Project title:	Using molecular quantification of *Verticillium dahliae* in soil to identify risk of strawberry verticillium wilt
Project number:	SF 097a
Project leader:	Tim O'Neill, ADAS
Report	Final Report, March 2015
Previous reports:	Annual Report, April 2014
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Date project commenced:	1 January 2013
Expected completion:	31 March 2015

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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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CONTENTS

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.

SCIENCE SECTION

Introduction

Background

Verticillium wilt is one of the most serious diseases of strawberries and is capable of causing significant yield losses. Many varieties grown in the UK such as Elsanta, Sonata and Figaro are susceptible. The causal pathogen, *V. dahliae*, can exist as microsclerotia that can persist in soil for many years. *Verticillium dahliae* has a very wide host range, many of which are common agricultural crops in the UK (e.g. potato, linseed). This means that there is a real risk that strawberries may be planted on land infested with pathogen propagules and yield could be severely limited.

Harris soil test

Soil testing offers one way to inform decisions about planting in fields potentially at risk from the pathogen. A pre-planting wilt risk assessment service, the Harris soil test, has been available to growers since the early 1990s (Harris *et al.,* 1993; Harris and Yang, 1996). This test is based on the detection and enumeration of *V. dahliae* microsclerotia in soil using a sieving technique to isolate the microsclerotia. As an incubation stage is required, the test takes typically six to eight weeks to complete.

PCR soil tests

A rapid real-time PCR assay coupled with a robust soil DNA extraction technique has the potential to offer a viable alternative to the Harris test with enhanced specificity and the possibility of a result within days compared with six to eight weeks with the Harris test. Designing species-specific assays for *Verticillium dahliae* is often problematic due to the range of closely related species, including *Verticillium albo-atrum* and *Verticillium longisporum*, the latter being a hybrid of *V. dahliae* and another, as yet unknown, *Verticillium* species. In 2012, Bilodeau and co-workers developed a sensitive assay designed to the ribosomal DNA Intergenic Spacer Region (IGS). This assay has been found to be highly sensitive compared to other assays (Gramaje *et al*., 2013). The IGS region exists in multiple copies within the genome of fungal nuclei and therefore this allows assays designed to this region to be highly sensitive. However, testing of this assay at Fera under standard conditions suggested it was not specific for *V. dahliae*, and was capable of also detecting a range of DNA from cultures of closely related *Verticillium* and *Gliocladium* species (SF 97). This would prove highly problematic for a soil test since it could lead to a large number of false positives. Therefore in a recent HDC funded project (SF 97), a

species-specific assay was developed for *V. dahliae*. Unlike the Bilodeau *et al*. assay, it was designed to the single copy elongation factor (EF) gene. Whilst this resulted in a more specific assay, it was at a cost to sensitivity. Using the assay, limited tests indicated a good relationship between the Harris test and the molecular test. However, an increase in sensitivity of the molecular assay was required for it to equal the sensitivity of the Harris test.

The assay developed in SF 97 did not react with the *V. longisporum* isolates tested in that project. *V. longisporum* is a pathogen of oilseed rape and vegetable brassicas whose microsclerotia are indistinguishable from *V. dahliae* on agar culture plates. Verticillium wilt of oilseed rape caused by *V. longisporum* has become an increasing problem in UK crops over the last five years; increased occurrence of *V. longisporum* in arable soils could possibly lead to erroneous estimates of verticillium wilt risk in strawberry from the traditional soil-sieving and agar plate *V. dahliae* assay. To our knowledge, the pathogenicity of *V. longisporum* to strawberry has not been tested. Previous work (Zeise & Von Tiedemann, 2008) has found that isolates belonging to VCG2B and 4B showed high aggressiveness to strawberry plants, whilst *V. longisporum* isolates produced little or no disease. However this was with just OSR *V. longisporum* isolates collected in 1997 and since then much greater diversity has been found in the *V. longisporum* group, with three distinct lineages present (Inderbitzin *et al.*, 2013).

The assay developed in SF 97 is able to detect *V. dahliae* down to approximately 1 microsclerotium per gram (ms/g) soil. As a result the reproducibility of results on soils with lower levels was poor. It is desirable to improve the sensitivity of the test down to levels close to 0.1 ms/g as some commonly grown strawberry varieties (e.g. Elsanta) are still very susceptible at this low infestation density.

Sub-samples

The qPCR test used in SF 97 gave results more consistent with the observed symptom development in strawberry fields when multiple qPCR tests were done on a soil sample and a mean of the results was taken. The same effect was reported in recent work on qPCR for quantification of *V. dahliae* in work on strawberry wilt in California where it was recommended that four separate sub-samples should be taken (Bilodeau *et al*., 2012). Work is needed to optimise the soil sub-sampling and testing strategy in order that a result most accurately reflects the risk of verticillium wilt from that soil.

Correlation with field symptoms

In SF 97, the correlation of soil infestation density measured by qPCR and verticillium wilt symptoms in field grown crops was moderately good, especially in the second cropping year. However, work was restricted to five fields. The test correctly identified two fields which developed a high incidence of verticillium wilt (>10%) and did not detect *V. dahliae* in two fields which developed a low incidence of confirmed verticillium wilt (<2%); results at a fifth site were unclear. This was most likely due to low levels (<1 ms/g) of microsclerotia in these three soils.

This project therefore aims to enhance the sensitivity of the molecular test. This will be done by developing new specific primers or using approaches to boost sensitivity of the existing test such as the use of FLAPs (Afonina *et al*., 2007) and TINA primers (Schneider *et al*., 2012), nested approaches and droplet digital PCR (ddPCR).

Potential risk vs symptom occurrence

Although it is a basic tenet of plant pathology that disease risk increases with inoculum level, and such relationships have been demonstrated for soil inoculum level of *V. dahliae* and some other verticillium wilt diseases, the relationship is not necessarily linear and can vary with many factors. For example, soil moisture, temperature, microbiology and type can all influence infection and disease development and might lead to between-year and between-site differences for the same inoculum level; this may also explain why the results of pot tests can differ from those of field experiments. The inoculum level in a soil may change between sampling and planting (e.g. through cultivations and debris decay releasing microsclerotia; and through microbial degradation reducing numbers of viable microsclerotia). Quantification of soil inoculum should thus be taken as a measure of the *potential* risk of infection arising from the soil, and not as a level of verticillium wilt that will necessarily occur.

The objectives of this project were:

1. To improve the sensitivity and reliability of a qPCR soil test for *V. dahliae;*
2. To validate the test by investigation of the relationship between soil infestation densities of *V. dahliae* measured by the improved test and verticillium wilt symptoms in commercial strawberry crops.

The specific objectives in Year 2 were:

1. To further improve the sensitivity and reliability of a qPCR test for *V. dahliae* through improved assay design;

2. To complete validation of the test assay by qPCR testing of soil samples from 50 fields and determining levels of verticillium wilt in strawberry crops planted in these fields in 2013.

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

Introduction

In the previous year, it was determined that specificity was possible with IGS primers but at a cost to sensitivity. Therefore, this year work focused on developing primers designed to other loci to determine if specificity could be achieved for *V. dahliae* and investigating approaches to boost overall assay sensitivity. In the previous year, two methods to boost sensitivity were evaluated, Tina and Flaps primers. In both these instances sensitivity was not improved. Therefore, this year efforts focused on investigating nested qPCR, which involves using an additional set of outer primers in a first PCR round, followed by inner primers in a second PCR round at a different temperature. The combination of two rounds of PCR with different primers is expected to enhance sensitivity.

Droplet digital PCR (ddPCR) was also evaluated. This is a recently commercialised method to enable the precise quantification of target nucleic acids in a sample (Pinheiro *et al*., 2012). Whilst traditional PCR quantifies the amount of target in a single reaction, ddPCR involves many PCR reactions and the number of positives of those reactions is used to determine the relative levels of target present. The premise for ddPCR is the water-oil emulsion droplet technology which is used to create the thousands of individual reactions required. Typically a sample is fractionated into 20,000 droplets, and PCR amplification of the template molecules occurs in each individual droplet. This method has high potential for quantification in soil. One of the key attributes is its tolerance to inhibitors. Recently Kim *et al*. (2014) showed the method was ten times more sensitive than standard qPCR for detection of bacteria in soils.

Materials and methods

Primer design and standard qPCR

Primers were designed using Primer Express 3 (Life Technologies). All qPCR was undertaken with environmental master mix (Life Technologies) using standard Fera conditions with 2 µl template DNA purified using a Wizard Food DNA extraction kit (Promega).

Nested qPCR and droplet digital PCR (ddPCR)

Nested qPCR was conducted by adapting the VCG1 primers. This was done by designing additional outer primers outside the existing forward and reverse primer sites. Each outer primer had an annealing temperature of 63°C. The existing primers were adapted to inner primers by removing bases from the 5-prime end until the primer had an annealing temperature of 54°C (the original primers had an annealing temperature of 60°C). Nested qPCR conditions consisted of 10 minutes at 95°C, followed by 10 cycles of 95°C for 15 s and 65°C for 30 s, then 40 cycles of 95°C 15 s, 55 for 20 s and 72°C for 30 s. The ddPCR was conducted using a BioRad QX100 according to the manufacturer's instructions. Primers and probe used were as Bilodeau *et al.* (2012). A fivefold dilution series of *V. dahliae* DNA from pure culture was used each time. All samples were tested in triplicate.

