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Project leader:	Tim O'Neill, ADAS
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Location of project:	ADAS Boxworth, Fera and commercial sites in England
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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

- A new molecular assay for *V. dahliae* designed in this project shows excellent specificity and moderate sensitivity.

Background and expected deliverables

Soil-borne *Verticillium dahliae* is a serious threat to profitable production of soil grown strawberry, especially where suitable land with irrigation is in short supply and crops are grown on a tight rotation. The major main-season 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 taxonomy 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 expected deliverables from this project are:

- A molecular test to quantify *V. dahliae* infestation density in soil with a sensitivity equivalent to 0.1 microsclerotia per gram of soil following DNA extraction from a 50 g soil sample.

- A data set relating *V. dahliae* infestation density in soil determined by qPCR to occurrence of verticillium wilt in field crops of strawberry.

The specific objectives in Year 1 were:

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

Summary of the project and main conclusions

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

Sensitivity of test

Four 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 will aim to boost the sensitivity of this assay.

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

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

Ct = 40 denotes a negative result; a low Ct value denotes good sensitivity. Nt = not tested.

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 seven naturally infested soils. For most samples variance decreased considerably with four sub-samples 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 (0.3)	32.4 (1.0)	32.7 (1.0)	32.2 (1.4)	32.3 (1.3)
3a	30.5 (0.9)	30.7 (0.8)	30.7 (0.6)	30.9 (0.7)	31.1 (0.8)
14	34.5 (3.2)	35.7 (3.1)	35.6 (2.5)	35.6 (2.2)	35.6 (2.0)
24	31.3 (0.1)	31.3 (0.1)	30.5 (1.5)	30.4 (1.3)	30.5 (1.2)
2b	34.5 (3.2)	35.7 (3.1)	35.6 (2.5)	35.6 (2.2)	35.6 (2.0)
3b	31.3 (0.1)	31.3 (0.1)	30.5 (1.5)	30.4 (1.3)	30.5 (1.2)
43	31.5 (3.4)	32.5 (3.0)	33.1 (2.7)	33.2 (2.3)	33.1 (2.1)

*Standard deviation 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 45 fields due to be planted with strawberry; an additional four fields were each sampled by taking and individually testing 50 soil cores to gain information on distribution of the pathogen. The samples were supplied to Fera for determination of *V. dahliae* by qPCR. The samples comprised sites in England (42), Scotland (4) and Wales (3) and covered major soft-fruit production counties including the South east (19), East Anglia (6) and West midlands (9).

When crops with soil sampled by the standard method were examined in autumn 2013 after the end of fruiting, symptoms of verticillium wilt were observed at 34 sites out of 41 sites, with an incidence >5% at 15 sites; four of the sites could not be assessed due to grubbing of the crop. Laboratory tests confirmed *V. dahliae* in symptomatic plant samples taken from five out of nine sites showing a low incidence of verticillium wilt (<5%). A verticillium wilt incidence above 10% was recorded in crops of cvs Camarillo, Eilan, Sonata and Symphony.

Association of soil infestation with verticillium wilt symptoms

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 sampled by the standard 50-core bulk soil method and assessed for wilt, *V. dahliae* was detected in five and 29 soils by the Fera and Bilodeau tests respectively (Table 4). There was not a good correspondence of incidence or severity of Verticillium wilt symptoms with soil density of *V. dahliae* determined by either the Fera or Bilodeau molecular assays.

Of the 15 sites with obvious verticillium wilt (symptoms present in >5% of plants), *V. dahliae* was detected in soil from two and 13 of these sites by the Fera and Bilodeau 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, occurrence of verticillium wilt symptoms will be further examined in the 41 crops assessed in 2013. Additional crops planted in 2014 will also be soil sampled and assessed to increase the data set on soil levels of *V. dahliae* and associated levels of verticillium wilt.

The soils from all 41 sites assessed in 2013 and additional sites sampled in 2014 will be tested for *V. dahliae* infestation density using one of the new qPCR assays developed in this project.

Table 4. Occurrence of verticillium wilt symptoms in strawberry crops in 2013 and associated levels of *V. dahliae* in soil pre-planting as determined by qPCR tests

Incidence of Verticillium wilt symptoms (% plants)	No. crops in this category	Number of sites where *Verticillium* was detected in soil	
		EF primers	Bilodeau primers
0	7	0	3
0.1 – 1	12	0	6
1.1 – 5	7	3	7
5.1 – 10	7	0	6
>10	8	2	7
Total	41	5	29

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 was possible for three of the four sites and this analysis will inform the development of a sampling strategy.

Main conclusions

- In 2013, using a molecular assay developed in California, the presence or absence of verticillium wilt symptoms in strawberry was correctly predicted by pre-plant soil test results for *Verticillium dahliae* at 29 out of 41 sites.

- The level of verticillium wilt symptoms was greater in crops grown in soils where *V. dahliae* was detected (8.8% of plants) than in soils where it was not detected (2.2% of plants).
- Some sites where no *V. dahliae* was detected had symptoms of verticillium wilt and *vice-versa*; such results are not unexpected given the differences between sites in variety and cropping factors, potential errors associated with assessing wilt symptoms and taking representative soil samples, and specificity of the molecular assay.
- A new molecular assay for *V. dahliae* designed in this project shows excellent specificity and moderate sensitivity. Soils from around 50 sites will be tested using this assay in 2014 to determine if it more accurately predicts verticillium wilt than either the Californian assay or the EF assay developed in SF 97.

Financial benefits

Verticillium wilt, caused primarily by *V. dahliae*, is one of the most serious diseases of field grown 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

- There are no action points at present.

SCIENCE SECTION

Introduction

Background

Verticillium wilt is one of the most serious diseases of strawberries and is capable of causing significant yield losses. Many cultivars 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 growers' 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* species and *Gliocladium* (SF 97). This would prove highly problematic for a soil test since it could lead to a large number of false negatives. 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, a small 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 *V. longisporum*, 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.

The assay we 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 cultivars (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 they recommended four separate sub-samples to be taken (Bilodeau *et al*., 2012). Work is needed to optimize 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 enhancements to DNA extraction methodology from soil.

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 test for *V. dahliae*
2. To validate the test by investigation of the relationship between soil infestation density of *V. dahliae* measured by the improved test and verticillium wilt symptoms in commercial strawberry crops.

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

Materials and methods

Assay design

DNA sequences for *V. dahliae* and related species were obtained from GenBank. Alignment of the resulting sequences allowed potential primer/probe sites to be identified and putative species specific primer and probe sequences were determined using Primer Express 2.0 (Life Technologies, Warrington, UK). All primers and probes were synthesized by MWG Biotech except locked nucleic acid (LNA) primers (Eurogentec Ltd, Southampton, UK) and Minor GB probes (Life Technologies). The forward and reverse primers from the single copy elongation factor assay were FLAPS modified as described in Afonina *et al*.,

2007. This was by adding 6-mer or 12-mer non-complimentary of adenine (A) or thymine (T) bases to the 5' end of each primer.

