Project title	Detection and quantification of *Verticillium dahliae* and *V. albo-atrum* in soils to determine risk of verticillium wilt in strawberry
Project number:	SF 97
Project leader:	Dr Jeff Peters, Fera
Report:	Final report
Previous report	May 2011
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Location of project:	Fera, York ADAS Boxworth, Cambs. Growers holdings – Cheshire, Lancs, Oxon
Project coordinator:	Dr Neil Boonham
Date project commenced:	1 April 2009
Date project completed (or expected completion date):	May 2012

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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

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

Background and expected deliverables

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

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

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

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

assay using current soil sampling methods used by growers.

With the loss of methyl bromide for soil disinfestation and increasing concern over the future use of alternative soil sterilants (e.g. chloropicrin), sound knowledge of levels of *V. dahliae* in soil are increasingly important to aid economically-sound planting decisions.

The overall aim of the project is to refine quantitative DNA assays for the rapid quantification and detection of *V. dahliae* and *V. albo-atrum* and to establish the risk of strawberry wilt disease based on soil inoculum densities.

Specific project objectives were:

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

Summary of the project and main conclusions

Objective 1: Refine and validate PCR assays

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

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

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

DNA extraction from large volumes of soil

A method was devised to extract total DNA from large volumes of soil. Replicate sub-samples each of 50 g were taken from a sample of 500 g and extracted by adaptations of a

published method. Results based on the detection of ubiquitous soil inhabiting bacteria, *Streptomyces* spp. in replicate samples from each of five soils showed a coefficient of variation of between 1.7% (high levels of target DNA) and 18.5% (low levels of target DNA). This shows that the soil test gave consistent extractions, particularly where there are abundant target species. All soils were tested using the *V. dahliae* and *V. albo-atrum* QPCR assays. A *Streptomyces* internal control assay was also carried out to ensure that soil extractions had performed efficiently.

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

Verticillium dahliae

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

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

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

Verticillium albo-atrum

In 2010 a pot experiment was set up, as above, to investigate the relationship between growing strawberry plants in soils artificially amended with differing levels of long-lived hyphae of *V. albo-atrum* with wilt symptoms. Six weeks after inoculation the mean level of wilt that developed in Elsanta generally increased with increasing levels of *V. albo-atrum* inoculum. The assay failed to detect the pathogen at the two lowest inoculum levels (corresponding to 1:80,000 and 1:160,000 sand maize-meal culture to compost). Of those, only the 1:80,000 inoculum level was sufficient to cause low levels of leaf necrosis.

There was no relationship between inoculum level of *V. albo-atrum* and severity of wilt symptoms in Florence and Symphony at six weeks after planting in infested soil.

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

Effect of natural soil inoculum level of V. dahliae on verticillium wilt – pot experiment

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

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

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

Effect of soil inoculum level of V. dahliae on verticillium wilt – field experiment

The aim of this experiment was to determine if pre-planting soil infestation density of *V. dahliae* on field sites measured by the conventional agar plate test and by QPCR was predictive of verticillium wilt risk. Replicated plots of three varieties (Elsanta, Symphony and Florence) differing in susceptibility to wilt were established in spring 2010 in five fields that ranged in *V. dahliae* infestation density from <0.1 to 5.7 cfu/g (<250 to 560 fg/g). All plants used were from the same supplier and a sample examined pre-planting was found to be free of *V. dahliae*.

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

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

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

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

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

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

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

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

Considering just the plots with levels of wilt above 10% in July (on the possibility that lower levels may be wilt caused by a factor other than *Verticillium*), then the QPCR test accurately identified the only plot in 12 at site A11 with a high risk of wilt. QPCR also accurately identified four plots at high risk of verticillium wilt at site A12. However no *V. dahliae* was

detected pre-planting at another seven of the 12 plots where high levels of wilt (over 10% and up to 49% in Elsanta) developed.

