



Grower Summary

CP 099a

Validation of the club-root
lateral flow in UK commercial
Brassica cropping systems

Final 2015

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Project Number: CP 099a

Project Title: Validation of the club-root lateral flow in UK commercial Brassica cropping systems

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GROWER SUMMARY

Headline

- The UK horticulture industry now has an optimised and validated molecular qPCR test for the assessment of fields for club-root disease. This test can be carried out quickly (within days of laboratory receipt) with an accuracy of >90%. It is quantitative and has a detection sensitivity of 1000 spores per gram of soil tested.
- This molecular test provides capability to accurately detect disease at low level and at specific points in the field. Using this approach the application of tailored and cost effective treatments can be made for management of club-root.
- This approach provides growers with an integrated disease management system for control of club-root disease and meets the criteria of the European Sustainable Use Directive (SUD).
- A lateral flow test has been developed and validated with 67% accuracy* for the measurement of club-root in soils. The test has a detection sensitivity of >10,000 spores per gram of soil. It provides a club-root risk of either zero to low ($\leq 10,000$ spores g^{-1} soil) or medium to high risk ($> 10,000$ spores g^{-1} soil).
- Further work is planned by the University of Worcester scientists to improve the efficiency of the lateral flow test.

* compared to grower field observation

Note: The lateral flow test is less accurate than the qPCR as a result of varied soil types and sample size.

Background

Brassica crops are of great economic importance in the United Kingdom. One of the most important diseases affecting Brassica crops is club-root, caused by the soil borne organism *Plasmodiophora brassicae*. Mild club-root infections lead to slowed growth and delayed harvesting. Severe infections result in total crop failure. Infection is easily recognisable by swelling of root tissue causing galls and club shaped structures. Club-root resting spores are capable of inducing disease in vegetable Brassica crops years after initial infestation of the soil.

Once soil has been contaminated club-root spores can remain viable for up to 18 years. In the UK, growers of horticultural crops frequently rent land on an annual basis, often with limited knowledge of previous cropping histories. The capability to forecast club-root disease risk prior to contractual agreements being made, would be beneficial. The level of soil infestation by club-root resting spores has been shown to directly affect the amount of club-root infection. Resting spore concentrations in excess of 100,000 spores per gram of soil have been reported for severe and uniform disease expression on bait plants (Buczacki & Ockendon, 1978). The soil, and prevailing environmental conditions during key cropping periods, will also affect the risk of disease development and the severity of crop symptoms.

As *Plasmodiophora brassicae* only grows within living tissues it is not possible to use standard dilution plating techniques to quantify numbers of pathogenic propagules within soil samples. The traditional technique used to assess potential risk of the disease in soils has been to infect bait plants grown under optimal disease conditions. It takes six to eight weeks for disease symptoms to be visible on bait plants, which makes the technique time consuming and expensive. Growers have reported that this type of test tends to 'over predict risk' compared to what actually happens in the field. With the development of new detection methods based at the molecular level (DNA, Antibody, Aptamers) these approaches may prove to be more accurate, quicker and less expensive. Whilst many molecular tests require a high degree of precision, knowledge, and are generally laboratory based, the lab on a stick (lateral flow / in-field test) offers an inexpensive alternative that could easily be used by a grower or agronomist.

The lateral flow device has the potential to be used by growers using field soils, as well as in water based systems such as reservoirs and irrigation lines used by vegetable brassica propagators. A quantitative measurement of club-root resting spore infestation can be made using the lateral flow test device when used in conjunction with a lateral flow reader and standard curve data. This means that a prediction of whether the crop is at risk can be made as well as the level of risk i.e. low, medium or high.

Determining the concentration of club-root resting spores in soils is an essential component in the development of an integrated disease management programme. Club-root spores are reported as having a half-life of approximately four years. If resting spore numbers can be maintained below the disease threshold (10,000 spores g⁻¹ soil) then rotation and an increased pH level ≥ 7.2 through liming should prove useful in reducing disease risk. Where an increased risk of disease is identified (>10,000 spores g⁻¹ soil) the application of targeted

treatments such as Limex, a by-product of the British sugar industry, could be applied. The use of club-root resistant varieties has also been shown to be useful in reducing the severity of disease expression when growing in infested soils.