Isolates

A range of *Verticillium* spp. isolates and a related genus (*Gliocladium*) were obtained to test each assay. These were representative of most of the species likely to cause an assay to cross-react; tests for cross-reaction with isolates of *V. longisporum* are planned for Year 2. Isolate details are given in Table 5. Cultures were grown on potato dextrose for 10-14 days and DNA was extracted using a 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. DNA was stored at -35°C until required.

Table 5. Isolates used to determine assay specificity

Species	Abbreviation	Isolate name	Original host	Geographical origin
Verticillium dahliae	Vd1	V97	Olive	Southern Europe
Verticillium dahliae	Vd2	1809	Not known	UK
Verticillium tricorpus	Vt	830	Not known	UK
Verticillium nigrescens	Vn	1506	Not known	UK
Verticillium albo-atrum	Vaa	341	Hop	UK
Verticillium longisporum	Vl	Vl145	Oilseed rape	UK
Gliocladium roseum	Gr	203	Compost	UK

DNA extraction from soil and real-time PCR

Environmental Master Mix 2.0 (Life Technologies) was used for all real-time PCR and consisted of half the total reaction volume of 25 µl, and 2 µl of DNA from fungal culture. Primers and probes were added to a final concentration in the reaction of 300 nM and 100 nM respectively with the remaining volume made up with molecular grade water. Cycling conditions typically consisted of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. However, in some assays tested the 60°C step was adjusted to 62 or 64°C for just 35 s to increase assay specificity (see results). The cycle threshold (Ct) value for each reaction was assessed using the Sequence Detection Software's default threshold setting of 0.2 ΔRn units. For specificity testing each sample was tested in two replicates and an average Ct value was determined. A low Ct value indicates high sensitivity; a Ct value of 40 denotes a negative result.

Tests on multiple 50 g sub-samples

2 kg soil samples were taken at seven sampling locations where *V. dahliae* was known to be present at five different sites. The 1 kg was homogenised and DNA was extracted from six 50 g subsamples. Real-time PCR was undertaken in triplicate using the Bilodeau assay under standard Fera conditions.

Results

Assay specificity

Details of all primer/probe sets used in this study are shown in Table 6. All assays were tested against the bank of DNA from representatives of species likely to cross-react with assays. Under Fera standard conditions, all assays cross-reacted with the closely related species (Table 7). The EF assay showed the highest specificity yet cross-reaction occurred above a Ct of 33 for all isolates. Whilst it was surprising this cross-reaction occurred, DNA levels of the cultures used in the reaction were likely to be at higher levels than may have been used before (between 25-35 ng). However, sensitivity was 10 times lower than some of the assays designed to multi-copy genes (e.g. Bilodeau assay).

Increasing the annealing temperature to 64°C with the multi-copy IGS v2 assay, which appeared most specific in earlier tests (data not shown), indicated that it was possible for this assay to become entirely specific but at a cost of approximately 100 fold sensitivity, based on difference in Ct values (a difference of 3 Ct indicates a ten-fold difference in the concentration of DNA detected). The rDNA ITS assay used, despite being multi-copy, had low sensitivity and only detected one of the two isolates of *Verticillium dahliae*. This is because the primary aim of this assay was for specificity rather than sensitivity. Due to polymorphism between some isolates of *V. dahliae* in this DNA region, it would appear that this assay cannot detect all *V. dahliae* isolates under these conditions.

Table 6. Details and source of real-time PCR assays evaluated in the study

Assay name	Loci	Multiple/Single copy	Source
Current assays			
EF	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

Table 7. Specificity testing of *Verticillium dahliae* assays: Ct for each isolate tested

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

Ct = 40 denotes a negative result. Nt = not tested.

Assessing the ability of FLAPs and TINA primers to enhance assay sensitivity

FLAPs primers were designed for the EF assay. These appear to make little or no difference to Ct or ΔRn values although they did influence the shape of the reaction curves (Figure 1) when tested with DNA from two isolates from pure culture. Consistent results were obtained for both isolates with all sets of primers. Related species (Table 4) were also tested and no difference was observed in terms of cross reaction.

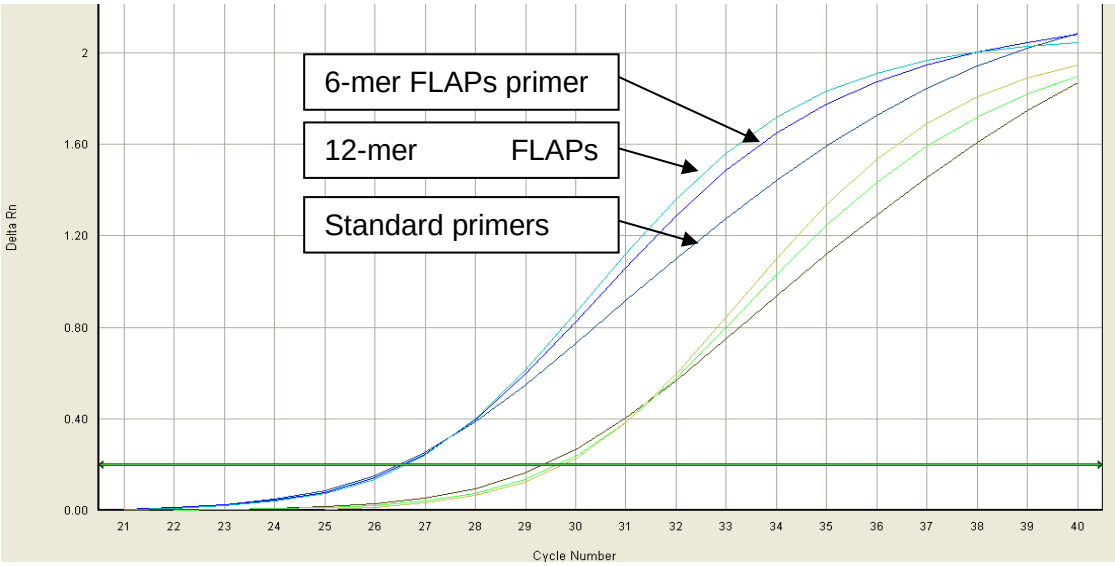

Figure 1. Effect of 6-mer and 12-mer FLAPs on reaction progress for two *Verticillium* isolates at different DNA concentrations.

Effect of TINA primers

No different in Ct was observed with TINA primers and no change in cross-reactivity between related species was observed.

Tests on multiple 50 g sub-samples

Results are presented below for the replicated soil DNA extractions from each of seven locations.

Table 8. 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 (0.3)	32.4 (1.0)	32.7 (1.0)	32.2 (1.4)	32.3 (1.3)
3a	30.5 (0.9)	30.7 (0.8)	30.7 (0.6)	30.9 (0.7)	31.1 (0.8)
14	34.5 (3.2)	35.7 (3.1)	35.6 (2.5)	35.6 (2.2)	35.6 (2.0)
24	31.3 (0.1)	31.3 (0.1)	30.5 (1.5)	30.4 (1.3)	30.5 (1.2)
2b	34.5 (3.2)	35.7 (3.1)	35.6 (2.5)	35.6 (2.2)	35.6 (2.0)
3b	31.3 (0.1)	31.3 (0.1)	30.5 (1.5)	30.4 (1.3)	30.5 (1.2)
43	31.5 (3.4)	32.5 (3.0)	33.1 (2.7)	33.2 (2.3)	33.1 (2.1)

*Standard deviation given in parenthesis.

