Grower Summary

SF 097a

Using molecular quantification of Verticillium dahliae in soil to identify risk of strawberry verticillium wilt

Final 2015

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GROWER SUMMARY

Headline

- The qPCR soil assays for detection and quantification of *V. dahliae* in soil as used in this project are not currently suitable for routine testing of soils for *V. dahlia*e.

Background and expected deliverables

Soil-borne *Verticillium dahliae* is a serious threat to profitable production of strawberry, especially where suitable land with irrigation is in short supply and crops are grown on a tight rotation. The major mainseason variety Elsanta is highly susceptible to verticillium wilt and leading new varieties being introduced appear to be susceptible (e.g. Sonata, Figaro). Strawberry verticillium wilt is difficult to control using fungicides applied to the growing crop and there are few effective products. Chemical fumigation options are limited. Alternative, non-chemical methods of soil disinfestation are not yet available. Host resistance to verticillium wilt is the most effective and sustainable approach, especially when used in combination with other disease management practices. At present genetic resistance plays a minor role in control of strawberry verticillium wilt as varieties are usually selected by growers for characteristics other than verticillium wilt resistance. Previous studies have demonstrated a positive relationship between soil infestation density of *V. dahliae* and occurrence of verticillium wilt in strawberry (Harris & Yang, 1996). A soil test that quantifies soil inoculum levels of *V. dahliae* prior to planting can have a significant role in the management of strawberry verticillium wilt. A soil sieving and agar plate culture test (the Harris test) has been used for this purpose for over 30 years. A quantitative real-time PCR (qPCR) test developed in HDC Project SF 97 offers several advantages over the traditional test, namely: speed (a few days), a high level of specificity and no dependence on expensive and increasingly rare taxonomic expertise. A rapid test is advantageous both for variety/field selection by growers when decision time is short (e.g. with rented land) and to minimise any change in soil inoculum that might occur between soil sampling and planting (e.g. change of microsclerotia distribution in the soil profile with cultivations; decay of crop debris in the soil to release microsclerotia; decline in microsclerotia number with microbial degradation).

The objectives of this project were:

1. To improve the sensitivity of the molecular diagnostic test developed in SF 97 and quantify *V. dahliae* in soils down to 0.1 g microsclerotium of *V. dahliae*/g soil.
2. To validate the test by monitoring the relationship between soil inoculum levels of *V. dahliae* (measured by qPCR) and the development of verticillium wilt in strawberry.

Summary of the project and main conclusions

Objective 1 – Improve sensitivity and reliability of qPCR test for *V. dahliae*

Sensitivity of test

Eight new real-time PCR assays with putative sensitivity to *Verticillium dahliae* were designed (Table 1). None of these assays, nor the EF assay developed in SF 97, nor a published Californian assay (Bilodeau *et al*., 2012), had complete specificity for *V. dahliae* under standard Fera PCR conditions. However, by manipulating the reaction conditions, assays designed to the rDNA IGS region showed excellent specificity, yet sensitivity was relatively poor (Table 2). This result is encouraging as it shows specificity within the rDNA IGS region can be obtained; future work can boost the sensitivity of this assay and the sensitivity of the VD+VL assay which best detected *Verticillium dahliae* in this project.

Three assays (VD+VL, Ko and Trypsin) had specificity for *V. dahliae* and *V. longisporum*. One assay (VCG1) appeared to just detect olive isolates of *Verticillium dahliae*; it did not detect isolates from UK strawberry or raspberry that Fera had in its culture collection. Since VCG1 is typically associated with olive isolates, this isolate was considered to have putative specificity for this vegetative compatibility group (VCG). Further work will need to obtain isolates of known VCG to confirm its specificity to VCG1.

The VD+VL assay and the putative VCG1 assay were taken forward for testing on a range of strawberry plants and soils due to the specificity of the VCG1 assay, plus the VD+VL assay had the best sensitivity of all the assays which detected *V. dahliae* and *V. longisporum* (approximately 10-100 more sensitive than the Trypsin and Ko assays but still 10 times less than Bilodeau). The limit of detection for the Bilodeau assay appeared to be at 0.1 microscelrotia per g of soil. In three soils tested at this level, only two of them were determined to be positive. As the other assays were ten times less sensitive, then the theoretical limit of detection is likely to be 1 microsclerotia using the current DNA extraction methodology.