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

Introduction

The aim was to investigate the relationship between soil infestation density of *V. dahliae* measured using qPCR and verticillium wilt in field-grown strawberry crops. As development of verticillium wilt symptoms can vary from season to season, being dependent on temperature and cropping factors as well as soil infestation density, crops were assessed for the disease in both their first (2013) and second (2014) year after planting.

Materials and methods

Site selection and soil samples

Sites were selected by contacting and recruiting growers who were planting in spring 2013, initially approaching growers who had already commissioned a soil analysis (by Harris test) through the Fera plant clinic or directly to ADAS Gleadthorpe, then by contacting growers planting in the soil in spring 2013 identified by ADAS fruit consultants and carrying out specific soil sampling for this project. Ten additional sites all growing cv. Sonata were identified, soil-sampled and monitored in 2014 to supplement the data set. Sites were selected to include a range of soil types and levels of expected verticillium wilt based on previous tests and observation, and included a range of different susceptible varieties planted. In order, over time, to capture any trends or associations between levels of wilt and cropping conditions the following information was collected for each sampled location:

- Grower name and field location;
- Soil type;
- History of soft fruit, potato or linseed production on this site (in last five years);
- Strawberry variety;
- Planting date;
- Soil sampling date;
- If the crop will be tunneled or left open field;
- If crop is irrigated;
- Details and dates of any soil sterilisation activities carried out (2012 or 2013).

The field sites were managed completely as normal with no restrictions on treatments to be applied by the grower post-planting. We preferentially targeted sites that had not been soil sterilised prior to planting. However it was not possible to get 50 unsterilised sites so sterilised sites were included where levels of wilt were predicted to be high and or where highly susceptible varieties were to be planted, as previous work has shown that soil fumigation is likely to result in a 90% reduction of *V. dahliae* soil infestation density, not eradication. On a few sites plants were in the ground prior to sampling; in this situation soil was sampled from between newly planted plants, being careful not include any root or growing media material.

Where a soil sample had not previously been taken and therefore a subsample was already available for analysis, soil sampling was carried out according to the standard sampling method for the Harris test. This was done on a bulked soil sample collected over an area less than 2 ha; larger areas were subdivided. This area was divided into a grid of approx. 10 m x 20 m squares and 50 subsamples were taken with a 25 mm auger from each grid square, to a depth of 200 mm. This gave a sample weighing approx. 2 kg per hectare sampled. The area sampled was dependent on the history of the site. If the field had had a uniform cropping history and was growing one cultivar only, it was considered as one unit; if not, separate samples were taken. Samples were sent to Fera after sampling or else kept in a cool dark place until dispatch.

DNA extraction and quantitative real-time PCR tests on soil samples

For each site (or individual sampling point in the field in the case of Sites 3, 13, 14 and 15) DNA was extracted from one 50 g subsample of soil. Soil samples were air dried for up to two days at room temperature on receipt and then stored at 4ºC in the dark. DNA was then extracted using the method adapted from Woodhall *et al*. (2012). This method is summarized as follows: 50 g of soil was weighed into a 250 ml Nalgene wide mouth environmental bottle with six 25.4 mm stainless steel ball bearings and 100 ml grinding

buffer (120 mM sodium phosphate buffer pH8, 2% cetrimonium bromide, 1.5 M sodium chloride) and 3 ml Antifoam B. The Nalgene bottles were then placed in a commercial paint shaker (Merris Dispensing & Mixing Equipment, Czech Republic) and shaken for four minutes. A 50 ml sub-sample was then transferred to a 50 ml tube and centrifuged at 5000 x g for five minutes. Then 20 ml of the resulting supernatant was added to a clean 50 ml tube containing 2 ml of 5M potassium acetate. The tubes were incubated on ice for 10 minutes. This was followed by five minutes centrifugation at 12 000 x g.

The supernatant was then added to a clean 50 ml tube containing 15 ml isopropanol and 800 µl 1% silicon dioxide suspension (Sigma) and placed on a flatbed shaker for 15 minutes at 100 rpm followed by five minutes centrifugation at 12 000 g. The supernatant was discarded and 2 ml Buffer A (Promega Wizard Food Kit) added to the remaining silicon dioxide particles and the tubes placed in a shaking incubator on their side for 10 minutes at 65°C at 100 rpm. The silica particles were then separated by centrifugation for five minutes at 12 000 g. 1000 µl of the resulting supernatant was processed according to the manufacturer's instructions for the Wizard Food Kit (Promega) in conjunction with a Kingfisher ML magnetic particle processor (Thermo Electron Corporation) with DNA eluted into 200 µl of TE buffer.

Environmental Master Mix 2.0 (Life Technologies) was used for all real-time PCR with soil samples and consisted of half the total reaction volume of 25 µ and 5 µl of the soil DNA sample. Each soil sample was tested in three replicate PCRs with both the Bilodeau *et al*., (2012) and the Elongation Factor assay. Cycling conditions were as described above with the anneal/extension step of 60ºC used for both assays. Target DNA in soil samples was quantified by including five DNA standards on each PCR run. The standards consisted of a DNA sample of known concentration taken from the appropriate culture which was used to produce a dilution series of four ten-fold dilutions. Target DNA was then determined by linear regression. Soil DNA was determined to be of suitable quality for PCR using the eubacterial assay of Yang *et al*. (2002) as an internal control. Soil samples which gave a high Ct value (over 23) were discarded and re-extracted.

Field assessment of verticillium wilt

Assessments were made in September - October 2013 after the crops had finished fruiting and in August – October 2014. At each of the sites the whole field was monitored for symptoms of verticillium wilt. This was done by walking five equidistant transects the full length of the field. Along each transect the number of plants showing verticillium wilt symptoms in one bed was counted and scored according to the 1-3 scale shown in the photographs below (Figure 2).

Alongside this the planting density was recorded (i.e. number of plants per linear metre in one bed) and the length of transect walked in order to determine the approximate total number of plants assessed and hence calculate the % of plants affected. This resulted in visual assessment of over 1000 plants for symptoms of verticillium wilt at each site.

At each site in 2014, five plant samples were taken of:

i) healthy plants i.e. no symptoms and

ii) symptomatic plants i.e. those which have score 2 on the below scale.

At one site 10 plants of each score (1 – 3) plus healthy plants were sampled to assess accuracy of scoring.

Figure 2. Field symptoms of verticillium wilt showing (1) early wilt symptoms (collapsing plants, RHS); (2) obvious wilt with a halo of dead leaves; (3) dead plants with all leaves fully collapsed. Samples of affected plants were examined for dark brown/black staining in vascular tissue of crowns (4).

Results

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

Four additional qPCR assays were designed this year. These included three designed from sequenced RAPD fragments present on GenBank and one from the Trypsin protease

(VTP1) gene (GenBank Y354459). Assays termed VD+VL, VCG1 and Ko were designed from sequence RAPD fragments placed on GenBank with the accession numbers AF363251, AJ302674 and U23151. These four assays were tested under standard Fera conditions against a bank of *Verticillium* species and one *Gliocladium* isolate (Table 5). Previously designed assays are shown for comparison. The VD+VL, Ko and Trypsin assay also detected *V. longisporum* but were otherwise specific. The VCG1 assay was highly specific. It only detected one *V. dahliae* isolate. A further analysis with all *Verticillium* isolates present in the Fera culture collection showed this assay only detected olive isolates (which were tentatively identified as one VCG of *V. dahliae* – VCG1), it did not pick up other *V. dahliae* isolates in the collection which were collected from raspberry, strawberry and potato. Isolates from these crops typically belong to other VCGs. Therefore this assay is likely to be VCG specific. However, it will need to be tested with a range of VCG determined isolates to determine its true specificity. Of the four new assays, the VCG1 appeared most sensitive along with the VD+VL assay as the lowest Ct was observed compared to the other assays and these two assays were taken forward for testing on plant and soil material.

Table 5. 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 belonged to VCG1.

UK strawberry soils with known amounts of microsclerotia, as determined by Harris testing, were tested with the Bilodeau *et al* assay. Table 6 shows approximate microsclerotia levels corresponding with pg of DNA per g of soil detected by PCR.

Table 6. Index used for quantification of *V. dahliae* in soil by qPCR and estimated equivalent quantities of microsclerotia

Index	Quantity of *Verticillium* spp. by qPCR (pg/g soil) (Bilodeau test)	Approximate equivalent of *V. dahliae* microsclerotia (no/g soil)
0	Not detected	n/a
1	<0.1	<1
2	0.1 - 5	1-10
3	5.1 - 10	10 - 20
4	10.1 - 100	10 -150
5	>100	150+

In order to attempt to boost sensitivity of the assay, nested PCR and ddPCR approaches were tried. The VCG1 assay was adapted for nested PCR. Despite changing the assay conditions and adding additional primers, the assay still remained highly specific and did not cross react with non-target species of any of the isolates in Table 5. The nested assay was tested against the standard VCG1 assay in a fivefold dilution series (Table 7). Despite Ct generally being lower for the nested assay, there was no difference in sensitivity observed. However, a nested approach at improving Ct may still be of use. A lower Ct may improve confidence in detection at the limit of detection.

