwick
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Location of project:	University Warwick, Wellesbourne, Campden BRI
Industry Representative:	James Bean, Crystal Heart Salad Company
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The results and conclusions in this report are based on an investigation conducted over a one year 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.

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GROWER SUMMARY

Headline

- The predicted time required to control club root resting spores is 110 minutes at 70ºC and 45 minutes at 80ºC. At 90ºC complete control was achieved after treatment for 17.5 minutes.

Background and expected deliverables

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 thermal death point of 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

- 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 the project.
- To control clubroot spores within a reasonable time requires high temperatures.
- At 90ºC it took 17.5 minutes to control clubroot spores.
- At 70ºC, one hour and 40 minutes was not quite sufficient to control clubroot spores (97% control)
- At 80ºC, 40 minutes was also not quite sufficient to control clubroot spores (97% control).
- Provisional experiments on the disinfectants Jet 5 and Aquaform suggested that very high concentrations can control clubroot spores following short exposure.

- The project is on track to determine the time needed to control clubroot resting spores at temperatures between 70ºC and 90ºC and the exposure times needed to control the spores in different dilutions of the disinfectants Jet 5 and Aquaform.

Financial Benefits

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

- Development of robust and reliable decontamination options will mean that time and money can be saved by eliminating ineffective treatments and 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 so far indicates that clubroot resting spores will need to be treated for 110 minutes at 70ºC, 45 minutes at 80ºC, or 17.5 minutes at 90ºC in order to obtain control. Shorter times at these temperatures are very unlikely to give 100% spore control.

- Accurate exposure times of clubroot spores to different concentrations of the disinfectants Jet 5 and Aquaform are being determined. This information will provide propagators with a broad range of options for using disinfectants to decontaminate trays.

SCIENCE SECTION

Introduction

- The project objectives 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 can cause perhaps 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.
- 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 (FV337). Bioassays also established in FV337 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) after seven weeks. 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 by 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, 70ºC, 80ºC and 90ºC, 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 were 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 FV337. 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 time required to control clubroot resting spores between 70ºC and 90ºC.

Quantified aliquots of clubroot spores combined with 0.05g of soil 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 70ºC, 80ºC or 90ºC for the appropriate time. 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 and 3 are shown in Tables 1-3 below. The treatments tested in Experiment 1 were based on the outcomes of Project FV337 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. We tested 30 batches of spores per experimental treatment, though due to poor bioassay plant germination this number was not achieved in the preliminary experiment.

Following treatment each batch of spores was inoculated on to a 10-day-old broccoli seedling (cv Marathon) 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 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 PCR test (detects *P. brassicae* 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).

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

Temperature, Treatment duration	Number of spore batches / plants in bioassay
70ºC, 20 minutes	20
70ºC, 40 minutes	20
80ºC, 12.5 minutes	20
80ºC, 25 minutes	20
90ºC, 7.5 minutes	13
90ºC, 15 minutes	13
Untreated spores	30
Autoclave-killed spores	20
Healthy Root Solution	20

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

Temperature, Treatment duration	Number of spore batches / plants in bioassay
70ºC, 20 minutes	30
70ºC, 40 minutes	30
80ºC, 12.5 minutes	30
80ºC, 25 minutes	30
90ºC, 7.5 minutes	30
90ºC, 15 minutes	30
Untreated spores	30
Autoclave-killed spores	30
Healthy Root Solution	30

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

Temperature, Treatment duration	Number of spore batches / plants in bioassay
70ºC, 60 minutes	30
70ºC, 80 minutes	30
70ºC, 100 minutes	30
80ºC, 30 minutes	30
80ºC, 35 minutes	30
80ºC, 40 minutes	30
90ºC, 17.5 minutes	30
90ºC, 20 minutes	30
Untreated spores	10
Autoclave-killed spores	10
Healthy Root Solution	10

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. 'Z' values are the degrees of temperature over which a 90% change in the decimal log reduction time for the numbers of viable organisms at any given temperature is observed. For example, a 'z' value of 8ºC means that for every change of 8ºC, there is a 10-fold change in the decimal log reduction time for the numbers of viable organisms at any given temperature. This 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 soil were aliquoted into Falcon tubes and treated for various times with Jet 5 and Aquaform. In preliminary experiments, the strongest concentrations of disinfectant solutions to be tested (Jet 5, 400ml/L; Aquaform, 80%) were added to the spore solution, 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 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 neutralizer 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 confirm that spores are not harmed by the neutraliser itself.

The combination of treatment times and disinfectant concentrations tested in the preliminary experiment (Table 4) were based on the results from Project FV337.

Table 4. List of treatments and number of spore batches tested in preliminary disinfectant Experiment 1.

Disinfectant, Concentration, Treatment duration	**No. of spore batches/ plants in bioassay**
Aquaform, 80%, 1 minute	30
Aquaform, 80%, 2 minutes	30
Aquaform, 80%, 4 minutes	30
Jet 5, 400ml/L, 5 minutes	30
Jet 5, 400ml/L, 10 minutes	30
Jet 5, 400ml/L, 15 minutes	30
Neutraliser + Aquaform, 10 secs followed by spores 10 mins	30
Neutraliser + Jet 5, 10 secs, followed by spores 10 mins	30
Untreated spores	10
Jet 5, 400ml/L, 5 days (killed spore control)	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) 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 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 PCR test (detects *P. brassicae* 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).