The development and expression of the disease in the crop will be dependent on a number of factors: the resting spore concentration, the conducive or suppressive nature of the soil type, the environmental conditions over the growing season, and the brassica cultivar planted.

The deliverables from this project are:

- Validation of assay systems to test soils for club-root prior to planting the crop
- Adoption of integrated pest management systems: field mapping of club-root resting spore distribution and application of targeted treatments

Summary

Club-root testing services and co-ordination of soils for sampling: A laboratory molecular based test and a 10 minute 'in field' test, were assessed for their ability to measure and predict risk of club-root disease in UK horticulture grade soils. Over a two year period a total over 100 soils across the UK were sampled for the measurement of *Plasmodiophora brassicae* infestation (club-root resting spores). At each sampling site, two hectare blocks were identified and spatially assessed by the collection of 48 soil samples at points across a W grid format. Soil cores were taken to a depth of 6-8" (15-20 cm). At each point a core soil sample of approx. 50 g was collected. In Year 1 of the project, the collected soils were individually air dried, sieved and mixed ahead of the test processes. This was to ensure that any disease present would be evenly distributed throughout the sample. However this process was labour intensive, time consuming and with high sample volumes required considerable space. Depending on the moisture content of the soils the process could take upwards of three weeks.

Assessment of soils for club-root resting spore concentration

In each of the two years, the molecular (laboratory quantitative PCR) and immunological test (in field test) gave some disparity in the prediction of club-root risk for the soils tested. It is known however that soil type can influence DNA extraction and subsequently the outcome of PCR amplification, as a result of the presence or absence of inhibitory substances. Humic and fulvic acids, soil clay content and the presence of heavy metals have all been reported to affect DNA extraction of a target organism from soil. One of the most common ways to

identify soil inhibitors is to dilute the samples in molecular grade water. By doing this, the extraction of club-root DNA for the soils tested in 2013 (Year 1) either remained the same or was improved. These results provided an opportunity to also improve the soil preparation process. Bringing the soil water content up to 50% created a slurry type suspension which could be stirred by hand. This was a simple, quick and robust process used to mix the soil sample uniformly before testing. The addition of water to samples was also useful in reducing the effect of soil inhibitors.

Using this system in Year 2 of the project enabled soil samples to be processed quickly and the results reported within days of sample receipt. The risk of club-root was reported via an interactive web page which the co-ordinators had access to via a personal login. In Year 1, the soils processing had resulted in growers receiving results between three and five weeks after sample receipt. Modification of the molecular test in Year 2 not only resulted in faster sample analysis time but with an improved accuracy. Compared to grower observations, the PCR (molecular DNA) was 92% accurate in predicting club-root disease risk. This was further improved to 96% when compared to the industry standard (bait test data). The rationale for this is that high numbers of resting spores are required in soils ($>10000\text{ g}^{-1}$ naturally infested soil) before above ground disease symptoms on infected plants are observed i.e. stunting, yield loss, wilting of plants during hot and prolonged dry weather. The industry bait test scores club-root risk based on below ground disease symptoms, i.e. root swellings / clubbing. For this reason the bait test will identify low level plant infection of *Plasmodiophora brassicae* (club-root) even though symptoms are not apparent to the plant above ground level. This is why the bait test is often seen as over predicting disease risk when compared to the grower outcome. In fact, both the molecular test and the bait test are able to measure risk well in advance of the crop outwardly exhibiting disease symptoms. Based on the data set provided, the bait test provided an accuracy of 70.5% compared to the grower outcome. When the bait test was compared to the results of the molecular and lateral flow test the accuracy of predicting club-root resting spores was improved to 88.5%.

Early detection of club-root infestation ahead of above ground symptom expression is critical if the disease is to be controlled successfully. This is amply demonstrated by a 'club-root negative' soil (S11). This soil was designated by the grower as showing no evidence of club-root. The PCR molecular test however designated $3040\text{ spores g}^{-1}$ soil. A club-root positive result was provided by the bait test but at a low level (8.3%). This spore concentration would be indicative of the healthy crop outcome observed. However, successive plantings of brassicas would, over a short time, result in the occurrence of club-root disease symptoms, as the spore concentration was close to the disease threshold ($>10000\text{ g}^{-1}$ naturally infested