Table 7. Relative sensitivity of a nested VCG1 assay and standard qPCR VCG1 assay

DNA concentration (pg)	Standard qPCR (Ct)	Nested qPCR (Ct)
1600.0	23.08	22.14
320.0	25.16	24.36
64.0	27.50	27.33
12.8	30.02	28.79
2.56	33.28	31.79
0.512	37.18	35.06
0.1024	40.00	40.00

The ddPCR method was tested against standard qPCR using the Bilodeau *et al*. (2012) assay in a fivefold dilution series (Table 8). It was determined that ddPCR was more sensitive than standard qPCR as it could detect 320 fg whilst the limit of detection for qPCR in this instance was 1600 fg. The ddPCR technique was also tested in the presence of humic acid, a potent inhibitor of PCR. Detection was possible at 5 mg/ml humic acid with ddPCR whilst detection was not possible at this level with standard qPCR.

Table 8. Relative sensitivity of standard qPCR and ddPCR using the Bilodeau *et al*. (2012) assay.

Verticillium DNA (fg)	qPCR (Ct)	ddPCR (copies detected/µl)
200000	24.7	477.7
40000	30.8	122.0
8000	33.3	16.2
1600	34.5	2.2
320	Not detected	0.4
64	Not detected	Not detected
13	Not detected	Not detected

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Details of sites

A total of 55 soils were sampled in 2013 and 2014, and four additional samples were sourced from stored samples previously taken and submitted to Fera in August 2012 for the Harris test soil analysis.

Table 9 details the sampling locations and site details. There is no sample 46 as this site was not planted with strawberries. Samples were taken from 21 counties including samples from Wales, Scotland, the North, the Midlands, South East and the far South West. No assessment was possible at five sites due to damage by herbicide or early grubbing of the crop.

Table 9. Details of sites soil sampled for *Verticillium dahliae* in 2013 (SS1 – SS50) and 2014 (SS 51-SS 60)

Ref no.	County	Soil type	Cultivar	Cropping history	Date soil sampled
SS 1	Cheshire	Sandy loam	Symphony, Eros, Malwina	Mixed	20/03/2013
SS 2	Essex	Sandy loam	Camarillo	Susceptible	10/04/2013
SS 3	Essex	Sandy loam	Fenella	Moderately susceptible	10/04/2013
SS 4	Essex	Clay loam	Serena	Susceptible	10/04/2013
SS 5	Essex	Clay loam	Serena and Finesse	Mixed	10/04/2013
SS 6	Surrey	loamy sand	Fenella, Symphony	Mixed	16/04/2013
SS 7	Kent	Clay loam	Trial site	Mixed	16/04/2013
SS 8	Kent	Clay loam	Diamond	Susceptible	16/04/2013
SS 9	Kent	Clay loam	Sonata	Susceptible	16/04/2013
SS 10	Kent	Clay loam	Sonata	Susceptible	16/04/2013
SS 11	Kent	Clay loam	Amesti	Susceptible	16/04/2013
SS 12	Cheshire	Sandy Loam	Symphony	Moderate resistance	14/04/2013
SS 13	Cambridgeshire	Silty loam	Sonata	Susceptible	01/05/2013
SS 14	Cambridgeshire	Silty loam	Fenella	Moderately susceptible	01/05/2013
SS 15	Kent	Silty loam	Elegance	Moderate resistance	10/05/2013
SS 16	Kent	Silty loam	Fenella	Moderately susceptible	10/05/2013
SS 17	Kent	Sandy loam	Vibrant	Susceptible	10/05/2013
SS 18	Kent	Sandy loam	Driscolls Diamond	Susceptible	10/05/2013
SS 19	Oxfordshire	Sandy loam	Sonata	Susceptible	17/06/2013
SS 20	Oxfordshire	Sandy loam	Florence, Fenella, Symphony	mixed	17/06/2013
SS 21	Oxfordshire	Sandy loam	Fenella	Moderately susceptible	17/06/2013
SS 22	Oxfordshire	Sandy loam	Symphony	Moderate resistance	17/06/2013

Ref no.	County	Soil type	Cultivar	Cropping history	Date soil sampled
SS 23	Staffordshire	Loamy sand	Elegance	Moderate resistance	21/06/2013
SS 24	Staffordshire	Loamy sand	Buddy	Susceptible	21/06/2013
SS 25	Staffordshire	Sandy loam	Sonata	Susceptible	21/06/2013
SS 26	Staffordshire	loamy sand	Fenella	Moderately susceptible	21/06/2013
SS 27	Staffordshire	loamy sand	Amesti	Susceptible	21/06/2013
SS 28	Swansea	Sandy loam	Malwina, Judi bell, Symphony, Cupid, Rumba	Mixed	26/06/2013
SS 29	Merseyside	Sandy loam	Eros, Florence, Judi bell, Symphony	Mixed	26/06/2013
SS 30	Wrexham	Sandy loam	Christine, Eros, Darselect, Amelia, Pegasus	Mixed	26/06/2013
SS 31	Buckinghamshire	Sandy loam	Malwina, Symphony	Mixed	16/07/2013
SS 32	Cornwall	Sandy loam	Symphony, Malwina	Mixed	10/07/2013
SS 33	Warwickshire	Sandy loam	Vibrant, Fenella, Florence, Symphony, Malwina	Mixed	02/05/2013
SS 34	Worcestershire	Clay loam	Vibrant, Eros Symphony, Malwina	Mixed	18/04/2013
SS 35	Powys	Silty Loam	Fenella, Malwina	Mixed	10/06/2013
SS 36	Devon	Sandy loam	Symphony, Elsanta, Malwina	Mixed	15/05/2013
SS 37	Cheshire	Sandy loam	Symphony, Eros, Malwina	Mixed	26/06/2013
SS 38	Leicestershire	Clay loam	Korona, Marshmellow, Elegance	Mixed	30/06/2013
SS 39	West midlands	Sandy loam	Malwina, Symphony, Florence, Cupid (trial), Alice, Korona	Mixed	30/06/2013
SS 40	Buckinghamshire	Sandy loam	Symphony	Moderate resistance	11/07/2013
SS 41	Buckinghamshire	Clay Loam	Malwina	Good resistance	11/07/2013
SS 42	Cornwall	Sandy Loam	Vibrant Symphony Florence Malwina	Mixed	17/07/2013
SS 43	Berkshire	Loamy sand	Symphony	Moderate resistance	08/08/2013
SS 44	Berkshire	Loamy sand	Elan	Moderately susceptible	08/08/2013
SS 45	York	Loamy sand	Vibrant	Susceptible	12/08/2013

Ref no.	County	Soil type	Cultivar	Cropping history	Date soil sampled
SS 46					
SS 47	Kincardineshire	Sandy Loam	Sonata	Susceptible	Autumn 2012 by BG
SS 48	Kincardineshire	Sandy Loam	Sonata	Susceptible	Autumn 2012 by BG
SS 49	Kincardineshire	Sandy Loam	Sonata	Susceptible	Autumn 2012 by BG
SS 50	Kincardineshire	Sandy Loam	Sonata	Susceptible	Autumn 2012 by BG
SS 51	Kent	Silty Loam	Sonata	Susceptible	08/05/2014
SS 52	Kent	Sandy Loam	Sonata	Susceptible	08/05/2014
SS 53	Kent	Sandy Loam	Sonata	Susceptible	08/05/2014
SS 54	Kent	Clay Loam	Sonata	Susceptible	08/05/2014
SS 55	Kent	Clay Loam	Sonata	Susceptible	08/05/2014
SS 56	Cambridgeshire	Silty Loam	Sonata	Susceptible	01/08/2014
SS 57	Herefordshire	Clay Loam	Sonata	Susceptible	01/08/2014
SS 58	Shropshire	Clay Loam	Sonata	Susceptible	01/08/2014
SS 59	Surry	Sandy Loam	Sonata	Susceptible	01/07/2014
SS 60	Oxfordshire	Sandy Loam	Sonata	Susceptible	02/07/2014

Note: there is no Site SS 46. * - no verticillium wilt symptoms present.

Density of V. dahliae in soil and incidence of verticillium wilt symptoms

In 2014, results of soil tests for *Verticillium dahliae* by three qPCR assays, incidence of verticillium wilt symptoms in crops grown on soil-sampled sites, and confirmation of verticillium infestation in individual plants taken from these sites are given below (Table 10). The results for 2013 are shown alongside those for 2014 in Appendix 1.

A full set of results was not available for Sites 46 (never planted with strawberry), 23, 24, 47 and 48 (crop grubbed before it could be assessed) and Sites 1, 28, 32, 37, 38, 49 and 50 (nil or incomplete plant sample for laboratory testing).

Symptoms of strawberry verticillium wilt occurred in crops at 34 out of 41 sites in 2013 and at 45 out of 54 sites in 2014. At the 34 sites where verticillium wilt was recorded in 2013, it occurred again at all of these sites in 2014. *Verticillium dahliae* was confirmed in plants, by the Bilodeau assay, from samples at 35 out of 52 sites sampled in 2014. It was detected in symptomatic plants at 34 sites, often showing higher levels (>5 pg/g) where soil levels were also highest. Surprisingly, it was detected in asymptomatic plants at 15 sites although this was generally at a much lower level (<1 pg/g).