Greater variation is observed in this dataset compared to a comparable experiment described in Bilodeau *et al.* (2012) where the largest value for standard deviation observed

was 0.7 in Ct compared to 3.2 observed here. This could be due to different soil extraction methodology used and/or differences in soil type. The soils processed by Fera in this experiment had a high clay content, which may have prevented complete homogenisation. Bilodeau et al. recommended four replicate DNA extractions. Despite the higher variability observed with UK soils, it would appear that four replicates are again advisable as variability decreased considerably from three to four replicates in four of the seven samples tested. The greater the number of sub-samples tested, the closer will be the value to the true mean. However, cost constraints will in practice limit the number that can be tested.

Discussion

A range of DNA loci in alternative conditions were tested. No assay was completely specific for *V. dahlia*; the EF assay appeared to be the most specific but was not as sensitive as multi-copy assays. Manipulation of the annealing temperature for one multi-copy assay (IGS v2) meant that complete specificity could be obtained, despite using high DNA concentrations in the DNA from related isolates. This result is encouraging; it shows that specificity for assays within the IGS region is feasible. Furthermore, this assay did not contain locked nucleic acid (LNA) primers or an MGB probe. Both of these can be used to enhance specificity of a reaction with little or no reduction in sensitivity. This will be tried in further work in the remaining part of the project. This assay could also be used in conjunction with a nested approach to increase specificity and/or sensitivity.

Also, realistic field concentrations of cross-reacting DNA will be determined in the remaining part of the project. In the experiments described above, relatively high DNA concentrations were used as the DNA originated from fungal cultures. These levels would not likely be present within plant or soil material, meaning cross reaction is less likely to occur when non-culture DNA is used. To determine what levels of DNA are likely to be present in the soil, soil will be spiked with realistic field levels of *V. albo-atrum* (e.g. 50 ms/g) to determine if this can cause cross reaction when tested with the new molecular test.

The FLAPS and TINA primers appeared to make little or no difference on cross-reactivity with related species or sensitivity when tested on pure culture DNA. It could be that in the presence of PCR inhibitors these differences could be more profound. Future work should test if sensitivity with FLAPS or TINA primers is better in the presence of inhibitors such as humic acid, which is commonly found in soil.

Summary of future work

- Using LNA and MGB primers; create a more specific, yet sensitive version of the IGS v2 primer/probe set.

- Develop a nested assay from the rDNA IGS region
- From experiments with *V. albo-atrum* spiked soils, determine if soil DNA concentrations of soil infested with *V. albo-atrum* are likely to cause cross reaction.
- Test TINA and FLAPS primers in the presence of a PCR inhibitor.
- Check for cross-reaction with *V. longisporum* isolates.

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 will be 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. 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 are 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 variety only, it was considered as one unit; if not, then separate samples were taken. Samples were sent to Fera as soon as possible after sampling or else kept in a cool dark place until dispatch.

At four of the sites (sites 3, 13, 14 and 15) the 50 sub-samples were taken in a grid pattern and GPS located and the soil samples individually bagged (rather than bulked) and analysed separately to examine how *Verticillium dahliae* is distributed to aid sampling methodologies.

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 flat-bed 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. 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 meter 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 1,000 plants for symptoms of verticillium wilt at each site.

At the four sites where individual soil cores were GPS located, all the plants in a 2 m radius around the centre of the GPS coordinate were assessed, recording the total number of plants, the number of plants showing wilt symptoms and scoring severity to the same 1 to 3 scale.

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). At nine of the sites where there was a low incidence of wilt symptoms (usually <1%), and symptoms were not fully typical of the disease, plant samples were collected and sent to Fera to test of *V. dahliae*.

Results

Details of sites

A total of 45 soils were sampled between March and August 2013, 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. Table 10 details the percent of plants showing symptoms at the autumn 2013 assessment and the results of the PCR assays. 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 four sites due to damage by herbicide or early grubbing of the crop.

Table 9. Details of sites soil sampled for *Verticillium dahliae* in 2013

Ref no.	County	Soil type	Cultivar	Cropping history	Date sampled
SS 1	Cheshire	Sand silt loam	Symphony, Eros, Malwina	Soft fruit	20/03/2013
SS 2	Essex	Sandy loam	Camarillo	Top and soft fruit; potatoes and OSR in rotation history	10/04/2013
SS 3	Essex	Sandy loam	Fenella	Top and soft fruit; potatoes and OSR in rotation history	10/04/2013
SS 4	Essex	Clay loam	Serena	Soft fruit	10/04/2013
SS 5	Essex	Clay loam	Serena and Finesse	Soft fruit	10/04/2013
SS 6	Surrey	Loamy sand	Fenella, Symphony	Soft fruit potatoes in history	16/04/2013
SS 7	Kent	Brick earth	Trial site	Soft fruit, OSR	16/04/2013
SS 8	Kent	Brick earth	Diamond	Soft fruit, OSR	16/04/2013
SS 9	Kent	Brick earth	Sonata	Soft fruit, OSR	16/04/2013
SS 10	Kent	Brick earth	Sonata	Soft fruit, OSR	16/04/2013
SS 11	Kent	Brick earth	Amesti	Soft fruit, OSR	16/04/2013
SS 12	Cheshire	Sandy loam	Symphony	Soft fruit, rhubarb	14/04/2013
SS 13	Cambs	Silty clay loam	Sonata	Top and soft fruit potatoes and OSR in rotation history	01/05/2013
SS 14	Cambs	Silty clay loam	Fenella	Top and soft fruit potatoes and OSR in rotation history	01/05/2013
SS 15	Kent	Silty clay loam	Elegance	Soft fruit	10/05/2013
SS 16	Kent	Silty clay loam	Fenella	Soft fruit	10/05/2013
SS 17	Kent	Sandy loam	Vibrant	Soft fruit, OSR	10/05/2013
SS 18	Kent	Sandy loam	Driscolls Diamond	Soft fruit	10/05/2013
SS 19	Oxon	Sandy loam	Sonata	Soft fruit	17/06/2013
SS 20	Oxon	Sandy loam	Florence, Fenella, Symphony	Soft fruit	17/06/2013