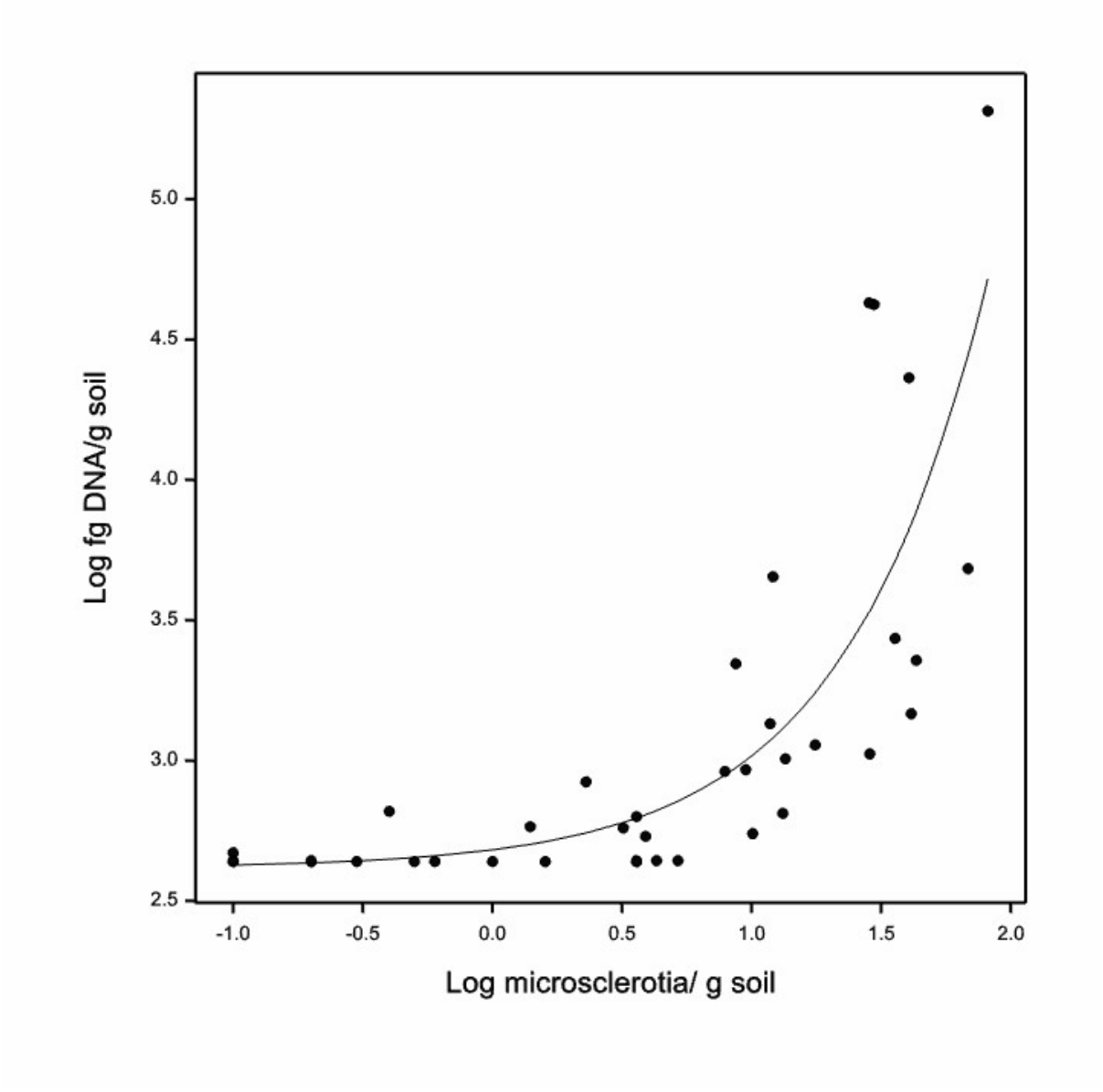
These results suggest that the current QPCR test is not sufficiently sensitive to detect low soil infestation densities of *V. dahliae*. Low soil infestation densities may result in a relatively low incidence of verticillium wilt in tolerant varieties but a high incidence in highly susceptible varieties,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

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

Parallel testing of QPCR and wet sieving

Although not one of the original objectives of this project, it was decided to directly compare the enumeration of *V. dahliae* inoculum in commercial soil samples using the traditional wet sieving (Harris) method with the new QPCR test. The data presented in Figure 1 show that there was a reasonable agreement (R^2=0.67) between the two methods. However, the data show that detection of pathogen DNA by QPCR drops off sharply after approximately 1.0 microsclerotia/g (i.e. log 0.0). This is consistent with the hypothesis that the occurrence of verticillium wilt symptoms post-planting in field plots where no *V. dahliae* was recorded in the soil pre-planting may have been due to insufficient test sensitivity.