The results were collated and further experiments designed based on the results.

Results

Objective 1. Determining the time required to control clubroot resting spores between 70ºC and 90ºC.

Complete spore control was not achieved at any temperature in Experiment 1 (Table 5). 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 test plants showing visible signs of clubroot infection. The least effective treatment was the shortest time (20 mins) at the lowest temperature (70ºC), with almost half of the inoculated plants showing symptoms.

Table 5. Number of broccoli plants infected with clubroot after treatment at 70-90ºC for a range exposure times.

Temperature, Treatment duration	Number of broccoli plants infected with clubroot/number of plants inoculated
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 6; a repeat of Experiment 1 with full replication). The same trend of increased spore control at higher temperatures with longer exposure times was also observed in this experiment. Complete control was nearly achieved at 90ºC with 15 minutes exposure. The least effective treatment was 12.5 minutes exposure at 80ºC, with 11 out of 30 test plants infected by clubroot. These results showed that longer exposure times needed to be tested in Experiment 3.

Table 6. Number of broccoli plants infected with clubroot after treatment at 70-90ºC for a range exposure times.

Temperature, Treatment duration	Number of broccoli 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 7). Very little infection was found in treatments at 70ºC and 80ºC (2/30 plants tested for 60 minutes at 70ºC showed infection whilst all other treatments at these temperatures resulted in only 1/30 plants infected with clubroot).

Table 7. Number of broccoli plants infected with clubroot after treatment at 70-90ºC for a range exposure times.

Temperature, Treatment duration	**Number of broccoli 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	30 / 30
Autoclave-killed spores	0 / 30
Healthy root preparation	0 / 30

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

The ‘z’ value for clubroot was calculated by extrapolating from the above results. Using the data, it is possible to predict that to obtain complete control (100% reduction of infection) would require a z value of 25°C. From this, we can predict that it would take at least 110 minutes at 70ºC and at least 45 minutes at 80°C to achieve the complete control achieved at 90ºC after 17.5 minutes exposure.

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

Both disinfectants gave 100% spore control at all treatment durations tested (Table 8). However, it was clear that the neutralising solution was not effective in neutralising either disinfectant; neutraliser added to the disinfectants prior to spore treatment failed to prevent spores being killed (no infection of any of the 30 bioassay plants detected). Given that the neutraliser was not effective, the exposure times of the disinfectant treatments were significantly (~10 minutes) longer than indicated in Table 8. Nonetheless, both disinfectants were very effective against clubroot spores, albeit with exposure times of in excess of 11

minutes for Aquaform and 15 minutes for Jet 5. This is promising for our future disinfectant experiments that will be performed in the coming months. In the absence of the disinfectant, the neutraliser had no discernible adverse effect on the clubroot resting spores.

Table 8. Number of broccoli plants infected with clubroot after disinfectant treatment at a range exposure times (preliminary experiment).

Disinfectant, Concentration, Treatment duration	Number of broccoli plants infected with clubroot/number of plants in bioassay
Aquaform, 80%, 1 minute	0 / 30
Aquaform, 80%, 2 minutes	0 / 30
Aquaform, 80%, 4 minutes	0 / 30
Jet 5, 400ml/L, 5 minutes	0 / 30
Jet 5, 400ml/L, 10 minutes	0 / 30
Jet 5, 400ml/L, 15 minutes	0 / 30
Neutraliser + Aquaform for 10 secs, followed by spores 10 mins	0 / 30
Neutraliser + Jet 5 for 10 secs, followed by spores 10 mins	0 / 30
Untreated spores	9 / 10
Jet 5, 400ml/L, 5 days (killed spore control)	0 / 10
Neutraliser only + spores, 10 minutes	10 / 10

Discussion

The results of the heat treatment experiments have provided thermal death point data, giving propagators a range of predicted treatment times at 70ºC (110 mins), 80ºC (45 mins) and 90ºC (17.5 mins) that will control clubroot on propagation trays. Over the coming months we will test these predictions.

The results of the preliminary disinfectant experiment suggested that both Jet 5 and Aquaform are capable of controlling clubroot spores. The next steps in the project will be focused on providing information on the efficacy of lower Jet 5 and Aquaform concentrations in controlling clubroot resting spores. This will provide propagators with more options for tray sterilisation.

Conclusions

- Robust, reliable and reproducible bioassays for assessing the efficacy of heat and disinfectant treatments in controlling clubroot resting spores have been successfully improved and deployed in this project.
- The predicted time required to control spores is 110 minutes at 70ºC and 45 minutes at 80ºC. At 90ºC complete control was achieved after treatment for 17.5 minutes.
- The preliminary disinfectant experiment indicated that Jet 5 and Aquaform will control clubroot. Although very high concentrations of each disinfectant were used, contact times were in excess of 11 minutes for Aquaform and 15 minutes for Jet 5.
- Further experiments over the coming months will provide propagators with a range of effective treatments with lower and hence cheaper disinfectant concentrations.
- 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.

Knowledge and Technology Transfer

John Walsh gave a presentation on progress of the project to Plant Propagators Ltd. In October at the NFU headquarters, Stoneleigh Park.

References

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