Ref no.	County	Soil type	Cultivar	Cropping history	Date sampled
SS 21	Oxon	Sandy loam	Fenella	Soft fruit, potatoes in history	17/06/2013
SS 22	Oxon	Sandy loam	Symphony	Potatoes in history	17/06/2013
SS 23	Staffs	Loamy sand	Elegance	Soft fruit	21/06/2013
SS 24	Staffs	Loamy sand	Buddy	Soft fruit, melons	21/06/2013
SS 25	Staffs	Sandy loam	Sonata B	Soft fruit	21/06/2013
SS 26	Staffs	Loamy sand	Fenella	Soft fruit	21/06/2013
SS 27	Staffs	Loamy sand	Amesti	Soft fruit	21/06/2013
SS 28	Swansea	Sandy clay loam	Malwina, Judi Bell, Symphony, Cupid, Rumba	Soft fruit, potatoes in history	26/06/2013
SS 29	Merseyside	Sandy clay loam	Eros, Florence, Judi Bell, Symphony	Soft fruit, potatoes in history	26/06/2013
SS 30	Wrexham	Sandy clay loam	Christine, Eros, Darselect, Amelia, Pegasus	Soft fruit, potatoes in history	26/06/2013
SS 31	Bucks	Sandy loam	Malwina, Symphony	Soft fruit, potatoes in history	16/07/2013
SS 32	Cornwall	Sandy clay loam	Symphony, Malwina	Strawberries in arable in rotation	10/07/2013
SS 33	Warwicks	Sandy loam	Vibrant, Fenella, Florence, Symphony, Malwina	Raspberries	02/05/2013
SS 34	Warwicks	Clay loam	Vibrant, Eros Symphony, Malwina	Strawberries	18/04/2013
SS 35	Powys	Silty loam	Fenella, Malwina	Raspberries	10/06/2013
SS 36	Devon	Sandy lLoam	Symphony, Elsanta, Malwina	Raspberries	15/05/2013
SS 37	Cheshire	Sand silt loam	Symphony, Eros, Malwina	Strawberries	26/06/2013
SS 38	Leics	Clay loam	Korona Marshmellow Elegance	Strawberries	30/06/2013
SS 39	West midlands	Sandy loam	Malwina, Symphony, Florence, Cupid (trial), Alice, Korona	Strawberries	30/06/2013

Ref no.	County	Soil type	Cultivar	Cropping history	Date sampled
SS 40	Bucks	Sandy loam	Symphony	Soft fruit	11/07/2013
SS 41	Bucks	Clay loam	Malwina	Soft fruit	11/07/2013
SS 42	Cornwall	Sandy silty loam	Vibrant Symphony Florence Malwina	Strawberries	17/07/2013
SS 43	Berks	Loamy sand	Symphony	Strawberries	08/08/2013
SS 44	Berks	Loamy sand	Eilan	Strawberries	08/08/2013
SS 45	North Yorks	Loamy sand	Vibrant	Linseed, potatoes in history	
SS 47	Kincardineshire	Sandy loam	Sonata	Arable rotation including OSR, potatoes in history	Autumn 2012 by BG
SS 48	Kincardineshire	Sandy loam	Sonata	Arable rotation including OSR, potatoes in history	Autumn 2012 by BG
SS 49	Kincardineshire	Sandy loam	Sonata	Arable rotation including OSR, potatoes in history	Autumn 2012 by BG
SS 50	Kincardineshire	Sandy loam	Sonata	Arable rotation including OSR, potatoes in history	Autumn 2012 by BG

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

Table 10. Results of the Fera *Verticillium dahliae* EF assay developed in SF 97, the published Bilodeau assay, autumn in-field verticillium wilt assessment and confirmation of *V. dahliae* in plants sampled – 2013

Ref no.	EF assay (pg/g)	Bilodeau IGS assay (pg/g)	% plants showing symptoms	% plants with symptom severity			Plants with *V. dahliae* confirmed by laboratory tests
				1	2	3	
SS 1	0.00	0.00	0.00	*	*	*	-
SS 2	51.00	103.20	10.64	0.27	8.27	2.11	-
SS 3 [a]	23.81	24.50	0.93	0.10	0.81	0.02	-
SS 4	0.00	12.00	7.53	1.73	4.72	1.08	-
SS 5	229.20	252.20	1.80	0.50	1.10	0.20	-
SS 6	0.00	3.81	0.66	0.00	0.53	0.18	Y
SS 7	0.00	0.81	0.26	0.06	0.17	0.03	Y
SS 8	0.00	1.66	0.58	0.15	0.24	0.18	Y
SS 9	0.00	0.00	0.87	0.17	0.33	0.37	N
SS 10	0.00	0.00	0.60	0.14	0.22	0.17	Y
SS 11	0.00	1.53	0.52	0.15	0.15	0.21	N

Ref no.	EF assay (pg/g)	Bilodeau IGS assay (pg/g)	% plants showing symptoms	% plants with symptom severity			Plants with *V. dahliae* confirmed by laboratory tests
				1	2	3	
SS 12	0.00	4.00	0.00	*	*	*	-
SS 13 [a]	0.00	1.50	52.94	0.34	3.12	2.14	-
SS 14 [a]	11.92	7.80	0.02	0.02	0.00	0.00	-
SS 15 [a]	0.00	0.10	4.19	0.23	2.29	1.78	N
SS 16	0.00	1.36	0.00	0.00	0.00	0.00	-
SS 17	0.00	4.00	2.52	0.45	1.52	0.55	N
SS 18	0.00	2.29	2.22	0.29	1.59	0.33	Y
SS 19	0.00	1.72	16.83	7.92	5.00	3.92	-
SS 20	0.00	0.77	6.20	5.70	1.23	0.07	-
SS 21	0.00	1.15	9.23	6.62	2.62	0.00	-
SS 22	0.00	0.00	8.80	7.73	1.07	0.00	-
SS 23	0.00	1.24	0.12	0.08	0.04	0.00	-
SS 24	334.90	766.87	40.0	0.00	20.00	20.00	-
SS 25	0.00	0.00	0.02	0.02	0.00	0.00	-
SS 26	0.00	10.28	0.24	0.24	0.00	0.00	-
SS 27	0.00	0.00	0.02	0.02	0.00	0.00	-

Ref no.	EF assay (pg/g)	Bilodeau IGS assay (pg/g)	% plants showing symptoms	% plants with symptom severity			Plants with *V. dahliae* confirmed by laboratory tests
				1	2	3	
SS 28	0.00	5.78	66.17	2.61	37.81	25.75	-
SS 29	0.00	15.41	66.67	1.82	45.05	19.80	-
SS 30	0.00	6.01	11.14	5.04	5.84	0.27	-
SS 31	0.00	6.59	6.00	1.63	9.85	0.83	-
SS 32	0.00	0.00	0.10	0.10	0.00	0.00	-
SS 33	0.00	5.65	4.06	3.79	0.27	0.00	-
SS 34	0.00	4.00	5.18	2.59	2.59	0.00	-
SS 35	0.00	0.00	0.00	0.00	0.00	0.00	-
SS 36	0.00	0.00	0.63	0.59	0.04	0.00	-
SS 37	0.00	9.00	0.00	*	*	*	-
SS 38	0.00	0.00	Crop grubbed - herbicide damage - replanted Sept	*	*	*	-
SS 39	0.00	3.96	2.65	2.65	0.00	0.00	-
SS 40	0.00	7.01	7.67	0.67	6.92	0.08	-
SS 41	209.18	271.13	4.44	3.41	1.04	0.00	-
SS 42	653.38	893.73	3.39	0.33	3.06	0.00	-

Ref no.	EF assay (pg/g)	Bilodeau IGS assay (pg/g)	% plants showing symptoms	% plants with symptom severity			Plants with *V. dahliae* confirmed by laboratory tests
				1	2	3	
SS 43	0.00	109.40	10.43	0.00	9.60	0.83	-
SS 44	0.00	0.00	15.68	0.00	14.68	1.00	-
SS 45	0.00	0.00	herbicide damage – gapped up	*	*	*	-
SS 47	0.00	0.00	0.00	*	*	*	-
SS 48	0.00	0.00	0.00	*	*	*	-
SS 49	0.00	0.00	pulled out before assessment - replanted Sept	*	*	*	-
SS 50	0.00	4.00	pulled out before assessment - replanted Sept	*	*	*	-

[a] Soils sampled as 50 individual cores for the mapping investigation, no bulk sample was analysed. The figure reported is the average of the 50 cores; these four sites will be re-sampled in 2014.