Nil *V. dahliae* was detected in soil at 12 sites by any of the 2014 qPCR assays yet verticillium wilt symptoms occurred in crops at seven of these sites, but confirmed in plant by qPCR in just two of these, suggesting some limitations to the field symptom scores. At these same 12 sites in 2013, nil *V. dahliae* was detected by either the Fera-EF or the Bilodeau assay at 11 of them; the exception was Site 15 where a very low density was detected by the Bilodeau assay in 2013.

Nil verticillium wilt symptoms occurred in crops at six sites in 2014, yet *V. dahliae* was detected in soil at three of these sites. In 2013 some assessments are missing for these sites and two fall within the additional 10 sites sampled only in 2014 but nil verticillium symptoms were also recorded for two of the same sites and these correspond to nil soil detection – Sites 1 and 32

Fifteen sites tested negative for *V. dahliae* by all soil tests in 2013 and in 2014. Nine of these sites showed symptoms of verticillium wilt in 2013 and 2014, but only one of four of these sites showed symptoms where plants were positive for *V. dahliae* when tested by PCR suggesting again some limitations to the field scoring system.

Table 10. Results of soil tests for *Verticillium dahliae* by qPCR tests, incidence of verticillium wilt symptoms in strawberry crops and detection of *Verticillium* species in visibly healthy and affected plants from these crops – 2014

Site	qPCR assay (pg/g)			% plants with verticillium wilt symptoms by severity*						qPCR plant assay (pg/g)	
	Bilodeau	VCG1	VD+VL	0 (healthy	1	2	3	Gaps	≥2 + gaps	Healthy	With symptoms
1	0	0	0	100	0	0	0	0	0	-	-
2	103.2	0	2.0	99.7	0	0.3	0	2.0	2.3	0.00	41.12
3	125.3	0	0	99.7	0	0.3	0	1.3	1.6	0.00	2604.15
4	12.0	0	11.6	96.3	0	3.1	0.5	11.0	14.7	0.00	1577.75
5	252.2	0	17.8	95.7	0.2	3.4	0.7	4.7	8.8	0.00	6566.87
6	3.8	37.8	3.2	97.7	1.3	1.0	0	5.1	6.1	0.46	15.99
7	0.8	0	0	87.6	1.8	8.0	2.8	3.2	14.0	0.00	0.00
8	1.7	0	0	86.8	0.5	11.2	1.5	2.4	15.1	0.08	3.28
9	0	0	0	89.3	0.2	8.5	2.0	0.4	10.9	0.00	0.00
10	7.7	0	0	88.9	0.7	7.7	2.7	0.5	10.9	0.00	30.31
11	1.5	0	0	98.8	0.5	0.5	0.2	0.3	1.0	0.00	61.82
12	4.0	0	2.3	100	0	0	0	0	0	0.94	365.89
13	1.6	0	2.2	90.2	3.0	0.1	0.7	0.5	7.4	0.08	29.66
14	15.0	0	5.6	97.3	0.9	1.6	0.2	2.5	4.3	0.00	43.34
15	0	0	0	96.5	0.5	2.5	0.5	0	3.0	0.00	4.46
16	1.4	0	0	97.5	1.2	1.3	0.1	0.8	2.2	0.01	29.11
17	4.0	0	0	98.8	0.1	0.7	0.4	0.3	1.5	0.00	8.06
18	2.3	0	0	98.6	0.1	0.7	0.6	0.1	1.4	0.00	35.97

Table 10. (cont'd)

Site	qPCR assay (pg/g)			% plants with verticillium wilt symptoms by severity*						qPCR plant assay (pg/g)	
	Bilodeau	VCG1	VD+VL	0 (healthy)	1	2	3	Gaps	≥2 + gaps	Healthy	With symptoms
19	1.7	0	0	96.5	1.1	2.2	0.2	0.3	2.7	0.00	0.36
20	0.8	0	0	90.7	0.9	7.1	1.3	6.9	15.2	0.00	0.29
21	1.2	0	2.2	97.9	1.0	1.1	0	1.5	2.6	0.00	0.00
22	0	11.7	2.2	98.7	0.5	0.3	4.8	0	5.6	0.00	0.00
23	1.2	0	0	-	-	-	-	-	-	0.00	2.08[a]
24	766.9	0	7.8	-	-	-	-	-	-	0.00	0.00 [a]
25	0	0	0	98.3	0.2	0.8	0.7	0	1.5	0.00	0.00
26	10.3	0	9.1	97.3	0.8	0.9	1.0	0.7	2.6	0.00	0.00
27	0	0	0	-	-	-	-	-	-	-	-
28	5.8	9.6	2.4	99.2	0.2	0.4	0.2	0	0.6	-	-
29	15.4	0	10.7	97.0	0.2	1.5	1.3	0	2.8	10.16	542.60
30	6.0	0	0	99.2	0.4	0.4	0.1	0	0.4	0.00	0.19
31	6.6	0	1.8	95.7	0	4.3	0	0	4.3	27.47	117.80
32	0	0	0	100	0	0	0	0.2	0.2	0.00	No sympt.
33	5.7	9.7	0	99.2	0.6	0.1	0.1	0.3	0.5	0.00	107.65
34	4.0	0	2.0	98.2	1.7	0	0	2.8	2.8	0.01	55.20
35	0	0	0	99.3	0.7	0	0	0.9	0.9	0.00	0.00
36	0	0	0	99.6	0.4	0	0	4.3	4.3	0.00	0.00
37	9.0	0	2.0	100	0	0	0	0	0	-	-

Table 10. (cont'd)

Site	qPCR assay (pg/g)			% plants with verticillium wilt symptoms by severity*						qPCR plant assay (pg/g)	
	Bilodeau	VCG1	VD+VL	0 (healthy)	1	2	3	Gaps	≥2 + gaps	Healthy	With symptoms
38	0	0	0	98.1	0.5	0.6	0.8	0	1.4	-	-
39	4.0	760.4	0	98.4	0.7	0.9	0	10.8	11.7	0.03	7.79
40	7.0	0	1.5	96.1	1.0	2.8	0.2	13.9	16.8	4.71	0.88
41	271.1	0	7.1	99.6	0.3	0	0.2	17.2	17.3	0.00	-
42	893.7	56.1	101.2	99.7	0.3	0	<0.1	1.6	1.6	0.01	105.97
43	109.4	0	2.4	94.4	2.1	3.4	0.1	7.9	11.4	0.00	78.54
44	0	14.3	0	79.8	1.7	14.1	4.4	2.8	21.2	0.00	0.02
45	0	12.3	0	96.3	0.4	0.2	1.0	0	3.3	0.55	118.66
46	-	-	-	-	-	-	-	-	-	-	-
47	0	0	0	-	-	-	-	-	-	-	-
48	0	0	0	-	-	-	-	-	-	-	-
49	0	0	0	100	0	0	0	0	0	0.00	0.00
50	4.0	14.5	2.2	100	0	0	0	0	0	0.00	0.00
51	0.3	0	1.1	93.2	0.8	3.6	2.3	0.3	6.2	0.01	0.04
52	0.2	-	-	97.2	0.1	1.3	1.4	0.2	2.7	0.00	0.00
53	0	0	2.1	98.9	0.3	0.3	0.6	0.1	0.9	0.00	0.07
54	<0.1	0	0	95.0	0.5	2.3	2.1	0	4.4	0.01	0.01
55	0	0	8.4	97.2	0.2	1.6	1.0	0.	2.6	0.00	0.00
56	7.5	0	2.7	99.8	0	0	0.2	0.3	0.5	0.00	0.68

Table 10. (cont'd)

Site	qPCR assay (pg/g)			% plants with verticillium wilt symptoms by severity*						qPCR plant assay (pg/g)	
	Bilodeau	VCG1	VD+VL	0 (healthy)	1	2	3	Gaps	≥2 + gaps	Healthy	With symptoms
57	5122.4	0	0	97.3	1.1	1.0	0.6	0.8	2.5	0.00	0.00
58	0	0	0	96.4	1.3	1.1	1.2	0	2.2	0.35	0.00
59	3.7	0	0	92.1	0	7.1	0.8	0.4	8.2	0.00	0.00
60	0	0	0	95.9	0	4.1	0.1	0.1	4.2	0.00	39.95

* See methods section for severity scale

[a] Samples taken prior to assessment visit, at which time crops had been grubbed.

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 in soil*

The proportion of sites where soil tests for *V. dahliae* and field observations for verticillium wilt symptoms corresponded on a presence/absence basis (i.e. nil symptoms occurred where nil *V. dahliae* was detected in soil, and symptoms did occur where *V. dahliae* was detected in soil) was examined.

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 (Table 11). There was a low correspondence of soil test results with field observations for all three assays (50%, 47% and 42% for Bilodeau, VCG1 and VD+VL respectively). This low level of correspondence between soil test results and verticillium wilt symptom occurrence was not increased when the data set was restricted to just the cultivar Sonata (n = 12), a cultivar known to be very susceptible to verticillium wilt (Table 11a); the correspondence of soil test results with field observation for the three qPCR assays was 47%, 25% and 38% for Bilodeau, VCG1 and VD+VL respectively at cv. Sonata sites. Looking at the correspondence between soil test and plant tests agreement increases to 54%, 38% and 50% for Bilodeau, VCG1 and VD+VL respectively and 64% 47% and 48% for the Sonata sites.