Table 1. Details and source of real-time PCR assays evaluated in the study

Assay name	Loci	Multiple/Single copy	Source
Established assays			

EF (SF 97)	Elongation factor	Single	HDC project SF 97
Bilodeau	IGS	Multiple	Bilodeau et al., 2012
New assays			
IGS v1	IGS	Multiple	This study
IGS v2	IGS	Multiple	This study
ITS	ITS	Multiple	This study
MtDNA	MtDNA intergenic spacer region	Multiple	This study
VD+VL	RAPD Fragment AF363251	Single	This study
VCG1	RAPD Fragment AJ302674	Single	This study
Ko	RAPD Fragment U23151 (Li et al., 1999)	Single	This study
Trypsin	Trypsin protease (VTP1) AY354459	Single	This study

Table 2. Specificity testing of *Verticillium dahliae* assays showing Ct values when tested against isolates of *V. dahliae* (Vd)*, V. tricorpus* (Vt), *V. nigrescens* (Vn), *V. albo-atrum* (Vaa), *V. longisporum* (Vl) and *Gliocladium roseum* (Gr)

Assay	Annealing Temperature used	Vd1*	Vd2	Vt	Vn	Vaa	Vl	Gr
EF	60°C	18.5	21.3	36.5	35.5	33.9	40	37.8
Bilodeau	62°C	16.6	16.2	31.8	Nt	30.3	40	33.3
IGS v1	62°C	22.5	18.1	33.9	Nt	34.4	35	36.7
IGS v2	64°C	22.8	24.2	40	40	40	40	40
ITS	62°C	31.0	40	40	40	40	40	40
MtDNA	60°C	15.8	17.3	33.7	34.6	31.5	Nt	34.5
VD+VL	60°C	19.0	18.8	40	40	40	26.5	40
VCG1	60°C	19.2	40	40	40	40	40	40
Ko	60°C	25.3	24.7	40	40	40	26.5	40
Trypsin	60°C	25.4	27.3	40	40	40	27.8	40

Ct = 40 denotes a negative result; a low Ct value denotes good sensitivity. Nt = not tested.*Vd1 isolate belonging to VCG1.

Reliability of test

The effect of testing multiple 50 g sub-samples from a 2 kg field soil sample on variation in test results was examined for six naturally infested soils. For four samples variance decreased

considerably with four sub-samples (3a, 14, 26 and 43) but showed little further decrease thereafter (Table 3).

Table 3. Evaluation of the variation in qPCR results for *Verticillium dahliae* quantification in soil when replicated DNA extractions are compared

Soil sample	Ct value mean of				
	Two replicates*	Three replicates	Four replicates	Five replicates	Six replicates
2a	33.0 (0.2)	32.4 (0.6)	32.7 (0.5)	32.2 (0.6)	32.3 (0.5)
3a	30.5 (0.6)	30.7 (0.5)	30.7 (0.3)	30.9 (0.3)	31.1 (0.3)
14	34.5 (2.3)	35.7 (1.8)	35.6 (1.3)	35.6 (1.0)	35.6 (0.8)
24	31.3 (0.1)	31.3 (0.1)	30.5 (0.8)	30.4 (0.6)	30.5 (0.5)
2b	34.5 (2.3)	35.7 (1.8)	35.6 (1.3)	35.6 (1.0)	35.6 (0.8)
43	31.5 (2.4)	32.5 (1.7)	33.1 (1.3)	33.2 (1.0)	33.1 (0.9)

*Standard error given in parenthesis.

Objective 2 – Validate test by assessment of verticillium wilt symptoms in commercial strawberry crops

Soil sampling and occurrence of verticillium wilt symptoms

In spring and summer 2013, soil samples (2 kg) were taken by ADAS staff using the standard sampling method for *V. dahliae* from 49 fields due to be planted with strawberry; soil samples from an additional 10 fields due to be planted with strawberry were taken in spring/summer 2014 to supplement the data set. The samples were supplied to Fera for determination of *V. dahliae* by qPCR. The samples comprised sites in England (52), Scotland (4) and Wales (3) and covered major soft-fruit production counties in the South East (26), East Anglia (7) and West Midlands (11).

When crops from the soil-sampled sites were examined in autumn 2013 after the end of fruiting, symptoms of verticillium wilt were observed at 34 out of 41 sites, with an incidence >5% at 16 sites. Laboratory tests confirmed *V. dahliae* in symptomatic plants from five out of eight sites sampled.

When crops from the soil-sampled sites were examined in autumn 2014 after the end of fruiting, symptoms of verticillium wilt were observed at 49 sites out of the 54 remaining sites (5 sites were grubbed after the 2013 season), with an incidence >5% at 10 sites. Laboratory tests confirmed *V. dahliae* in symptomatic plant samples taken from 34 out of 52 crops sampled (it was not possible to sample all sites); the fungus was also detected in visibly healthy plants from 15 of these 50 crops.