Detection and incidence of verticillium wilt

Verticillium wilt symptoms were observed in 38 of the 45 crops examined, at levels ranging from 0.1 to 67% of plants. Examination of data for association of soil test results with verticillium wilt symptoms was confined to the 41 crops where soil was sampled and tested by the same method (50 cores bulked to from one sample). Results for the four sites where 50 individual cores were tested are discussed separately.

Data were examined initially to see how the presence or absence of *V. dahliae* in the soil related to occurrence of verticillium wilt symptoms in the crop. Assuming symptoms were correctly identified at all sites, the SF 97 EF assay gave no false positives and 29 false negatives (Table 11). No *V. dahliae* was detected in soils where the crops showed ≤1% of verticillium wilt.

Table 11. Association of incidence of verticillium wilt symptoms (autumn 2013) with quantity of *V. dahliae* in soil determined by SF 97 EF qPCR assay in 41 strawberry crops

Verticillium wilt incidence (% plants)	No. crops in this category	No. fields with *V. dahliae* detected in soil		% fields with *V. dahliae* detected
		ND	Present	
0	7	7	0	0
0.1 – 1	12	12	0	0
1.1 – 5	7	4	3	43
5.1 – 10	7	7	0	0
>10	8	6	2	25

ND – Not Detected

Relating the presence or absence of verticillium wilt symptoms to detection of *V. dahliae* in soil, the Bilodeau assay gave three false positive and eight false negatives (Table 12). The association of *V. dahliae* in soil with verticillium wilt symptoms in the crop increased from 50% of sites where wilt incidence was 0.1-1% to over 86% of sites where wilt incidence was >1%.

Table 12. Association of incidence of verticillium wilt symptoms (autumn 2013) with detection of *V. dahliae* in soil by Bilodeau qPCR assay in 41 strawberry crops

Verticillium wilt incidence (% plants)	No. crops in this category	No. of fields with *V. dahliae* detected in soil:		% fields with *V. dahliae* detected
		ND	Present	
0	7	4	3	43
0.1 – 1	12	6	6	50
1.1 – 5	7	0	7	100
5.1 – 10	7	1	6	86
>10	8	1	7	88

ND – Not Detected

Detection and severity of verticillium wilt

Data were then examined for association of verticillium wilt severity (mean % plants affected) with detection of *V. dahliae* in the soil. There was some evidence of a link between soil test results and the mean % plants showing symptoms. There were five sites where *V. dahliae* was detected in soil by the EF assay and they were associated with a higher mean level of % plants showing symptoms (Table 13) than the 36 sites when *V. dahliae* in the soil as determined by qPCR with the EF assay.

Table 13. Mean % strawberry plants with symptoms of verticillium wilt in 2013 grouped according to presence or absence of *V. dahliae* in the soil as determined by qPCR with EF primer

Detection of *V. dahliae* by EF primer	Number sites assessed	Mean % plants with Verticillium wilt	Range
0	36	7.04	0.12 – 66.67
1	5	13.20	3.39 – 40.00

Sites 28 and 29 were outliers in the data set and the regression was therefore re-run with these sites omitted. The means below show a larger difference between the % plants with symptoms whether the EF qPCR assay detected *V dahliae* in soil or not (Table 14).

Table 14. Detection of *V. dahliae* in soils with the EF assay and associated levels of verticillium wilt in 2013 (restricted data set)

Detection of *V. dahliae* with the EF assay	No. sites assessed	Mean % plants wilted
0	34	3.55
1	5	13.20

Association of Bilodeau primer soil test results with wilt symptoms

The results with the Bilodeau assay were an improvement on the EF assay as the test identified the two high disease level sites, Site 28 and 29. There were 30 sites where *V. dahliae* was detected in soil by the Bilodeau assay and they were associated with a higher mean level of % plants showing symptoms (Table 15).

Table 15. Detection of *V. dahliae* in soils with the Bilodeau assay and associated levels of strawberry verticillium wilt – 2013

Detection of *V. dahliae* with the Bilodeau assay	Number sites assessed	Mean % plants wilted	Range
0.0	11	2.42	0 – 15.68
1.0	30	9.67	0 – 66.67

If the two high disease level sites are removed from the data set there was very little difference between the % plants with symptoms according to detection of *V. dahliae* in soils by the Bilodeau assay (Table 16).

Table 16. Detection of *V. dahliae* in soils by the Bilodeau assay and associated levels of strawberry verticillium wilt (restricted dataset) – 2013

Detection of *V. dahliae* by Bilodeau primer	Number sites assessed	Mean % plants wilted
0.0	11	2.42
1.0	28	5.62

Soil level of *V. dahliae*

Data were also examined for evidence that the quantity of *V. dahliae* determined in soils by the two molecular tests was associated with the incidence of plants showing verticillium wilt symptoms. The quantity of *V. dahliae* DNA expressed as pg/g soil was grouped into four categories: nil, 0.1-100, 101-200 and >200 pg/g.

Only five soils tested positive of *V. dahliae* using the EF assay. Four of the five positive sites had relatively high level of *V. dahliae* (>200 pg/g), and these sites all showed a verticillium wilt incidence of >1% (Table 17).

Twenty-nine of the 41 soils tested positive for *V. dahliae* by the Bilodeau assay (Table 18). Most of these sites (23) had a relatively low level of *V. dahliae* (<100 pg/g). However, this low level of *V. dahliae* was not consistently associated with a low incidence of Verticillium wilt symptoms; there was a broad spread of verticillium wilt incidences from nil to >10% plants affected. Four of the sites had relatively high levels of *V. dahliae* (>200 pg/g) and these sites all showed verticillium wilt incidences of >1%. The mean incidence of plants recorded with verticillium wilt symptoms increased progressively from 2.4% of plants to 13.6% of plants as the quantity of *V. dahliae* detected in soil increased from not detected to >200 pg/g (Table 18).