Table 11. Detection of *V. dahliae* in UK field soils by four qPCR assays and occurrence of verticillium wilt symptoms in strawberry crops grown on these soils in 2013 and 2014 – all crops

qPCR assay	Number sites tested	Proportion (%) sites where field symptoms reflect soil results	Proportion (%) sites where plant PCR result reflects soil result	Occurrence of verticillium wilt symptoms					
				Soil test positive			Soil test negative		
				No. soils positive	No. crops with symptoms	No. crops nil symptoms	No. soils negative	No. crops with symptoms	No. crops nil symptoms
2013									
Bilodeau	41	30/41 (73%)	-	29	26	3	12	8	4
Fera-EF	41	12/41 (29%)	-	5	5	0	36	29	7
2014									
Bilodeau	54	37/54 (50%)	27/50 (54%)	36	32	4	18	13	5
VCG1	53	17/53 (32%)	19/50 (38%)	10	9	1	43	35	8
VD+VL	53	25/53 (47%)	25/50 (50%)	24	20	4	29	24	5

Table 11a. Detection of *V. dahliae* in UK field soils by four qPCR assays and occurrence of verticillium wilt symptoms in strawberry crops grown on these soils in 2013 and 2014 – Sonata crops only

qPCR assay	Number sites tested	Proportion (%) sites where field symptoms reflect soil results	Proportion (%) sites where plant PCR result reflects soil result	Occurrence of verticillium wilt symptoms					
				Soil test positive			Soil test negative		
				No. soils positive	No. crops with symptoms	No. crops nil symptoms	No. soils negative	No. crops with symptoms	No. crops nil symptoms
2013									
Bilodeau	4	1/4 (25%)	-	1	1	0	3	3	0
Fera-EF	4	1/4 (25%)	-	0	0	0	4	3	1
2014									
Bilodeau	17	8/17 (47%)	11/17 (64%)	8	7	1	9	8	1
VCG1	16	2/16 (25%)	8/17 (47%)	2	1	1	14	13	1
VD+VL	16	6/16 (38%)	10/17 (48%)	6	5	1	10	9	1

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

Results were examined to determine the mean incidence of verticillium wilt at sites when *V. dahliae* was detected in soil, and at sites where it was not detected. The mean incidence of verticillium wilt symptoms was similar in crops where *V. dahliae* was detected in the soil and in crops where the fungus was not detected in the soil was similar for all three qPCR assays (Table 12).

Table 12. Occurrence of *V. dahliae* in soil on a presence/absence basis as determined by three qPCR tests and associated incidence of verticillium wilt symptoms in strawberry crops – 2014

Detection of *V. dahliae* in soil by the test	Mean number of plants with verticillium wilt		
	Bilodeau	VCG1	VD+ VL
No	3.9 (n=18)	3.3 (n=43)	4.3 (n=29)
Yes	3.3 (n=36)	4.2 (n=10)	2.5 (n=24)

n – Number of crops in this category

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

When the soil test results were grouped into six categories according to soil infestation density of *V. dahliae* (not detected, <1, 1-5, 5.1-10, 10.1-100 and >100 pg *V. dahliae*/g soil) there was no evidence that the mean incidence of affected plants increased (or healthy plants declined) as the quantity of *V. dahliae* detected in the soil increased for any of the three qPCR assays used in 2014. This was true for both the full crops data set (n = 54, 53) and for the susceptible cultivar Sonata dataset (n = 12, 11) (Tables 13 and 13a).

Considering just plants with verticillium wilt severity index 2 (outer whorl of leaves collapsed) or index 3 (whole plant collapsed), there was no evidence that the mean proportion of plants in this category increased as the quantity of *V. dahliae* detected in the soil increased.

Assuming that all gaps were caused by plants lost due to verticillium wilt, there was some evidence that the mean number of gaps was relatively high (>5%) when *V. dahliae* levels in the soil were high (>5 pg/g). However this is based on a limited data set (Sites 6, 1 and 8 for the Bilodeau, VCG1 and VD+VL assays respectively) (Tables 13 and 13a).

When verticillium wilt severity index 2 + 3 combined with gaps, there was no evidence that the mean number of plants in this category increased with quantity of *V. dahliae* detected in the soil.

With plant PCR results there was a slight trend of an increasing proportion of the symptomatic plants showing a positive PCR result from soils with a higher level of *V. dahliae.*

Table 13. Detection of *V. dahliae* by three qPCR assays of soil samples and associated levels of verticillium wilt in strawberry crops grown on these sites – 2014 (all crops)

Quantity of *V. dahliae* in soil (pg/g)	Number of crops in this category	% plants with verticillium wilt by category							% healthy plants positive for Vd by PCR	% symptomatic plants positive for Vd by PCR
		Nil (healthy)	1	2	3	Gaps	2+3	2+3 + gaps		
Bilodeau assay										
Not detected	18	96.1	0.5	2.5	0.9	0.8	3.4	4.2	12.5	40
<1	5	92.7	0.9	4.5	2.0	2.1	6.5	8.6	40	60
1 - 5	14	96.5	0.8	2.3	0.3	1.8	2.7	4.5	46.7	80
5.1 - 10	7	98.5	0.3	1.1	0.1	2.1	1.2	3.3	40	100
10.1 - 100	4	97.0	0.5	1.8	0.8	3.6	2.5	6.1	25	75
>100	6	98.1	0.5	1.3	0.2	5.8	1.4	7.2	20	75
VCG1 assay										
Not detected	43	96.7	0.6	2.2	0.6	2.1	2.8	4.9	27.5	67.5
<1	0	-	-	-	-	-	-	-		
1 - 5	0	-	-	-	-	-	-	-		
5.1 - 10	3	95.8	0.5	2.7	1.0	0.3	3.7	4.0	0	100
10.1 - 100	6	95.4	0.7	3.0	1.0	2.4	4.0	6.4	50	66.6
>100	1	98.4	0.7	0.9	0	10.8	0.9	11.7	100	100
VD+ VL assay										
Not detected	29	95.7	0.6	2.9	0.8	1.3	3.7	5.0	21.4	64.2
<1	0	-	-	-	-	-	-	-		
1 - 5	16	97.5	0.7	1.5	0.3	2.5	1.8	4.3	50	78.5
5.1 - 10	4	97.9	0.6	1.0	0.6	5.1	1.6	6.7	0	20
10.1 - 100	3	96.3	0.1	2.7	0.8	5.2	3.5	8.7	33.3	100
>100	1	99.7	0.3	0	<0.1	1.6	<0.1	1.6	100	100

Table 13a. Detection of *V. dahliae* by three qPCR assays of soil samples and associated levels of verticillium wilt in strawberry crops grown on these sites – 2014 (cv. Sonata crops only)

Quantity of *V. dahliae* in soil (pg/g)	Number of crops in this category	% plants with verticillium wilt by category							% healthy plants positive for Vd by PCR	% symptomatic plants positive for Vd by PCR
		Nil (healthy)			3	Gaps	2+3	2+3 + gaps		
Bilodeau assay										
Not detected	6	97.6	0.5	1.3	0.6	0.2	1.9	2.1	11	33
<1	3	95.1	0.5	2.4	2.0	0.1	4.4	4.5	22	22
1 - 5	2	96.1	0	3.5	0.4	0.2	3.9	4.1	25	50
5.1 - 10	1	99.8	0	0	0.2	0.3	0.2	0.5	0	100
10.1 - 100	0	-	-	-	-	-	-	-	-	-
>100	0	-	-	-	-	-	-	-	-	-
VCG1 assay										
Not detected	10	96.6	0	2.1	0.9	0.2	3.0	3.2	25	43.7
<1	0	-	-	-	-	-	-	-	-	-
1 - 5	0	-	-	-	-	-	-	-	-	-
5.1 - 10	1	88.4	0.74	7.7	2.7	0.5	10.4	10.9	0	100
10.1 - 100	1	100	0	0	0	0	0	0	0	0
>100	0	-	-	-	-	-	-	-	-	-
VD+ VL assay										
Not detected	9	96.1	0.5	2.6	0.8	0.2	3.4	3.6	22	44
<1	0	-	-	-	-	-	-	-	-	-
1 - 5	5	98.0	0.3	1.0	0.8	0.2	1.8	2.0	40	80
5.1 - 10	1	97.2	0.2	1.6	1.0	0	2.6	2.6	0	0
10.1 - 100	0	-	-	-	-	-	-	-	-	-
>100	0	-	-	-	-	-	-	-	-	-

Examination of results by sites

In 2014 the Bilodeau, VCG1 and VD+VL assays detected verticillium in 36, 10 and 24 (66%, 19% and 45%) of soils respectively. The three assays all indicated verticillium was present in four samples (Sites 6, 28, 42 and 50) and all indicated verticillium was absent in 14 samples (Sites 1, 9, 15, 25, 27, 32, 35, 36, 38, 47, 48, 49, 58 and 60) (Table 14).

