Grower Summary

SF 97

Detection and quantification of *Verticillium dahliae* and *V. albo-atrum* in soils to determine risk of verticillium wilt in strawberry

Final 2012

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Project Number:	SF 97
Project Title:	Detection and quantification of *Verticillium dahliae* and *V. albo-atrum* in soils to determine risk of verticillium wilt in strawberry
Project Leader:	Dr Jeff Peters
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Headline

- A new rapid molecular test has been developed for testing field soils for the presence of *Verticillium dahliae* and *Verticillium albo-atrum*.

Background and expected deliverables

Verticillium wilt is one of the most serious diseases of strawberries causing significant yield loss. The major main season strawberry variety Elsanta is highly susceptible to verticillium wilt, and leading new varieties being introduced (e.g. Sonata, Figaro) appear to be as susceptible.

The causal pathogen, *Verticillium dahliae*, can exist as microsclerotia that can persist in soil for many years. *Verticillium dahliae* and *V. albo-atrum* (Vaa) have a wide host range (c. 300 plant species), including common agricultural crops such as potatoes, linseed and brassicas. If those crops become infected with *Verticillium* species the soil can become contaminated with pathogen propagules for up to 25 years.

A pre-planting wilt risk assessment service, the Harris soil test, has been available to growers since the early 1990s. This test is based on the detection and enumeration of *V. dahliae* microsclerotia in soil. The assay costs around £165 + VAT and takes six to eight weeks to complete. Such a time requires planning quite far in advance of planting a new crop in field soils. As the traditional method is reliant upon the detection of microsclerotia, it overlooks and underestimates the level of inoculum due to pathogenic *Verticillium* species, particularly *Vaa*, surviving in soil in the form of saprophytic mycelium. Currently no test exists for the detection and enumeration of Vaa in soil because the Harris test uses a sieving assay which detects only inoculum in the form of microsclerotia. The significance of Vaa in soft fruit crops is therefore poorly understood, due to the lack of reliable diagnostic assays. It is reported to infect strawberry, raspberry and blackberry.

This project seeks to develop a rapid PCR-based alternative to the Harris test for detection and quantification of both *V. dahliae* and Vaa in soil. Results using the molecular assay would take days to complete, compared with six to eight weeks using the conventional method. The project aims to develop a PCR-based soil test for detecting and quantifying both *V. dahliae* and *Vaa* concurrently. This test could be offered to growers by Fera and other laboratories. Fera has made advances in sample processing and DNA extraction from large volumes of soil (c. 1 kg). This permits the development of a quantitative PCR assay using current soil sampling methods used by growers.

With the loss of methyl bromide for soil disinfestation and increasing concern over the future use of alternative soil sterilants (e.g. chloropicrin), sound knowledge of levels of *V. dahliae* in soil are increasingly important to aid economically-sound planting decisions.
The overall aim of the project is to refine quantitative DNA assays for the rapid quantification and detection of *V. dahliae* and *V. albo-atrum* and to establish the risk of strawberry wilt disease based on soil inoculum densities.

Specific project objectives were:

1. To refine and validate real time PCR assays for the rapid detection and quantification of both *V. dahliae* and *V. albo-atrum*.
2. To refine and validate soil sample DNA extraction methods for large volumes of soil (c. 1 kg) and to determine the correlation between soil inoculum densities as measured by QPCR and disease levels under controlled environment conditions.
3. To correlate *V. dahliae* and *V. albo-atrum* data from soils tested by PCR with verticillium wilt development in strawberry crops grown at field sites.

Summary of the project and main conclusions

Objective 1: Refine and validate PCR assays

Specificity of V. dahliae and V. albo-atrum QPCR assays

Molecular PCR assays were developed at Fera to detect and quantify *Verticillium dahliae* and *V. albo-atrum*. Full validation was carried out using 30 isolates of *Verticillium* spp. (20 isolates of *V. dahliae*; six isolates of *V. albo-atrum*, three isolates of *V. longisporum* and one isolate of *V. tricorpus*) plus three non-*Verticillium* soil-inhabiting or saprophytic fungi. The assays have been shown to detect only the target pathogen species. The *V. dahliae* assay detected all 20 isolates of target species and did not detect the non-target species. The *V. albo-atrum* assay detected all six target species and did not detect any non-target species.

Objective 2: Refine and validate soil DNA extraction; correlate soil infestation density with disease in pot experiments

DNA extraction from large volumes of soil

A method was devised to extract total DNA from large volumes of soil. Replicate sub-samples each of 50 g were taken from a sample of 500 g and extracted by adaptations of a published method. Results based on the detection of ubiquitous soil inhabiting bacteria, *Streptomyces* spp. in replicate samples from each of five soils showed a coefficient of variation of between 1.7% (high levels of target DNA) and 18.5% (low levels of target DNA).