Table 17. Effect of quantity of *V. dahliae* detected in soil using the EF assay on the incidence of verticillium wilt symptoms – 2013

Quantity *V. dahliae* in soil (pg/g)	No. crops in this category	Mean % wilt	Wilt incidence by category (%)				
			0	0.1-1	1.1-5	5.1-10	>10
ND	36	7.3	7	12	4	7	6
0.1 - 100	1	10.6	0	0	0	0	1
101 - 200	0	-	-	-	-	-	-
>200	4	12.4	0	0	3	0	1

Table 18. Effect of quantity of *V. dahliae* detected in soil using the Bilodeau assay on the incidence of verticillium wilt symptoms – 2013

Quantity *V. dahliae* in soil (pg/g)	No. crops in this category	Mean % wilt	Wilt incidence by category (%)				
			0	0.1-1	1.1-5	5.1-10	>10
ND	12	2.4	4	6	0	1	1
0.1 - 100	23	9.4	3	6	4	6	4
101 - 200	2	10.5	0	0	0	0	2
>200	4	13.6	0	0	3	0	1

Region and soil type

Association of verticillium wilt incidence with region and soil type is summarised in Table 19. There was a significant effect (p = 0.039) from region, with least verticillium wilt (1.8%) in the midlands and higher levels (>5%) in the south-east and Wales. Soil type, raised bed (vs flat cropping) and irrigation (vs no irrigation) had no significant effect (Table 20).

Table 19. Association of region and soil type with observed incidence of strawberry verticillium wilt – autumn 2013[a]

Region	Mean % plants affected (no. of crops)				
	Clay loam	Loamy sand	Sandy loam	Silty loam	All soil types
East Anglia	4.7 (2)	-	10.6 (1)	-	6.7 (3)
Midlands	5.2 (1)	0.1 (3)	2.2 (3)	-	1.8 (7)
South East	1.2 (6)	8.9 (3)	7.4 (8)	0 (1)	5.2 (18)
South West	-	-	1.4 (3)	-	1.4 (3)
Wales + North	-	-	3.7 (4)	0 (1)	5.6 (2)
Scotland	-	-	0 (2)	-	-
All regions	2.4 (9)	4.5 (6)	4.4 (21)	0 (2)	3.7 (38)

[a] Excludes sites 28 and 29 due to their large influence. A further four sites were not assessed as plants had been grubbed.

Table 20. Stepwise analysis of variance examining the association of soil type, region and irrigation with incidence of verticillium wilt in strawberry – autumn 2013

Factor	Levels	Df	F prob.
Soil type	4	3	0.281
Regions	5	4	0.006
Irrigated	2	1	0.163
Residual		27	
Total		36	

Distribution of *V. dahliae* in fields

V. dahliae was detected in all four fields (sites 3, 13, 14 and 15) where testing was done on individual soil cores using the Bilodeau assay. The proportion of cores that were positive ranged from 4/50 (site 15) to 47/50 (site 03) (Table 21). For the three sites where most cores were positive, the majority of these cores had only a low quantity of *V. dahliae* (1-10 pg/g). A few individual cores at sites 3 and 14 had very high levels (>100 pg/g).

No assessments of verticillium wilt symptoms were done in these crops.

Table 21. Detection of *V. dahliae* in 50 individual soil cores sampled from four fields in 2013

Quantity of *V. dahliae* (pg/g)	No. of soil cores (of 50) in each category			
	Site 03	Site 13	Site 14	Site 15
ND	3	18	13	46
1 – 10	36	31	32	4
11 – 50	4	1	3	0
51 – 100	2	0	1	0
>100	5	0	1	0

Analysis of the distribution of *Verticillium* in four fields through Kriging

Kriging (a method of interpolation using Gaussian process regression) was attempted for the multiple samples taken at sites 3, 13, 14 and 15. The predicted field distribution of *V. dahliae* through this analysis is given in Figures 3 to 5. For site 15, Kriging was not possible due to the relatively low number of positive samples (Figure 6). These figures will be used to form a sampling strategy in the remainder of the project.

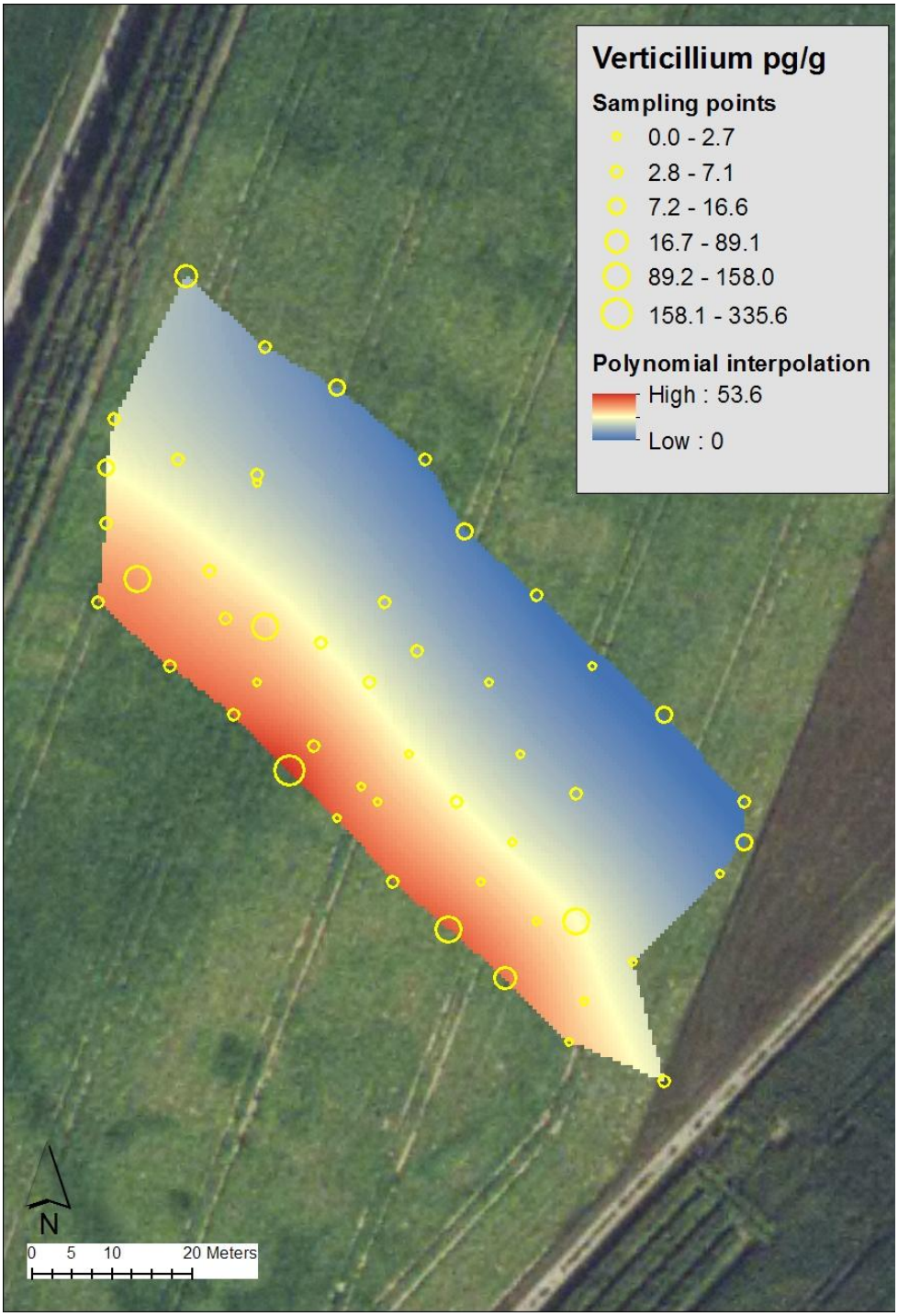

Figure 3. Predicted distribution of *Verticillium dahliae* at site 3.