soil). This was shown to good effect in soil (S8) where the molecular test identified disease at 1080 spores g^{-1} (bait test negative). The first cropping showed no signs of club-root but the second cropping recorded yield loss to club-root. By adopting a four year rotation period the concentration of resting spores could, before the first cropping, have been reduced by up to 50%. (Club-root resting spores are proposed to have a half-life of four years). Alternatively, at this spore concentration the application of lime to raise the pH to 7.2 may have had beneficial effect in disease control. Where resting spore concentrations are identified above this level ($\leq 10000 g^{-1}$), HDC project FV 349 showed that an application rate of Limex at or above 10 tons ha^{-1} could reduce club-root symptoms. The capability to accurately detect disease at low level and at specific points in the field provides growers with the opportunity to map soils on a year by year basis. This will provide a cost effective and tailored approach towards treatment of specific field areas for club-root disease risk.

An in-field ten minute test (lateral flow device) has also been developed and used for the measurement of club-root resting spores in water and directly in artificially infested soil samples (FV 259 & FV 349). A clear relationship has been established between the molecular PCR test and the lateral flow test for measurement of resting spores in artificially infested soil, (Figure 1). In water, a reliable test detection sensitivity of 1000 spores ml^{-1} is achieved.

The lateral flow test has been used by growers to detect club-root spores within 10 minutes at epidemiologically significant levels in artificially infested soil standards ($\geq 10,000$ spores / g soil detection sensitivity). At three grower group meetings the lateral flow tests have been used successfully by agronomists and growers to accurately measure resting spores in the soil standards. A good correlation between the predicted risk (10 minute lateral flow in-field test) and resting spore concentration in a club-root artificially infested soil was recorded at one the HDC Technology Transfer meeting in Edinburgh. However, when the test was used in naturally infested soils poor correlation was observed with the molecular PCR test.

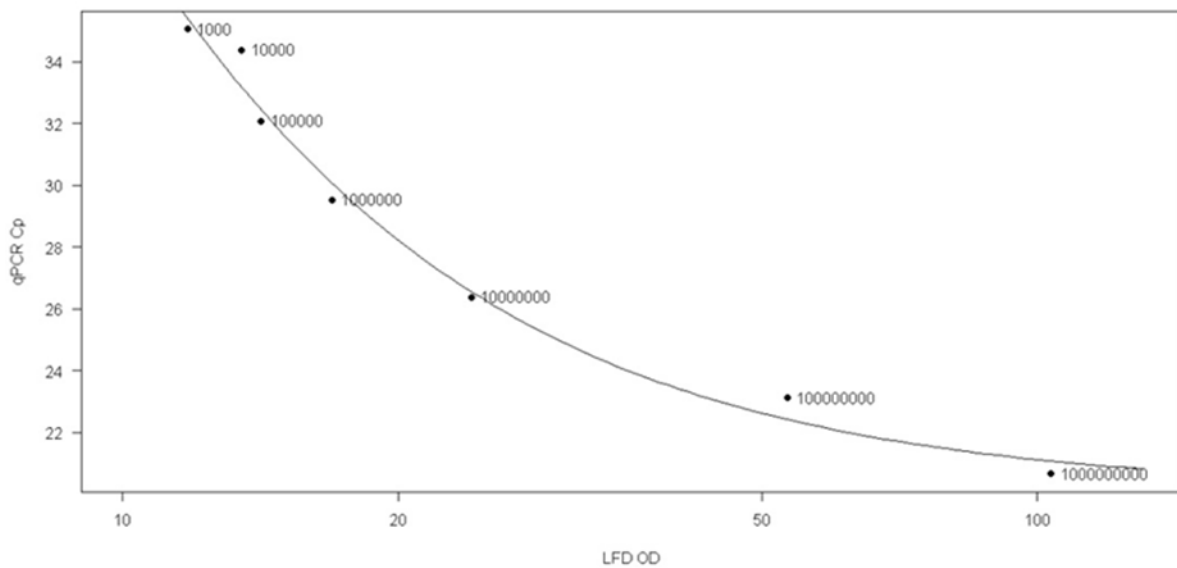


Figure 1. Relationship of the lateral flow test (LFD) and the molecular qPCR test for measurement of *Plasmodiophora brassicae* resting spores in artificially infested soil standards. Points on the graph relate to the number of club-root spores g⁻¹ soil.