Table 14. Examination of verticillium wilt occurrence in 2014 at sites where verticillium was detected in soils by all three assays, or not detected in soils by any of three assays

Site	Level of verticillium in soil (0-5 index) by assay:			Incidence of wilt symptoms (% plants ≥ index 2)	*Verticillium* confirmed in suspect plants	Varietal susceptibility
	Bilo	VCG1	VL+VD			
Verticillium detected in soil by all assays						
6	2	4	2	6.1	✓	Mixed
28	3	3	2	0.6	NT	MR
42	5	4	5	1.6	✓	Mixed
50	2	4	2	0	X	S
Verticillium not detected in soil by any assay						
1	0	0	0	0	NT	Mixed
9	0	0	0	10.9	X	S
15	0	0	0	3.0	✓	MR
25	0	0	0	1.5	X	S
27	0	0	0	NA	NT	S
32	0	0	0	0.2	X	MR
35	0	0	0	0.9	X	GR
36	0	0	0	4.3	X	Mixed
38	0	0	0	1.4	NT	Mixed
47	0	0	0	NA	NT	S
48	0	0	0	NA	NT	S
49	0	0	0	0	X	S
58	0	0	0	2.2	✓	S
60	0	0	0	4.2	✓	S

Assuming the soil assay results correctly show that *Verticillium dahliae* (and/or *V. longisporum* for the VL+VD assay) are present or absent in soils as indicated, and given the relative susceptibilities of the varieties grown at the sites, the levels of verticillium wilt symptoms observed in 2014 are as expected for Sites 6, 28 and 42 (high infestation sites), and 1, 32, 35 and 49 (low infestation sites). However, the observed levels of verticillium wilt

were contrary to soil test results at Sites 50, 9, 15, 25, 32, 35, 36, 38, 49, 58 and 60. It is particularly difficult to explain the lack of symptoms in cv. Sonata at site 50; and the occurrence of symptoms at Sites 9 (10.9% of plants where no verticillium was detected in soil); 15, 25, 28 and 60 (verticillium wilt symptoms observed and *Verticillium* sp. confirmed in plants).

The Bilodeau test is known to have a high sensitivity for *V. dahliae*, but it is also known to cross-react with *V. longisporum*, This probably explains by the Bilodeau assay gave a positive result in 66% of soils tested in 2014, a detection rate much greater than the other two assays.

Given the Bilodeau soil test detects non-pathogens and consequently is prone to give false positive results, the correspondence of soil test results and field symptoms of verticillium wilt was re-examined using the two new assays developed by Fera and shown to have a greater specificity for *V. dahliae* alone (VCG1 test) or *V. longisporum* and *V. dahliae* (VD+VL test) (Table 15). There was no obvious association of verticillium wilt symptom incidence in the field with density of *Verticillium* spp. detected in the soil using either assay. For both assays, there was a large variation in verticillium wilt symptoms incidence at each soil detection level; and there was little evidence of a marked increase in verticillium wilt symptom incidence with increasing density of verticillium detected in the soil. *Verticillium* spp. was confirmed in >70% of plants showing symptoms of the disease irrespective of the level of *Verticillium* spp. detected in soils.

Table 15. Association of soil levels of *Verticillium* spp. determined by two molecular assays and incidence of verticillium wilt symptoms in strawberry crops – 2014

Soil test and level of detection (pg/g)	Number of sites in this category	Mean % verticillium wilt (index ≥2) symptoms (and range)	% sites where *Verticillium* spp. confirmed in wilting plants
VCG assay			
Not detected	48	4.8 (0-17.3)	78.3
<0.1	0	0	-
0 - 1-5	0	0	-
5.1 - 10	4	4.4 (0.5-10.9)	100
10.1 - 100	5	7.7 (1.6-21.2)	100
>100	1	11.7	100
VD+VL assay			
Not detected	33	5.2 (0-15.1)	72.7
<0.1	0	0	-
0.1 - 5	16	4.2 (0-16.8)	93.2
5.1 - 10	5	6.7 (2.5-17.5)	75.0
10.1 - 100	3	8.8 (2.8-14.7)	100
>100	1	1.6	100

Comparing the site results for the two assays, *Verticillium* spp. was detected by neither, one or both assays for 28, 25 and 5 soils (48%, 52% and 9%) respectively. The VD+VL assay gave a positive result with more soils (25 sites) than the VCG1 assay (10 sites). This is consistent with VD+VL detecting both *V. longisporum* and all *V. dahliae* isolates, and VCG1 detecting just one VCG of *V. dahliae*. Detail of the 10 sites testing positive by the VCG1 assay are given below (Table 16). This result suggests that more than one VCG of *V. dahliae* is associated with verticillium wilt in the UK.

Table 16. Detail of results for sites testing positive for *V. dahliae* by VCG1 soil assay and occurrence of verticillium wilt symptoms at these sites in 2014

Site	Density of *V. dahliae* in soil (pg/g))	Incidence of verticillium wilt symptoms (index ≥2)	Verticillium confirmed in suspect plants	Cultivar	Susceptibility to verticillium wilt
6	37.8	6.1	✓	Fenella/Symphony	Mixed
10	7.7	10.9	✓	Sonata	S
22	11.7	5.6	✓	Symphony	MR
28	9.6	0.6	NT	Mixed	MR
33	9.7	0.5	✓	Mixed	Mixed
39	760.4	11.7	✓	Mixed	Mixed
42	56.1	1.6	✓	Mixed	Mixed
44	14.3	21.2	✓	Elan	MS
45	12.3	3.3	✓	Vibrant	S
50	14.5	0	NT	Sonata	S

S – Susceptible; MS – moderately susceptible; MR – moderately resistant; NT not tested.

Relatively high levels of *V. dahliae* (>5 pg/g) were detected at all sites. *Verticillium* sp. was confirmed in plants at all sites where a test was done. As far as can be ascertained, the levels of verticillium wilt symptoms observed are as expected (e.g. high levels in the susceptible cv. Sonata at Site 2 and the moderately susceptible cv. Elan at Site 44), for all sites except for Site 50, where no verticillium wilt symptoms were observed in cv. Sonata despite detection of 14.5 pg/g *V. dahliae* in soil (equivalent to around 25 MS/g).

In order to further reduce confounding factors, data were examined for sites with the susceptible cv. Sonata only, using VCG1 soil assay (Table 17). There were 19 sites where Sonata was grown, with relevant complete test results for 17 sites.

Table 17. Detail of results for sites growing cv. Sonata and tested by the VD+VL assay for *V. dahliae*

Site	Date soil sampled	Density of *V. dahliae* in soil (VD+VL assay) (pg/g)	Incidence of verticillium wilt symptoms		Detection of *Verticillium* sp. in suspect plants in 2014	County	Soil type
			2013	2014			
9	16-4-13	ND	0.7	10.9	X	Kent	Clay loam
10	16-4-13	ND	0.4	10.9	✓	Kent	Clay loam
13	1-5-13	2.2	5.3	7.4	✓	Cambs	Silty loam
19	17-6-13	ND	8.9	2.7	✓	Oxon	Sandy loam
25	21-6-13	ND	0	1.5	✓	Staffs	Sandy loam
49	Autumn 2013	ND	NA	0	NT	Kincards	Sandy loam
50	Autumn 2013	2.2	NA	0	NT	Kincards	Sandy loam
51	8-5-14	1.1	-	6.2	✓	Kent	Silty clay loam
52	8-5-14	NT	-	2.7	X	Kent	Sandy loam
53	8-5-14	3.1	-	0.9	X	Kent	Sandy loam
54	8-5-14	ND	-	4.4	✓	Kent	Clay loam
55	8-5-14	8.4	-	2.6	X	Kent	Clay loam
56	1-8-14	2.7	-	0.5	✓	Cambs	Silty clay loam
57	1-8-14	ND	-	2.4	X	Herford	Clay sandy silt
58	1-8-14	ND	-	2.2	✓	Salop	Clay loam
59	1-7-14	ND	-	8.2	X	Surrey	Sandy loam
60	2-7-14	ND	-	4.2	✓	Oxon	Sandy loam

ND – Not detected; NT – not tested.

Although *V. dahliae* was detected in soil pre-planting at only six of the 17 sites, symptoms clearly consistent with verticillium wilt (index ≥2) were observed at four out of the five sites assessed in 2013 and in 15 of the 17 sites assessed in 2014. *Verticillium* sp. was confirmed in plants from nine of 15 sites sampled and tested.

Thus, the VD+VL soil test does **not** appear to be a good indicator of whether or not verticillium wilt symptoms will occur in a susceptible cultivar grown on the site from which the soil was sampled. Possible explanations for this are:

a) The VD+VL test is not sufficiently sensitive for *V. dahliae* detection in soils;

b) There was release of *V. dahliae* from crop debris into soils between soil sampling and planting (this seems unlikely);

c) Verticillium wilt symptoms were not due to *V. dahliae* (this seems very unlikely given the experience of the consultants conducting the field assessments; and the positive results of tests for *Verticillium* sp. on individual plants);

d) *V. dahliae* was introduced into the field with the plants at planting and infection did not originate from the field soil.

Explanations (a) and (d) are considered the most likely to be the main reasons for a lack of a clear association between levels of *V. dahliae* detected in a soil pre-planting and occurrence of verticillium wilt symptoms in a subsequent strawberry crop with the VD+VL soil assay.