Comparison of soil infestation with verticillium wilt symptoms

Three methods were used to examine data for evidence of an association between occurrence of *V. dahliae* in soils and occurrence of verticillium wilt symptoms in crops. The aim was to assess the suitability of the qPCR tests for predicting risk of verticillium wilt.

1. *Presence or absence of verticillium wilt in crops compared to detection (yes/no) of* Verticillium dahliae *in soil*

In 2013 each soil sample was tested for *V. dahliae* by established qPCR assays using sets of primers from a UK test (Fera-EF assay) and a Californian test (Bilodeau assay). In the 41 fields assessed for verticillium wilt, the presence or absence of symptoms was correctly predicted at 73% of sites by the Bilodeau soil test and 50% of sites by the Fera-EF soil test (Table 4). The Bilodeau test showed the best correspondence between soil results and field symptoms. Of 16 sites with obvious verticillium wilt symptoms (>5% of plants), *V. dahliae* was detected in soil from 13 and 2 of these sites by the Bilodeau and Fera-EF tests respectively. The high level of apparently false negative results from the Fera-EF soil test may reflect the known lower sensitivity of this assay.

There were seven sites where no verticillium wilt symptoms were observed; three of these were reported to have *V. dahliae* present in the soil by the Bilodeau test, none by the Fera-EF test. The apparently false positive results from the Bilodeau test may reflect the lower specificity of this assay.

In 2014, soils were re-tested by the Bilodeau assay and also tested by two new assays (VCG1 and VL+VD) developed by Fera.

In the fields assessed for incidence of strawberry verticillium wilt, *V. dahliae* was detected in 36/54 soils using the Bilodeau assay, 10/53 soils using the VCG1 assay and 24/53 soils using the VD+VL assay. There was a relatively low correspondence of soil test results with field observations for all three assays (50%, 47% and 42%) (Table 4). This low correspondence of soil test results as an indication of whether or not verticillium wilt symptoms would be present in the crop was not increased when the data set was restricted to just the susceptible variety Sonata (Table 4).

2. *Incidence of verticillium wilt symptoms compared to detection (yes/no) of* Verticillium dahliae *in soil*

In 2013, the mean incidence of verticillium wilt symptoms was greater in soils where *V. dahliae* was detected than where it was not detected for both soil assays (Table 4). In 2014, the mean incidence of verticillium wilt symptoms was very similar in crops grown on soils where *V. dahliae* was detected and where it was not detected for all three assays. The levels of verticillium wilt recorded in 2014 were generally lower than those in 2013. Across all crops,

the mean incidence of affected plants was 6 % in 2013 and 3.5 % in 2014. This difference is probably due to the better growing conditions with less moisture stress on crops in 2014. Possibly a greater incidence of verticillium wilt symptoms may have been seen in crops where *V. dahliae* was detected in soil, compared with sites where it was not detected, in crops grown under more stressful conditions.

3. *Incidence of verticillium wilt symptoms related to density of* Verticillium dahliae *detected in soil*

In 2013, where soil test results were grouped into six categories of increasing soil density of *V. dahliae* (not detected, <1, 1.1-5, 5.1-10, 10.1-100 and >100 pg/g), there was a trend for the proportion of plants with verticillium wilt symptoms to increase (2.2, 3.6, 7.2, 13.6, 21.0 and 11.8 plants respectively). It should be noted however that the number of samples in each category was relatively small (n = 12, 3, 13, 7, 4 and 6 respectively), so one outlier could strongly influence results.

In 2014, when soil test results were grouped into the same six categories, there was no evidence from any of the three assays that the incidence of verticillium wilt in crops, or the proportion of plants with severe symptoms of verticillium wilt (severities 2 and 3 on a 0-3 scale), increased as the soil infestation density of *V. dahliae* increased (Table 4). Comparing the soil results with the plant qPCR results (rather than field symptoms) shows slightly greater agreement but still this is only around 50%.