Compared with the grower observations for incidence of club-root disease, a 67% test accuracy rate was recorded when the commercial soils were tested with the lateral flow test (negative to low risk <10,000 spores g⁻¹ soil; ≥10,000 medium to high risk club-root). However, like the molecular test, instances were recorded where the lateral flow test recorded positive for disease and this was subsequently confirmed by bait test. Examples of this are recorded for soils S10, S11 and Sb. Using the same analogy as described for the molecular test, an accuracy of 79% could therefore be assumed.

The S16 soil sample was recorded as a false negative result (0 < 1000 spores g⁻¹ soil recorded) by lateral flow. The grower observed only low instances of disease in the crop. This result may reflect the sample process (0.25g tested by lateral flow) and patchy nature of the disease at this low level rather than the test accuracy. The molecular test recorded a resting spore concentration of 10,000 spores g⁻¹ for this soil which is at or near the cusp of disease expression in the field. Also, only one of the two molecular tests identified disease presence.

When resting spore concentration was below the disease threshold for above ground symptom expression the bait test was able to detect disease presence at a low level in a number of the soils tested (S10, S11, Sb), S9 soil however remains as a bait false positive

(78.3% clubroot predicted risk). The molecular and lateral flow tests for this soil were clubroot negative. Grower observation recorded no sign of clubroot after the field was double cropped with brassicas. Nevertheless, the bait test when compared to the other diagnostic tests (lateral flow and molecular test) provides an accuracy of 88.5% for clubroot disease risk. For this reason, the bait test delivers a better measure of club-root disease potential than the lateral flow test but not as accurate as the molecular test. However the bait test requires large volumes of soil, a five to six week test turnaround and, is considerably more expensive than the lateral flow test.

Trouble shooting the on-site lateral flow test

In FV 349 the lateral flow test demonstrated different test sensitivities to different *P. brassicae* pathotypes, significantly race 5. (This pathotype was not considered to be a 'gene breaker' i.e. able to overcome Brassica club-root resistant varieties). Studies have shown that within a single club-root gall multiple pathotypes can be present. Differences shown by the lateral flow could in part result from the different *P. brassicae* pathotypes present in the soil. However it is more likely that the textural structure of the soil type and the small sample size will have influenced the accuracy of the test.

Studies reported in Year 1 showed that the interactions between silt, sand and clay were significant in reducing the capability of the qPCR molecular test for measurement of *P. brassicae* in soil. This effect on the lateral flow had not been considered. In variation to the PCR test, a complex extraction process is not followed or the removal of inhibitors made. Instead a simple flotation process is adopted where the larger soil particles are allowed to settle out of solution. This is needed to prevent the pores of the lateral flow (nitrocellulose membrane) from blocking, causing inhibition of the test. It is likely that this 'settling' process provides opportunity for resting spores to be taken out of solution. Also the positively charged resting spore has been reported to adhere to clay particles which are flat, plate-like and negatively charged. Too few results were available for a comprehensive study of soil textural significance on the efficiency of the lateral flow test. However sandy loam soils and those in association with clay might lend towards false positives or over prediction of disease. Gold complexes (to include colloids used in the lateral flow) have been shown to adsorb to organic matter, clays and iron. Each of these factors could affect the accuracy of the test. For this purpose, studies in year 2 continued to focus on isolation of the resting spores from the soil by immuno-magnetic extraction.

Immuno-magnetic fishing

Earlier studies at Warwick HRI, and later at NPARU, have shown that *P. brassicae* specific antibody coated magnetic beads can label and isolate resting club-root spores directly from a range of soil types. This process provided the capability for concentrating resting spores from 50g soil samples. In these studies the club-root resting spores were directly labelled with UW 249 monoclonal antiserum and indirectly isolated from soil with anti-mouse IgM super paramagnetic particles BE-M03/0.3 (Merck Chimie SAS). These particles were of suitable size for lateral flow test development (300nm). In Year 1 of the project, the super paramagnetic particles were withdrawn from the market place as a standard product, and work was conducted in Year 2 to produce a replacement. However, this area of work is likely to require significant investment of time to develop suitable alternative protocols.

Financial Benefits

- The use of the detection tests for risk assessment of club-root will improve the control of this pathogen.
- Knowledge of resting spore concentration in soils will provide cultivators with information on optimal crop rotation patterns, varietal selections and appropriate control measures to prevent yield loss.

Action Points

- Consider annual sampling and precision application of targeted club-root control treatments using GPS mapping systems.