Effect of various cropping factors on disease

The effect of various cropping factors on verticillium wilt was examined by regression analysis. The factors accounting for significant levels of the variation were soil sterilisation, tunnelling and soil type (Table 18). These accounted for 40.5% of the variance in results between sites. The mean effects of soil sterilisation, tunnelling and soil type are shown in Table 19. The lower incidence of verticillium wilt symptoms on sterilised sites (most sterilised by chloropicrin treatment) compared with non-sterilised sites, is probably as expected. The greater incidence in outdoor crops compared with tunnelled crops may reflect greater stress on plants from the weather. The greater incidence on loamy sand soils than on other soil types is consistent with known occurrence of verticillium wilt in strawberry.

The regression analysis indicated no significant effect on incidence of verticillium wilt from region, varietal susceptibility, growing system, irrigation or occurrence of linseed/potatoes in the cropping history.

Table 18. Stepwise analysis of variance examining the association of soil type, region and irrigation with incidence of verticillium wilt in strawberry – 2014

Factor	Levels	Df	F probability
Soil sterilisation	2	1	**<0.001**
Tunnelled	2	1	**0.002**
Soil type	4	3	**0.028**
Region	8	7	0.182
Wilt susceptibility	5	4	0.226
Raised beds	2	1	0.222
Residual	35	-	-
Total	52	-	-

Significant differences in bold.

Table 19. Association of soil sterilisation, tunnelling and soil type on incidence of verticillium wilt in strawberry – 2014

Mean % plants affected					
Soil sterilisation		Tunnelled		Soil type	
No	1.2 (0.80)	No	4.4 (0.59)	Clay loam	5.9 (1.0)
Yes	5.3 (0.64)	Yes	1.8 (0.85)	Loamy sand	7.1 (1.4)
				Sandy loam	2.5 (0.7)
				Silty loam	3.5 (1.4)

( ) – standard error

The effect of occurrence of potatoes and/or linseed in the cropping history, and varietal susceptibility was also examined.

Discussion

V. dahliae *soil infestation at monitored sites*

The sites chosen for soil sampling and assessment of verticillium wilt proved suitable for the project as *V. dahliae* was detected in soil by one or more of the qPCR assays, at 29/41 sites sampled in 2013 and at 45/54 sites in 2014. At all five sites where the Fera-EF assay detected *V. dahliae* in 2013, the Bilodeau assay result was also positive (Appendix 1). At three of these sites, high levels of *V. dahliae* (>100 pg/g) were recorded by both assays. At seven of the nine sites where the VCG1 assay detected *V. dahliae* in 2014, either the Bilodeau and/or the VD+VL assays were also positive. Thus, overall, the soil test results found *V. dahliae* present in soil at most of the sites where crops were monitored.

Effect of year on verticillium wilt symptoms

The mean incidence of verticillium wilt observed across all sites in 2014 (2.6% of plants) was less than that observed in 2013 (7.8% of plants). The occurrence of verticillium wilt at relatively high levels (>5% of plants) was also greater in 2013 (14/41 = 34.5% of crops) than in 2014 (7/54 sites = 12.9% of crops). Possibly the weather was more favourable to development of verticillium wilt symptoms in 2013 than in 2014. Both 2013 and 2014 were warmer than the 30 year average, however, in July and August the 2014 averages were lower than in 2013, and 2014 was also wetter during this period. This is the time when plants will be most stressed in peak harvest or just finishing therefore the warmer drier conditions in 2013 may have increased stress on plants and worsened the effects of the verticillium wilt symptoms.

Varietal susceptibility

As was expected with work in commercial crops, a range of strawberry cultivars were planted on the soil-sampled fields. Unfortunately this confounds efforts to draw relationships between soil infestation density and in-crop verticillium wilt incidence because cultivars are known to differ in their susceptibility to verticillium wilt. Nevertheless, symptoms of verticillium wilt were observed in all of the cultivars on at least one site where they were grown. These were: Amesti, Buddy, Camarillo, Diamond, Elan, Elegance, Fenella, Malwina, Sonata, Symphony and Vibrant. Infection was confirmed in samples of cvs. Diamond, Fenella, Sonata and Symphony. As paired results on soil infestation density determined by qPCR assays and level of verticillium wilt accumulate over the years, they will provide a data set for examination of the relationship between soil infestation density and level of wilt, and of the relative susceptibility to verticillium wilt of different varieties.

At 16 of the 41 sites examined in 2013, two or more varieties were planted. Consequently in 2014 further sites were identified for soil sampling and disease assessment, and at all sites the cultivar chosen for monitoring was cv. Sonata, an increasingly popular cultivar.

Soil results and crop observations in conflict

In 2013 there were 12 sites where neither the Fera EF nor the Bilodeau assay detected *V. dahliae* in the soil samples. At four of these sites no verticillium wilt symptoms were observed; at the other eight sites verticillium wilt was observed at levels of <1% (six sites), 8.8% (site 22) and 15.7% (site 44). In 2014, there were 15 sites where none of the three qPCR assays detected *V. dahliae* in soil samples, yet verticillium wilt symptoms occurred in crops at six of these sites, plant qPCR analysis only showed one positive result. There are several possible explanations for these discrepancies:

1) The plants were infected at planting;
2) The soil infestation density was very low and not detected by the assays;
3) The soil distribution of *V. dahliae* was very clustered and not detected by the sampling pattern;
4) Infection was caused by an isolate of *V. dahliae* (or *V. albo-atrum*) not detected by the assays;
5) Symptoms of verticillium wilt were caused by another pathogen or factor.

In 2014, samples of wilted plants were taken from most of the crops and tested for infection by qPCR to verify that verticillium wilt symptoms were being assessed accurately. Of 50 samples with presumptive symptoms of verticillium wilt tested in 2014, 34 of them (68 %) proved positive by laboratory tests. Interestingly, at 29 % of the sites, visibly healthy plants also tested positive for *Verticillium* spp. by the Bilodeau laboratory test but these often

showed very low or trace levels of the disease so this may not have had time to show symptoms at the time of sampling.

Choice of qPCR assay

In 2013 there was a positive association between detection of *V. dahliae* in soil and presence and absence of symptoms in the strawberry crops using the Bilodeau assay (73% of sites appeared correct) but not with the EF assay (29% of sites appeared correct). Assuming that all crops were correctly identified as affected by verticillium wilt or not, these results probably reflect the known greater sensitivity of the Bilodeau assay. However, assuming that many of the sites recorded with low levels of verticillium wilt symptoms were incorrectly identified (i.e. were actually free of the disease), the EF assay with its known greater specificity may be correct. A new assay was developed with both increased specificity to just *V. dahliae* and *V. longisporum* and with reasonable specificity and the soils were re-tested with this new assay. However, the correspondence of soil and crop results with this assay was only 47%.

Severity of wilt also showed a positive association between detection of *V. dahliae* in the soil, or not, and severity of verticillium wilt (mean % plants affected) using both the EF and the Bilodeau qPCR assays. Using the EF assay, the difference in mean % plants affected in negative and positive fields was approximately two-fold (7.0% versus 13.2%); using the Bilodeau assay it was approximately four-fold (2.4% versus 9.7%). However in 2014, the incidence of verticillium wilt symptoms in soils where verticillium was detected (3.1% of plants) and was not detected (3.9% of plants) were very similar.

The data set of *V. dahliae* positive infestation densities using the EF assay was too small (n = 5) to draw any conclusions on quantity of *V. dahliae* and % plants affected by verticillium wilt symptoms.

In 2013 the data set of *V. dahliae* positive infestation densities using the Bilodeau assay was larger (n = 29), but was spread very unevenly: 23 values were 0.1 - 16 pg/g, two values were 101 - 200 pg/g and four values were 200 - 893 pg/g. Accepting this limitation, there was evidence that the mean incidence of verticillium wilt symptoms increased progressively from 2.4 to 13.6% plants affected as infestation densities increased from not detected to >200 pg/g (Year 1 report, Table 17). A larger data set was obtained in 2014 for the Bilodeau assay (n = 36), and with a reasonable spread of infestation densities by category (5, 14, 7, 4, 6), but % infected plants varied little between the categories and with no consistent trend (this report, Table 13). Possibly a larger and more evenly distributed data set would allow firmer conclusions to be drawn with regard to soil infestation density and severity of verticillium wilt symptoms.

Conclusions

Year 1

1. Soil samples collected from 41 fields due to be planted with strawberries in 2013 were tested for *V. dahliae* by two established qPCR assays. The EF assay developed by Fera in SF 97 and the Californian Bilodeau assay detected *V. dahliae* in five and 29 samples respectively. The differing results are likely to be due to the greater specificity of the EF assay (fewer false positives) and the greater sensitivity of the Bilodeau assay (fewer false negatives).
2. Four new qPCR assays with putative sensitivity to *V. dahliae* were designed. One assay designed to the rDNA IGS region showed excellent specificity (no cross-reaction with *V. tricorpus, V. nigrescens, V. albo-atrum* or *Gliocladium roseum*) but sensitivity was only moderate.
3. In autumn 2013 symptoms of verticillium wilt were observed in strawberries at 34 out of the 41 sites soil sampled by the standard method; four sites could not be assessed as the crops had been grubbed. Levels of wilt were above 1% and 5% at 22 and 15 sites respectively. Wilt was confirmed in plants at four of six sites where the incidence was <1%. Assuming all field assessments of wilt symptoms were correct, correlation of verticillium wilt symptoms with presence/absence in soil by qPCR test result was greater with the Bilodeau assay (73%) (30/41 correct) than the EF assay (29%) (12/41 correct). It should be noted that soil quantification of *V. dahliae* broadly predicts potential risk of verticillium wilt in strawberry; the actual level of wilt that develops varies with cultivar, cropping practices and other factors.
4. Using the Bilodeau assay, there was limited evidence for a positive association between quantity of *V. dahliae* in the soil and % plants affected by verticillium wilt. The mean % plants affected increased from 2.4 to 13.6% as soil levels increased from none detected to >200 pg/g.