This shows that the soil test gave consistent extractions, particularly where there are abundant target species. All soils were tested using the *V. dahliae* and *V. albo-atrum* QPCR assays. A *Streptomyces* internal control assay was also carried out to ensure that soil extractions had performed efficiently.

Effect of adding different levels of artificial inoculum to soil on verticillium wilt – pot experiments

Verticillium dahliae

In 2010, a glasshouse pot trial was set up in June at Fera to investigate the relationship between *Verticillium* inoculum levels in soil and wilt symptoms in the strawberry varieties Elsanta, Florence and Symphony. Young cold stored runners were grown in loam-based compost which was artificially amended with differing levels of microsclerotia of *V. dahliae*. Approximately six weeks after inoculation the mean level of wilt, as measured by leaf necrosis, generally increased with increasing levels of *V. dahliae* inoculum. For Elsanta there was an excellent relationship between the amount of inoculum added to the soil and amount of DNA detected using the QPCR assay. However, the assay failed to detect the pathogen at the lowest inoculum level (corresponding to 1:160,000 sand maize-meal culture to compost estimated to be c. 0.25 microsclerotia/g soil). This inoculum level was sufficient to cause low levels of leaf necrosis after six weeks.

There was no relationship between inoculum level and severity of wilt symptoms in the varieties Florence and Symphony at six weeks after planting in infested soil.

In 2011, a glasshouse pot trial was again set up in June to investigate the relationship between growing young strawberry cold stored runners in loam-based compost that was artificially amended with differing levels of microsclerotia of *V. dahliae* with wilt symptoms. Eight weeks after inoculation the relationship between inoculum levels and wilt, as measured by leaf necrosis and wilt symptoms that developed in Elsanta, was poor, but disease symptoms were generally higher in plants growing in inoculated compost than in uninoculated compost. The assay detected the pathogen at 0.5, 1, 2, 5 and 10 ms/g but failed to detect the pathogen at 0.25 ms/g.

Verticillium albo-atrum

In 2010 a pot experiment was set up, as above, to investigate the relationship between growing strawberry plants in soils artificially amended with differing levels of long-lived hyphae of *V. albo-atrum* with wilt symptoms. Six weeks after inoculation the mean level of

wilt that developed in Elsanta generally increased with increasing levels of *V. albo-atrum* inoculum. The assay failed to detect the pathogen at the two lowest inoculum levels (corresponding to 1:80,000 and 1:160,000 sand maize-meal culture to compost). Of those, only the 1:80,000 inoculum level was sufficient to cause low levels of leaf necrosis.
There was no relationship between inoculum level of *V. albo-atrum* and severity of wilt symptoms in Florence and Symphony at six weeks after planting in infested soil.

In 2011 a glasshouse pot trial was set up in June to investigate the relationship between growing young strawberry cold stored runners in loam-based compost that was artificially amended with differing levels of melanised hyphae of *V. albo-atrum* with wilt symptoms. Eight weeks after inoculation, the relationship between inoculum levels and wilt, as measured by both leaf necrosis and wilt symptoms that developed in Elsanta, was poor but was generally higher in plants growing in inoculated compost than in uninoculated compost.

Effect of natural soil inoculum level of V. dahliae on verticillium wilt – pot experiment
Soil naturally infested with *V. dahliae* was collected from a soft fruit farm and diluted with John Innes soil to create five infestation densities ranging from 0.8 to 7.6 cfu/g as determined by the conventional agar plate test (<250 - 766 fg/g by molecular test, QPCR). Pots of these soils were planted with the strawberry variety Elsanta in May 2010 and grown in a polytunnel at ADAS Boxworth.

In 2010 no obvious symptoms of verticillium wilt developed, although by October the incidence of dead plants (5%) was greater in the only treatment where *V. dahliae* had been detected in soil by QPCR than all other treatments (0-1% plants dead). In 2011 soil infestation density had no significant effect on the incidence of leaf wilting or yellowing, leaf necrosis or dead plants; occurrence of wilting or yellowing symptoms was low (1 - 14%); data in 2011 were confounded by occurrence of vine weevil damage to roots. Verticillium wilt was not confirmed in a destructive assessment at the end of the experiment. Possibly production of plants in pots of soil with drip irrigation was not conducive to development of verticillium wilt at the soil infestation densities used.

Objective 3: Correlate V. dahliae soil infestation density with verticillium wilt

Effect of soil inoculum level of V. dahliae on verticillium wilt – field experiment
The aim of this experiment was to determine if pre-planting soil infestation density of *V. dahliae* on field sites measured by the conventional agar plate test and by QPCR was predictive of verticillium wilt risk. Replicated plots of three varieties (Elsanta, Symphony and

Florence) differing in susceptibility to wilt were established in spring 2010 in five fields that ranged in *V. dahliae* infestation density from <0.1 to 5.7 cfu/g (<250 to 560 fg/g). All plants used were from the same supplier and a sample examined pre-planting was found to be free of *V. dahliae*.