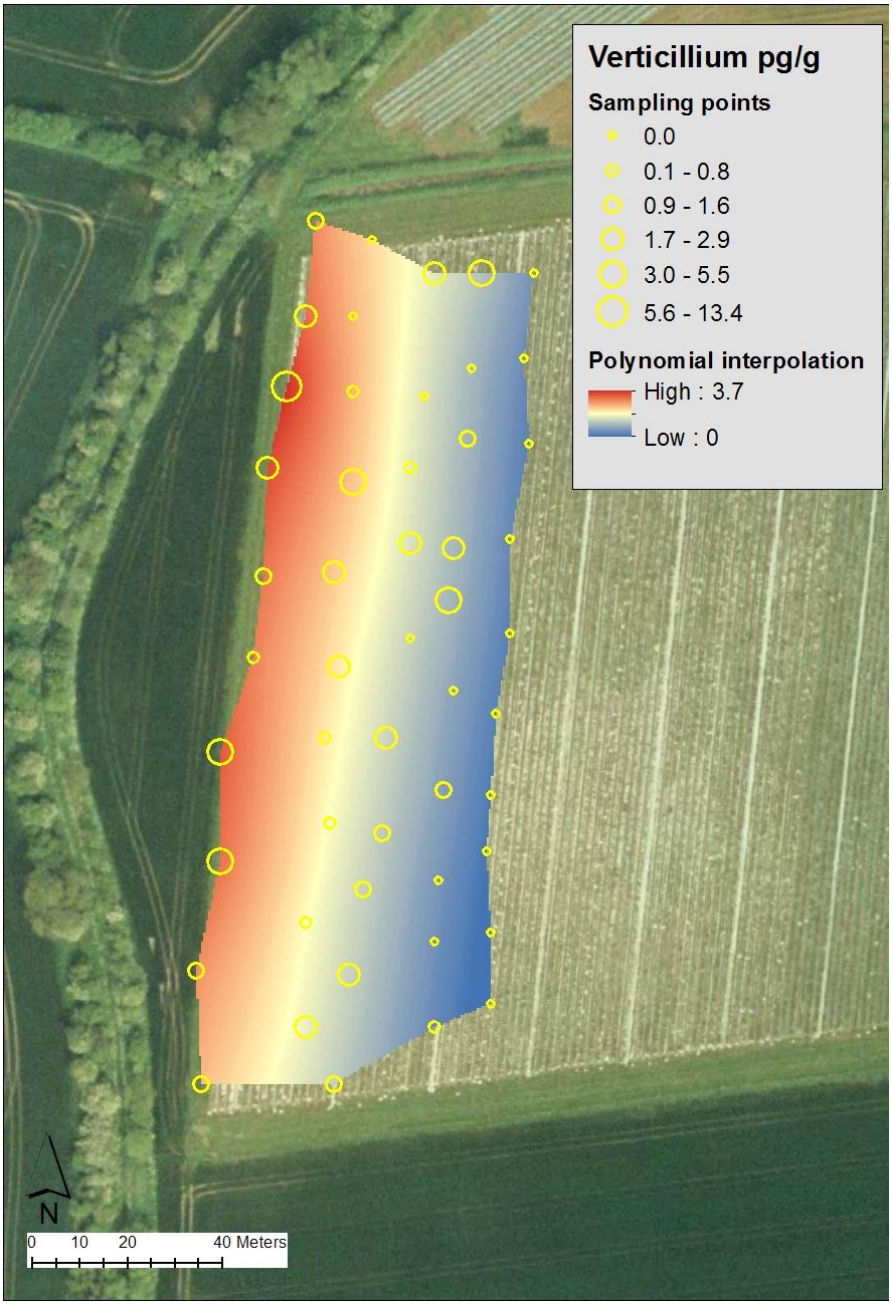

Figure 4. Predicted distribution of *Verticillium dahliae* at site 13.

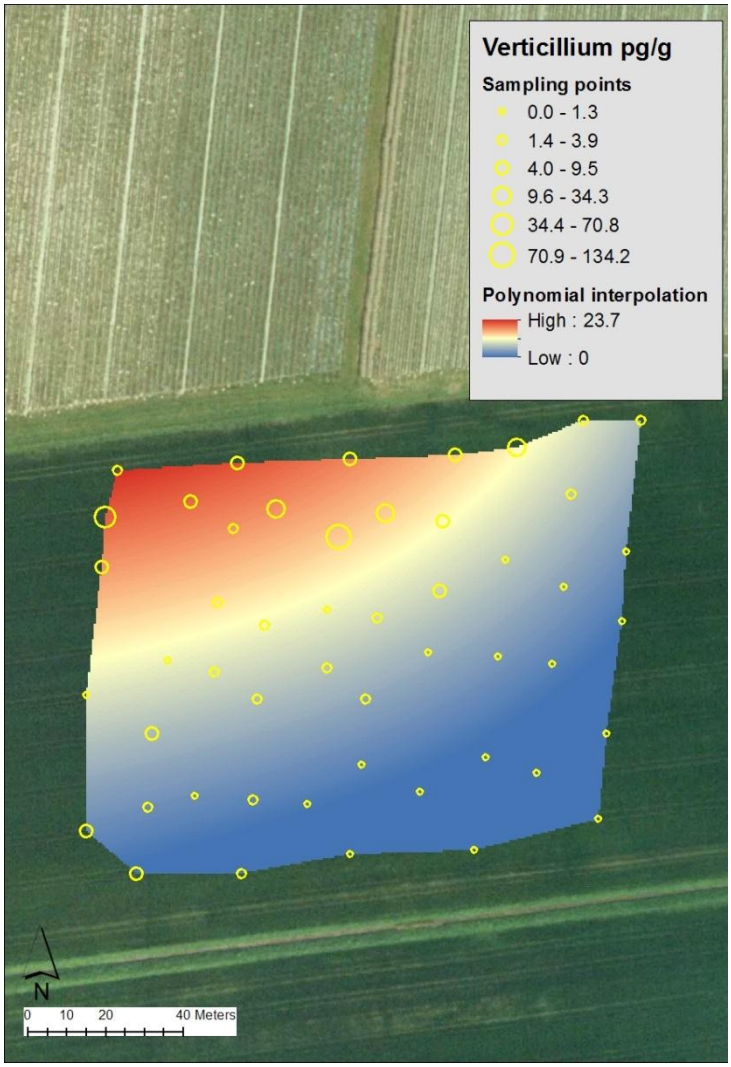

Figure 5. Predicted distribution of *Verticillium dahliae* at site 14.