Year 2

5 Four new assays were designed. Three assays detected *V. dahliae* and *V. longisporum*. One assay appeared highly specific, only detecting a subgroup of *V. dahliae* isolates which were suspected to belong to VCG1.

6 Two new qPCR assays (VCG1 and VD+VL) developed in this project showed increased specificity and sensitivity for detection of *V. dahliae* in soil compared with the assay developed in SF 97 (Fera-EF assay).

7 The four qPCR assays used in this project (Fera-EF, Bilodeau, VCG1 and VD+VL) to test soils were able to detect *V. dahliae* approximately 5, 0.1, 1 and 1 microsclerotia of *V. dahliae*/g soil respectively.

8 Based on the results of 41 paired samples of soil and crop assessments in 2013, and 54 in 2014, we found no evidence that any of the four qPCR assays as used at present to detect and determine density of *V. dahliae* in soil is currently suitable to accurately assess risk of verticillium wilt in strawberry planted in the sampled soils.

9 In 2014, the incidence of verticillium wilt symptoms was relatively low at most of the 54 sites; it was less than 1%, 1-10% and >10% at 22, 29 and 3 sites respectively. The lack of frequent sites with a high incidence of verticillium wilt symptoms (>10%) may in part account for the failure to find a relationship between density of *V. dahliae* in the soil and verticillium wilt severity in crops.

10 The ddPCR method was shown to increase the sensitivity of assays by a factor of five and has high potential to be used in future soil quantification work.

11 Results indicate that verticillium wilt in strawberry in the UK is caused by more than one Vegetative Compatibility Groups (VCG) of V. dahliae

Technology transfer

Project progress meeting, Fera, 17 February 2014.

Project progress meeting, ADAS Boxworth, 27 October 2014

Project conference call, 16 January 2015

Woodhall J, Boonham N, Roberts H & O'Neill TM (2014). Genetic tests root out wilts. *HDC News* **204**, 26-27.

HDC Agronomists Meeting, EMR, 12 February 2015.

O'Neill TM, Roberts H, Woodhall J & Boonham N (2015). Strawberry verticillium wilt proves difficult to predict. *HDC News* (in preparation)

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Appendix 1. Summary of results – 2013 and 2014

Soil site	*V. dahliae* detected in soil (index 0-5)					Verticillium wilt symptoms present in field (% plants index ≥2)		*V. dahliae* confirmed in wilting plants		Cultivar	Wilt susceptibility
	2013		2014								
	EF	Bilo	Bilo	VCG1	VL+VD	2013	2014	2013	2014		
1	0	0	0	0	0	NA	0	-	NT	Mixed	Mixed
2	4	5	5	0	2	10.3	2.3	NT	✓	Cam	S
3	4	4	5	0	0	0.8	1.6	NT	✓	Fen	R
4	0	4	4	0	4	5.8	14.7	NT	✓	Ser	S
5	5	5	5	0	4	1.3	8.8	NT	✓	Mixed	Mixed
6	0	2	2	4	2	0.7	6.1	✓	✓	Mixed	Mixed
7	0	2	2	0	0	0.2	14.0	✓	✓	Mixed	Mixed
8	0	2	2	0	0	0.4	15.1	✓	✓	Dia	S
9	0	0	0	0	0	0.7	10.9	X	X	Son	S
10	0	0	0	3	0	0.4	10.9	✓	✓	Son	S
11	0	2	2	0	0	0.4	1.0	X	✓	Ame	S
12	0	2	2	0	2	NA	0	NT	✓	Sym	MR
13	0	2	2	0	2	5.3	7.4	NT	✓	Son	S
14	4	4	4	0	3	0	4.3	NT	✓	Fen	
15	0	1	0	0	0	4.1	3.0	X	✓	Ele	MR
16	0	2	2	0	0	0	2.2	NT	✓	Fen	MS
17	0	2	2	0	0	2.1	1.5	X	✓	Vib	S

Soil site	*V. dahliae* detected in soil (index 0-5)					Verticillium wilt symptoms present in field (% plants index ≥2)		*V. dahliae* confirmed in wilting plants		Cultivar	Wilt susceptibility
	2013		2014								
	EF	Bilo	Bilo	VCG1	VL+VD	2013	2014	2013	2014		
18	0	2	2	0	0	1.9	1.4	✓	✓	DD	S
19	0	2	2	0	0	8.9	2.7	NT	✓	Son	S
20	0	2	2	0	0	1.3	15.2	NT	X	Mixed	Mixed
21	0	2	2	0	2	2.6	2.6	NT	✓	Fen	MS
22	0	0	0	3	2	1.1	5.6	NT	✓	Sym	MR
23	0	2	2	0	0	<0.1	NA	NT	✓	Ela	MR
24	5	5	5	0	3	40.0	NA	NT	✓	Bud	S
25	0	0	0	0	0	0	1.5	NT	✓	Son	S
26	0	4	4	0	3	0	2.6	NT	✓	Fen	MS
27	0	0	0	0	0	0	NA	NT	NT	Ame	S
28	0	3	3	3	3	63.6	0.6	NT	NT	Mixed	MR
29	0	4	4	0	4	64.9	2.8	NT	✓	Mixed	Mixed
30	0	3	3	0	0	6.1	0.4	NT	✓	Mixed	Mixed
31	0	3	3	0	2	10.7	4.3	NT	✓	Mixed	MR
32	0	0	0	0	0	0	0.2	NT	NT	Mixed	MR
33	0	3	3	3	0	0.3	0.5	NT	✓	Mixed	Mixed
34	0	2	2	0	2	2.6	2.8	NT	✓	Mixed	Mixed
35	0	0	0	0	0	0	0.9	NT	X	Mixed	GR
36	0	0	0	0	0	<0.1	4.3	NT	X	Mixed	Mixed

Soil site	*V. dahliae* detected in soil (index 0-5)					Verticillium wilt symptoms present in field (% plants index ≥2)		*V. dahliae* confirmed in wilting plants		Cultivar	Wilt susceptibility
	2013		2014								
	EF	Bilo	Bilo	VCG1	VL+VD	2013	2014	2013	2014		
37	0	3	3	0	2	NA	0	NT	NT	Mixed	Mixed
38	0	0	0	0	0	NA	1.4	NT	NT	Mixed	Mixed
39	0	2	2	5	0	0	11.7	NT	✓	Mixed	Mixed
40	0	3	3	0	2	7.0	16.8	NT	✓	Sym	MR
41	5	5	5	0	3	1.0	17.3	NT	NT	Mal	GR
42	5	5	5	4	5	3.1	1.6	NT	✓	Mixed	Mixed
43	0	5	5	0	2	10.4	11.4	NT	✓	Sym	
44	0	0	0	4	0	15.7	21.2	NT	✓	Ela	MS
45	0	0	0	4	0	NA	3.3	NT	✓	Vib	S
46	NT	NT	NT	NT	NT	NA	NA	NA	NA	NA	NA
47	0	0	0	0	0	NA	NA	NT	NT	Son	S
48	0	0	0	0	0	NA	NA	NT	NT	Son	S
49	0	0	0	0	0	NA	0	N	NT	Son	S
50	0	2	2	4	2	NA	0	NT	NT	Son	S
51	-	-	2	0	2	-	6.2	-	✓	Son	S
52	-	-	2	NT	NT	-	2.7	-	X	Son	S
53	-	-	0	0	2	-	0.9	-	X	Son	S
54	-	-	1	0	0	-	4.4	-	✓	Son	S
55	-	-	0	0	3	-	2.6	-	X	Son	S

Soil site	*V. dahliae* detected in soil (index 0-5)					Verticillium wilt symptoms present in field (% plants index ≥2)		*V. dahliae* confirmed in wilting plants		Cultivar	Wilt susceptibility
	2013		2014								
	EF	Bilo	Bilo	VCG1	VL+VD	2013	2014	2013	2014		
56	-	-	3	0	2	-	0.5	-	✓	Son	S
57	-	-	5	0	0	-	2.4	-	X	Son	S
58	-	-	0	0	0	-	2.2	-	✓	Son	S
59	-	-	2	0	0	-	8.2	-	X	Son	S
60	-	-	0	0	0	-	4.2	-	✓	Son	S
No. soils/sample positive			39	10	25	32	54	5	39	-	-
% soils/samples positive			67	17	43	80	91	56	81	-	-

NT – not tested; NA – not assessed

Varieties: Ame – Amesti; Bud – Buddy, Cam – Camarillo; DD – Driscoll's Diamond; Dia – Diamond; Ela – Elan; Ele – Elegance; Fen – Fenella; Mal – Malwina; Ser – Serena; Son – Sonata; Sym – Symphony; Vib – Vibrant.