In 2010 verticillium wilt only occurred at the two sites with the highest overall soil infestation densities of *V. dahliae,* as shown by the agar plate test (4.6 and 5.7 cfu/g). The incidence of verticillium wilt in August at both sites was significantly greater in Elsanta (10-15% plants affected) than in Symphony or Florence (0.9-2.3% plants affected), reflecting the higher susceptibility of Elsanta.

In 2011 verticillium wilt occurred at four of the sites, with high levels at the two sites affected in 2010 and lower levels at the other two sites (Table 1). *V. dahliae* was confirmed in wilted plants from these four sites, although not in all of the sampled plants.

Table 1. Occurrence of verticillium wilt symptoms in summer 2010 and 2011 in five fields of strawberry differing in soil infestation density of *V. dahliae* at planting in spring 2010. E= Elsanta; S = Symphony; F = Florence.

Site	Soil density of *V. dahliae*			Mean % plants affected in summer (July/August)					
	Field result		Means of plots	2010			2011		
	(cfu/g)	(fg/g)	(fg/g)	E	S	F	E	S	F
A8	<0.1	<250	<250	0	0	0	1.3	0.8	1.0
A1	0.2	560	<250	0	0	0	0[a]	0	0
A7	0.5	<250	<250	0	0	0	1.1	0.2	0.6
A11	4.6	<250	274	12.8	2.3	1.1	6.8	1.6	2.1
A12	5.7	468	624	10.3	1.9	0.9	33.7	16.3	46.9

[a] Majority of Elsanta died following winter cold damage.

The occurrence of verticillium wilt symptoms in these 60 individual plots (five sites x 12 plots/site) was examined with reference to the pre-planting soil levels of *V. dahliae* determined by QPCR. Data for site A1 in 2011 was excluded due to the high incidence of plant death over winter at this site. *V. dahliae* was detected by QPCR in five of the 60 plots and *V. albo-atrum* in one.

At sites A8 and A7 no *V. dahliae* was detected pre-planting by QPCR in any of the 24 plots. Although presumptive verticillium wilt symptoms were recorded in 14 of the 24 plots at one or both assessment dates in 2011 (none was recorded in 2010), they were only affecting 1-2% of plants. It is possible that most of this low level of wilting was due to a factor other than verticillium wilt; if so the QPCR test pre-planting was a good measure of verticillium wilt risk within two years of planting at these sites.

At sites A11 and A12, *V. dahliae* was recorded in five of the 24 plots pre-planting. At the final assessment in November 2011 the incidence of verticillium wilt in these five plots was 48, 38, 24, 33 and 17% respectively. However, verticillium wilt symptoms were also recorded in all 19 plots where *V. dahliae* was not detected by QPCR pre-planting.

Considering just the plots with levels of wilt above 10% in July (on the possibility that lower levels may be wilt caused by a factor other than *Verticillium*), then the QPCR test accurately identified the only plot in 12 at site A11 with a high risk of wilt. QPCR also accurately identified four plots at high risk of verticillium wilt at site A12. However no *V. dahliae* was detected pre-planting at another seven of the 12 plots where high levels of wilt (over 10% and up to 49% in Elsanta) developed.

These results suggest that the current QPCR test is not sufficiently sensitive to detect low soil infestation densities of *V. dahliae*. Low soil infestation densities may result in a relatively low incidence of verticillium wilt in tolerant varieties but a high incidence in highly susceptible varieties, such as Elsanta.