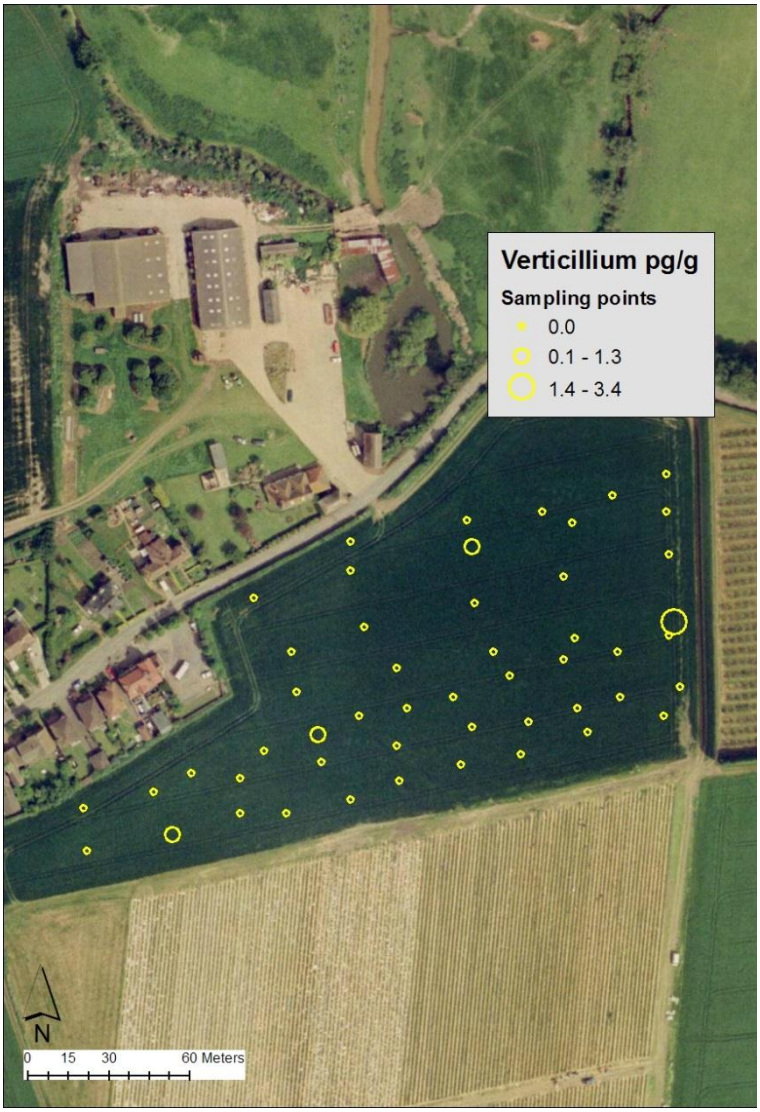

Figure 6. Presence of positive *Verticillium dahliae* soil samples at site 15.

Discussion

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 both of the two qPCR assays at 29/41 sites. At all five sites where the EF assay detected *V. dahliae*, 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.

As was expected with work in commercial crops, a range of strawberry varieties 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 varieties 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, Eilan, Elegance,

Fenella, Malwina, Sonata, Symphony and Vibrant. Infection was confirmed in samples of 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, two or more varieties were planted. Efforts will be made in 2014 to identify further sites for soil sampling and disease assessment, and where a sampled area is planted with two or three varieties, these will be individually assessed for verticillium wilt.

There were 12 sites where neither the 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% (6 sites), 8.8% (site 22) and 15.7% (site 44). 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, it is planned that samples of wilted plants will be taken from a greater proportion of crops and tested for infection by *V. dahliae*.

There was a positive association between detection of *V. dahliae* in soil and presence or absence of symptoms in the strawberry crops using the Bilodeau assay (29 out of 41 sites appear correct) but not with the EF assay (5 out of 41 sites appear 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. Should good progress be made in Objective 1, and a new assay is developed with both high specificity for and high sensitivity to *V. dahliae* it is preferable that soils are re-tested using such an assay.

There was also 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%).

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.

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 (Table 17). A larger and more evenly distributed data set is required before any firm conclusions can be drawn with regard to soil infestation density and severity of verticillium wilt symptoms.

Summary of future work

- If the IGS v2 modified assay proves to be more sensitive and more specific than the EF and Bilodeau assays, and does not cross-react with *V. longisporum* re-test the soil samples from the assessed sites using this new assay.
- Re-assess the 41 sites for verticillium wilt symptoms in 2014.
- Seek to identify, soil sample and assess 10 new sites at risk of verticillium wilt, preferably sites to be planted with one variety.
- Where a sampled site contains two or three varieties, assess incidence of verticillium wilt in each variety separately.
- Where verticillium wilt symptoms are observed in 2014 and the disease has not been confirmed in plants (in this project in 2013 or by other tests), collect samples of three plants with typical symptoms for testing by Fera.

Conclusions

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. Work in Year 2 aims to increase sensitivity.

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 variety, 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.

Technology transfer

Project progress meeting, Fera, 17 February 2014.

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Appendix 1. Summary of results - 2013

Results on the 41 sites soil sampled by the standard method and with strawberry crops assessed for verticillium wilt in 2013

Soil site	*V. dahliae* detected in soil		Verticillium wilt symptoms present	*V. dahliae*		Cultivar
	EF	Bilodeau		Plants tested	Vd confirmed	
1	-	-	-			Mixed
2	+	+	⊕			Camarillo
4	-	+	⊕			Serena
5	+	+	+			Ser/Fin
6	-	+	+	✓	Y	Fen/Sym
7	-	+	+	✓	Y	Trials
8	-	+	+	✓	Y	Diamond
9	-	-	+	✓	N	Sonata
10	-	-	+	✓	Y	Sonata
11	-	+	+	✓	N	Amesti
12	-	+	-			Symphony
16	-	+	-			Fenella
17	-	+	+	✓	N	Vibrant
18	-	+	+	✓	Y	Diamond
19	-	+	⊕			Sonata
20	-	+	⊕			Mixed
21	-	+	⊕			Fenella
22	-	-	⊕			Symphony
23	-	+	+			Elegance
24	⊕	⊕	⊕			Buddy
25	-	-	+			Sonata
26	-	+	+			Fenella
27	-	-	+			Amesti
28	-	+	⊕			Mixed
29	-	+	⊕			Mixed
30	-	+	⊕			Mixed
31	-	+	⊕			Malwina/Sym
32	-	-	+			Malwina/Sym
33	-	+	+			Mixed
34	-	+	⊕			Mixed
35	-	-	-			Malwina/Fenella
36	-	-	+			Mixed
37	-	+	-			Mixed
39	-	+	+			Mixed
40	-	+	⊕			Symphony
41	⊕	⊕	+			Malwina
42	⊕	⊕	+			Mixed
43	-	⊕	⊕			Symphony
44	-	-	⊕			Eilan
47	-	-	-			Sonata
48	-	-	-			Sonata
Total (of 41)	5	29	34	8	5	-

- not detected; + detected; ⊕ >100 pg/g / >5% wilt; Y – Yes, N – No; Ser – Serena; Fin – Finesse; Sym - Symphony