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



Main conclusions

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

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

Financial benefits

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

Action points for growers

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

SCIENCE SECTION

Introduction

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

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

A pre-planting wilt risk assessment service, the Harris soil test, has been available to UK growers since the early 1990s. This test is based on the detection and enumeration of *V. dahliae* microsclerotia in soil. The assay costs around £165 + VAT and takes six to eight weeks to complete. This means both that considerable forethought is required prior to planting and costs are prohibitive to permit regular pre-planting screening. In addition, as the traditional method is reliant upon the detection of microsclerotia, it overlooks and underestimates the level of inoculum due to pathogenic *Verticillium* species, particularly Vaa, surviving in soil in the form of saprophytic mycelium. Currently no test exists for the detection and enumeration of Vaa in soil because the Harris test uses a sieving assay which detects only inoculum in the form of microsclerotia. The significance of Vaa in soft fruit crops is, therefore, poorly understood due to the lack of reliable diagnostic assays. It is reported to infect strawberry, raspberry and blackberry

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

With the loss of methyl bromide for soil disinfestation and increasing concern over the future

use of alternative soil sterilants (e.g. chloropicrin), sound knowledge on levels of *V. dahliae* in soil are increasingly important to aid economically-sound planting decisions.

Aim and objectives

The overall aim of the project was to refine quantitative DNA assays for the rapid quantification and detection of *V. dahliae* and *V. albo-atrum* and to establish the risk of strawberry wilt disease based on soil inoculum densities. The specific project objectives were to:

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

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

Introduction

In year 1, quantitative PCR assays were developed that detect *Verticillium dahliae* and *V. albo-atrum*, respectively. Details of the assay sequences are given in Appendix 1. The assays were shown to be specific to each target species tested and did not detect non-target fungi tested. In years 2 and 3 this validation work continued by increasing the number of *Verticillium* species tested. A small selection of isolates of *V. dahliae*, *V. albo-atrum* and *V. longisporum* were cloned and sequenced to determine the amount of genetic variability between and within species. This was done to inform assay specificity.

Materials and methods

Twenty seven isolates identified as *Verticillium* species were selected on the basis of providing a suitable range of species and host range (including many from strawberry). Details of the isolates are given in Table 1.1.

Table 1.1 List of isolates used in developing and validating the assay and specificity of *V. dahliae* (VdC1) and *V. albo-atrum* (VaaC1) assays.