Table 4. Summary of comparisons between occurrences of *V. dahliae* in soil as determined by qPCR tests and verticillium wilt symptoms in strawberry crops – 2013 and 2014

Comparison	Occurrence of *V. dahliae* in soil determined by qPCR test				
	2013		2014		
	Bilodeau	Fera-EF	Bilodeau	VCG1	VD+VL
1. Presence/absence in soil vs presence/absence in crops					
Mean % sites where field symptoms reflect soil test results					
All crops	73	25	50	32	47
Sonata only	25	25	40	25	38
2. Presence/absence in soil vs mean wilt incidence					
Mean % plants with Verticillium wilt symptoms					
V. dahliae not detected	2.4	7.0	3.9	3.3	4.3
V. dahliae detected	9.7	13.2	3.3	4.2	2.5
3. Density in soil vs mean wilt incidence					
Density in soil (pg/g)	*Mean wilt incidence (number of sites)*				
ND	2.2 (n=12)	8.2 (n=38)	3.9 (n=18)	3.3 (n=43)	4.3 (n=29)
<1	3.6 (n=5)	-	7.3 (n=5)	-	-
1.1 – 5	7.2 (n = 13)	-	3.5 (n=14)	-	2.5 (n=16)
5.1 – 10	13.6 (n=7)	-	1.5 (n=7)	4.2 (n=3)	2.1 (n=4)
10.1 – 100	21.0 (n=4)	0.3 (n=3)	3.0 (n=4)	4.6 (n=6)	3.7 (n=3)
>100	11.8 (n=6)	12.4 (n=4)	1.9 (n=6)	1.6 (n=1)	0.3 (n=1)

Distribution of V. dahliae in fields

Examination of the distribution of *V. dahliae* in four fields by testing 50 soil samples taken on a grid pattern showed that infestation was highly clustered. Kriging (a statistical technique which gives the best linear prediction of intermediate values i.e. in this instance a prediction of the level of *V. dahliae* between sampling points) was possible for three of the four sites and this analysis could be used to inform the development of a new sampling strategy. Kriging was not possible at one site due to low levels of *V. dahliae* detected in that field.

Main conclusions

- In 2013, the presence or absence of verticillium wilt symptoms in strawberry crops was correctly predicted by the presence or absence of *Verticillium dahliae* in the soil as detected by a Californian (Bilodeau) molecular-based soil test at 73% of 41 sites; in 2014 using this assay, occurrence of verticillium wilt was correctly predicted by the soil test at 50% of 54 sites.
- Two novel assays (VCG1 and VD+VL) developed by Fera and used in 2014 to test soils for *Verticillium dahliae* detected the fungus in 32% and 40% of 53 sites, respectively. The single copy VD+VL appears to be insufficiently sensitive for pre-plant risk assessment as verticillium wilt symptoms occurred in crops grown on 44 of these soils. The VCG1 assay is likely to be specific for one vegetative compatibility group (VCG) and will not detect other VCGs of *V. dahliae*
- In 2013, the incidence of verticillium wilt symptoms was greater in crops grown in soils where *V. dahliae* was detected than in soils where it was not detected by the Bilodeau assay (9.7% vs 2.4%) and the Fera-EF assay (13.2% vs 7%). However, in 2014 there was very little difference in the incidence of verticillium wilt symptoms in crops grown in soil where *V. dahliae* was detected and where it was not detected. This was true for all three qPCR assays used. Apart from the Bilodeau assay in 2013, there was no evidence of a trend for increasing levels of verticillium wilt in crops with increasing density of *V. dahliae* in soil.
- Where *Verticillium dahliae* was detected in the soil, 56% of plants sampled tested positive for *Verticillium dahliae* by the Bilodeau test; where no *Verticillium* was detected in the soil, 10% of plants tested positive.
- The results from this project indicate that the three molecular soil assays for *Verticillium dahliae*, as used in this work, are not currently suitable for assessing the relative risk of verticillium wilt occurrence in strawberry.

Financial benefits

Verticillium wilt, caused primarily by *V. dahliae*, is one of the most serious diseases of strawberry causing significant yield losses, and is a significant driver to soft fruit production being shifted into substrate and table top systems. Quantifying soil inoculum prior to planting can be used as a tool to manage the disease. Depending on the levels found, fields and varieties can be selected to limit risk.

If a field is not tested for *V. dahliae* prior to planting a susceptible variety, and the fungus is present at levels sufficient to cause infection, potential losses are around £12,000/ha assuming 50% of the crop is affected. If a field is treated with Basamid (dazomet) or Custofume (chloropicrin) as a precaution against verticillium wilt, and the fungus is not present at levels sufficient to cause disease, unnecessary costs of £3-5,000/ha may be incurred. An accurate assessment of *V. dahliae* soil infestation density can thus provide significant savings.

Action points for growers

The qPCR soil assays for detection and quantification of *V. dahliae* in soil as used in this project are not currently suitable for routine testing of soils for *V. dahliae*, as a chargeable service to growers, due to the lack of evidence that these soil test results accurately predict the relative risk of verticillium wilt developing in strawberry.