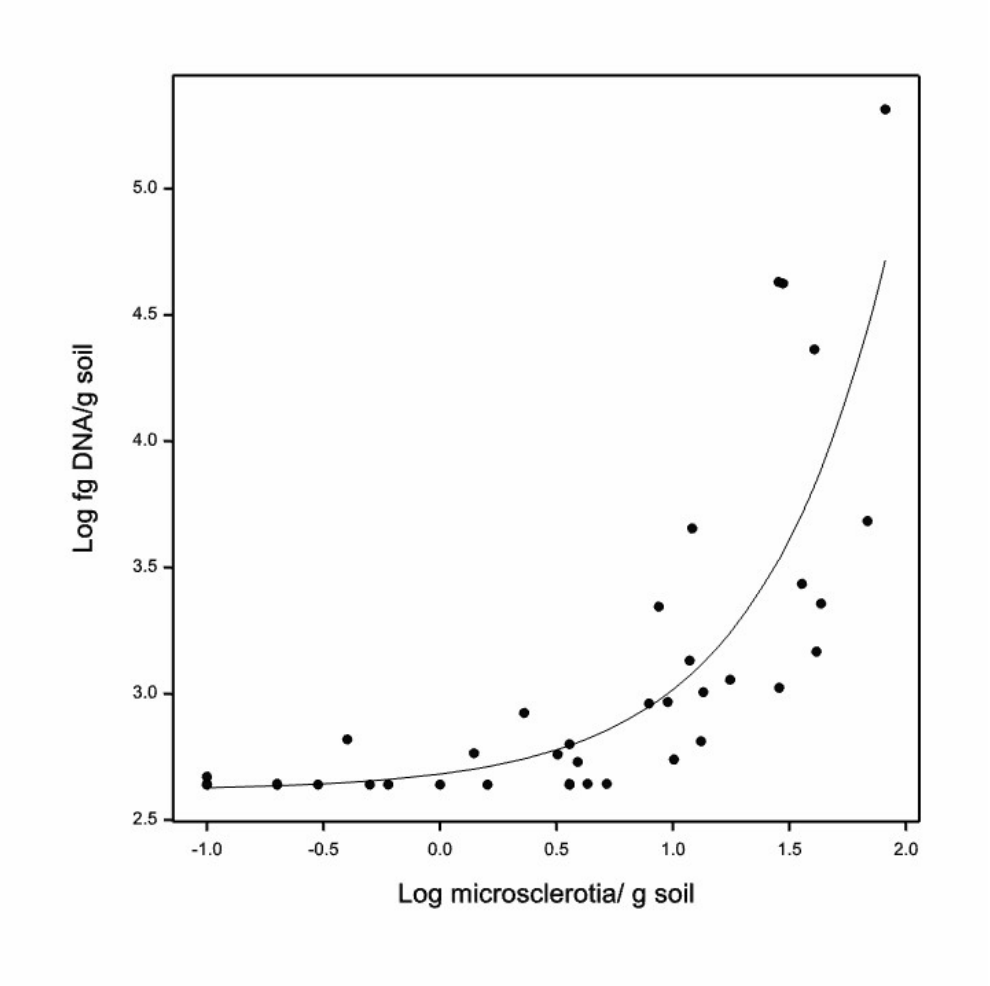
The experiment provided some evidence that measurement of soil infestation density of *V. dahliae* by QPCR is a useful predictor of verticillium wilt risk. Where average values across fields (means of plots) are considered (Table 1), the QPCR test correctly identified the two fields (A11 and A12) where the highest levels of wilt developed. This suggests that testing many soil samples in a field improves the reliability of the QPCR test. The agar plate test still looks the better method at present where single bulk samples are tested.

Parallel testing of QPCR and wet sieving

Although not one of the original objectives of this project, it was decided to directly compare the enumeration of *V. dahliae* inoculum in commercial soil samples using the traditional wet sieving (Harris) method with the new QPCR test. The data presented in Figure 1 show that there was a reasonable agreement (R^2=0.67) between the two methods. However, the data

show that detection of pathogen DNA by QPCR drops off sharply after approximately 1.0 microsclerotia/g (i.e. log 0.0). This is consistent with the hypothesis that the occurrence of verticillium wilt symptoms post-planting in field plots where no *V. dahliae* was recorded in the soil pre-planting may have been due to insufficient test sensitivity.

Figure 1. Correlation between microsclerotial counts and DNA levels for commercial samples tested by both QPCR (DNA/g soil)) and wet sieving (microsclerotia counts/g soil, log scale in 2011). R^2 = 67.0%.



Main conclusions

- PCR assays developed for *V. dahliae* and *V. albo-atrum* were shown to be specific for these fungi.
- A DNA extraction method was devised to extract total DNA from multiple samples of 50 g of field soil; tests on replicate sub-samples gave consistent results.
- A prototype QPCR test for correlating *V. dahliae* pre-plant soil infestation density with verticillium wilt is now available.

- As well as testing for *V. dahliae*, all soils are tested with an internal control based on the detection of ubiquitous soil inhabiting bacteria, *Streptomyces* spp., to ensure that extractions have performed efficiently.
- Whilst the QPCR test is currently less sensitive than the conventional (Harris) test, it detected *V. dahliae* at two sites where significant verticillium wilt developed in strawberry and not at two other sites where very little or no wilt developed; the results at a fifth were unclear.

Financial benefits

- These will depend on when a new QPCR test becomes available to growers and its cost compared to the traditional Harris test.
- The rapid turn-around time for this test will allow growers more time to take decisions on the suitability of field soils for new strawberry crops. The test will guide growers on the need to fumigate the soil prior to planting.

Action points for growers

- At present, the new QPCR test is being run by Fera in parallel to the Harris test to validate it.
- Growers are encouraged to submit samples to Fera to help to provide more material to allow validation to take place.