Isolate identifier	Identification	Supplied by	Isolated from	Reaction with VdC1 assay	Reaction with VaaC1 assay
12001	V. dahliae	Fera	Chrysanthemum	pos	neg
DC59	V. dahliae	DB, Warwick Uni	Soil	pos	neg
321-3	V. dahliae	DB, Warwick Uni	Strawberry	pos	neg
1875	V. dahliae	DB, Warwick Uni	Strawberry	pos	neg
1877	V. dahliae	DB, Warwick Uni	Strawberry	pos	neg
2341	V. dahliae	DB, Warwick Uni	Hop	pos	neg
332-1	V. dahliae	DB, Warwick Uni	Strawberry	pos	neg
12078	V. dahliae	DB, Warwick Uni	Soil	pos	neg
12079	V. dahliae	DB, Warwick Uni	Strawberry	pos	neg
12080	V. dahliae	DB, Warwick Uni	Soil	pos	neg
12085	V. dahliae	DB, Warwick Uni	Strawberry	pos	neg
12086	V. dahliae	DB, Warwick Uni	Strawberry	pos	neg
Vd olive 4	V. dahliae	L Tsor, Israel	Olive	pos	neg
Vd olive 11	V. dahliae	L Tsor, Israel	Olive	pos	neg
12008	V. dahliae	Fera	Potato	pos	neg
12701 symphony bx09/77a	V. dahliae	Fera	Strawberry	pos	neg
12701 Eros bx09/77b	V. dahliae	Fera	Strawberry	pos	neg
12701 Bx 09/76	V. dahliae	Fera	Strawberry	pos	neg
12702	V. dahliae	Fera	Strawberry	pos	neg
13526	V. dahliae	Fera	Potato	pos	neg
12005	V. albo-atrum	Fera	Unknown	neg	pos
1871	V. albo-atrum?	DB, Warwick Uni	Strawberry	neg	pos
Cc340	V. albo-atrum	Fera	Unknown	neg	pos
Cc341	V. albo-atrum	Fera	Unknown	neg	pos
Cc407	V. albo-atrum	Fera	Unknown	neg	pos
Cc435	V. albo-atrum	Fera	Unknown	neg	pos
12006	V. longisporum	Fera	Oil seed rape	neg	neg
Ve007 PFI	V. longisporum	DB, Warwick Uni	Oil seed rape	neg	neg
Ve009 WVL2	V. longisporum	DB, Warwick Uni	Oil seed rape	neg	neg
JDO1	V. tricorpus	JD, Belgium	Unknown	neg	neg
CC505	Gliocladium sp.	Fera	Strawberry	neg	neg
-	Colletotrichum coccodes	Fera	Soil	neg	neg
-	Rhizoctonia solani AG3	Fera	Soil	neg	neg

In total 20 *V. dahliae*, sic *V. albo-atrum*, three *V. longisporum* and one *V. tricorpus* isolate were tested. In addition three non-verticillium fungal isolates were tested. One,

Gliocladium sp. is a saprophytic fungus that is commonly isolated from strawberry tissue and is difficult to differentiate from *Verticillium dahliae* using morphological identification during the early stages of growth. All those isolates were used to validate the QPCR assays. A selection was also used for phylogenetic analysis.

The DNA sequences for the internal transcribed spacer (ITS) and intergenic spacer (IGS) regions were amplified using specific forward and reverse primers at a concentration of 10pmol. Each PCR mixture contained 1µl of each primer, 12.5µl of 2X ready PCR Master Mix (Thermo Scientific), 1µl of DNA and 9.5µl sterile distilled water (i.e. 25µl total volume). Amplification conditions consisted of a denaturation step of 94°C (2min) then 35 cycles with each cycle consisting of 94°C (30s), 55°C (30s) and 72°C (1min) plus a final 10min elongation stage of 72°C. Amplification of PCR products was checked on 1% agarose gel in 1X TBE Buffer.

The PCR products were cloned using the Promega pGEM®-T Easy Vector System kit. Cloned products were cleaned for sequencing using Exonuclease and shrimp alkaline phosphatise (Exosap). Sequencing was carried out on products using a 3130XL Genetic Analyser (Applied Biosystems).

Sequences were edited and manually trimmed using Sequence Scanner software, then compared with other sequences from the NCBI database using nucleotide blast. These new sequences were used to create a phylogenetic tree using ClustalW alignment, the Neighbor-joining and p-distance method using MEGA 4 software.

Results and discussion

Figure 1.1 shows the phylogenetic tree produced from cloned sequences for seven *V. dahliae* isolates, four *V. longisporum* isolates and two *V. albo-atrum* isolates. This shows that the three species form three distinct clusters, with the exception that one clone of *V. longisporum* from OSR, colony 7, aligned closer to *V. albo-atrum* than with the main *V. longisporum* clade. The assays VdC1 and VaaC1 detected only *V. dahliae* and *V. albo-atrum* respectively.

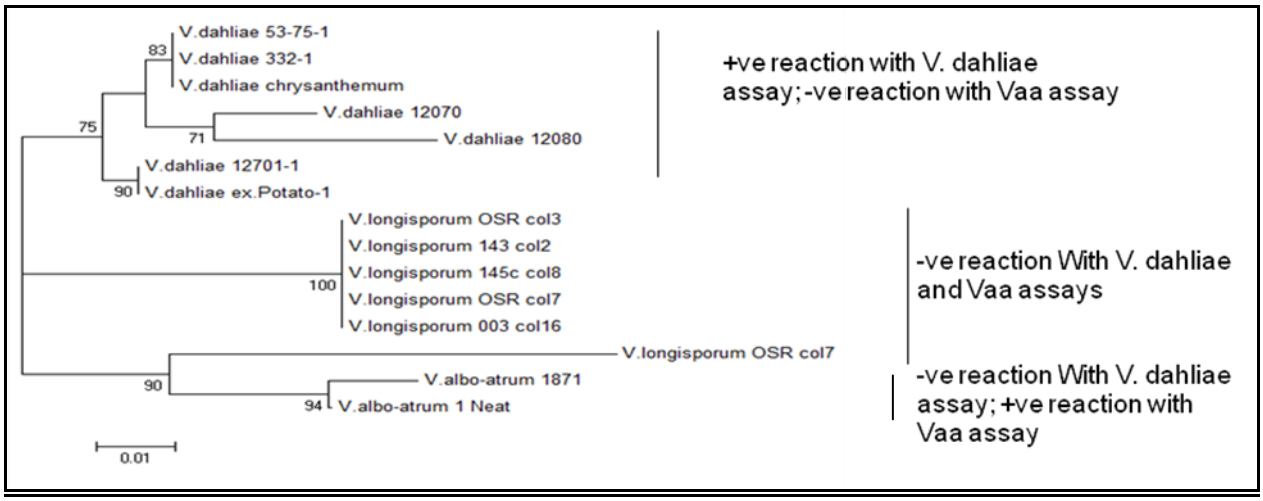


Fig 1.1. Phylogenetic tree showing the relationship between selected isolates of *V. dahliae*, *V. albo-atrum* and *V. longisporum* and the reaction with the *V. dahliae* (VdC1) and *V. albo-atrum* (VaaC1) QPCR assays.

2. QPCR conditions, assay design and sensitivity

Introduction

The main project objective was to improve disease control by developing a rapid, affordable and accurate molecular diagnostic test for verticillium wilt pathogens affecting strawberry. To this end the work focused on refining quantitative PCR assays for *Verticillium dahliae* and *V. albo-atrum* (Vaa), causes of strawberry wilt. This is particularly important for Vaa which survive in soil in the form of saprophytic mycelium. Currently no test exists for the detection and enumeration of Vaa in soil because the Harris test uses a sieving method which detects only microsclerotia.

Materials and methods

Sensitivity of the QPCR assays when tested using DNA extracts of Verticillium isolates

The target *V. dahliae* and *V. albo-atrum* specific TaqMan® forward and reverse primers (VdaF1, VdaR1 and VaaF1 and VaaR1) and probes (VdaP1 and VaaP1) were used to detect and quantify the pathogens in soil and plant material. Real time PCR were done in 96-well reaction plates using 12.5 µl of 2X PCR Master Mix (TaqMan Environmental Master Mix 2.0, Applied Biosystems) containing Taq polymerase, plus 1.0 µl (7.5 µM) of each primer, 0.5 µl of probe, 5.0 µl of nuclease-free water and 5 µl of DNA extract giving a final volume of 25 µl per reaction. Negative controls containing nuclease-free water instead of DNA were included in every run. Real time PCR was carried out on an ABI Prism 7900HT (Applied Biosystems) using generic cycling conditions (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). The Cycle threshold (Ct) value for each reaction was assessed using the Sequence Detection Software's default threshold setting of 0.02 ΔRn fluorescence units.

DNA templates from isolates of *V. dahliae* and *V. albo-atrum* (isolates obtained from strawberry) were used with a concentration of 14.8 ng/µl (*V. dahliae*) and 7.4 ng/uL (*V. albo-atrum*) determined on a ND-1000 NanoDrop spectrophotometer (NanoDrop Products). A 10-fold dilution series was prepared in TE buffer to provide concentrations in the range 1.4 x 10^7 to 148 fg DNA /uL (*V. dahliae*) and 7.4 x 10^6 to 740 fg DNA/uL (*V. albo-atrum*). The dilutions were run on QPCR cycling conditions as described above.

DNA extraction from large volumes of soil

Five agricultural field soils were obtained from East and North Yorkshire, Lincolnshire, Norfolk and Shropshire, for use in comparing and validating soil extraction methods. Soil samples were stored in clean plastic boxes at 4°C until required. Two of the five soils were used to compare the Fera extraction method, adapted from Budge et al. (2009), with two commercial extraction kits. Commercial DNA extraction kits were used according to manufacturer's instructions. The Fera method is based on CTAB buffer in the initial grinding step (120 mM sodium phosphate buffer pH8, 2% CTAB, 1.5 M NaCl). Alternatively, GTC buffer (100mM sodium phosphate buffer pH8 and 4M guanidinium thiocyanate) can be used. For the comparison of extraction methods, 20 ml of the buffer, 10 g soil and fifteen 10 mm diameter stainless steel ball bearings were added to a 120ml Nalgene bottle. Samples were homogenised and ground then processed using a Wizard Magnetic DNA Purification System kit (Promega UK, Southampton) according to the manufacturer's instructions in conjunction with a Kingfisher ML magnetic particle processor (Thermo Electron Corporation). For all methods tested, three replicate DNA samples were extracted per soil type. All resulting DNA extracts were stored at -30°C until required.

One of the field soils used for the extraction method experiment, originating from Norfolk, was used to determine the effect of increasing the soil sample weight from 1g to 250g, and the corresponding change in buffer volumes, on DNA yield. Soil samples ranging from 1 to 250 g weight were examined. For the different sample weights the size of Nalgene container size, the amount of buffer, size and number of ball bearings used was adjusted accordingly. For sample sizes of 50g and above extra protein precipitation and silica binding steps were used, as well as additional amendments to cope with larger volumes of liquid required for larger samples and were processed according to Budge et al. (2009).

To measure how consistent the preferred Fera method was able to extract DNA from soils, five soils (described above) were tested in triplicate. To ensure that all soils had detectable target DNA a surrogate microbial organism that is present in most (if not all soils), *Strepromyces* spp., was quantified by QPCR. The number of copies of the target organism obtained from each soil extract was compared and the coefficient of variation, for each soil, was used to determine the consistency of extraction.

DNA extracts from the extraction method and sample size experiments were analysed using a nanodrop spectrophotometer and real-time PCR. The nanodrop spectrophotometer (Thermofisher Scientific, Loughborough, UK) was used with absorbance values at 260 and 280 nm to assess DNA quality and quantity. Three readings were taken for each replicate and the mean calculated. Environmental Master Mix 2.0 (Applied Biosystems, Warrington,

UK) was used for all real-time PCR and consisted of half the total reaction volume of 25 µl, whilst 5 µl consisted of the soil DNA sample. Primers and probes (MWG Biotech, Germany) 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 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. The Ct value for each reaction was assessed using the Sequence Detection Software's default threshold setting of 0.2 ΔRn (fluorescence) units. Each sample was tested in two replicates and an average cycle threshold (Ct) was taken.

Results and discussion

Sensitivity of the QPCR assays when tested using DNA extracts of Verticillium isolates

The results from the real-time PCR quantification of DNA standards from *V. daliae* and *V. albo-atrum* are shown in Figures 2.1 and 2.2. Using the assay VdaC1, QPCR analysis of DNA extracts of *V. dahliae* (isolate 12080) was able to detect 147 fg DNA/uL at a Ct of 35.4. The cycle limit for the PCR reaction is set at 40. Assuming a ten-fold increase in detection occurs every three cycles, it should be possible to detect 10 times less than the amount detected (i.e. around 15 fg/µL for *V. dahliae*). Using the assay VaaC1, QPCR analysis of DNA extracts of *V. albo-atrum* (isolate 1871) was able to detect 740 fg DNA/µL at a Ct of 35.9. The theoretical limit of detection for *V. albo-atrum* using this assay should be around 70 fg/µL.

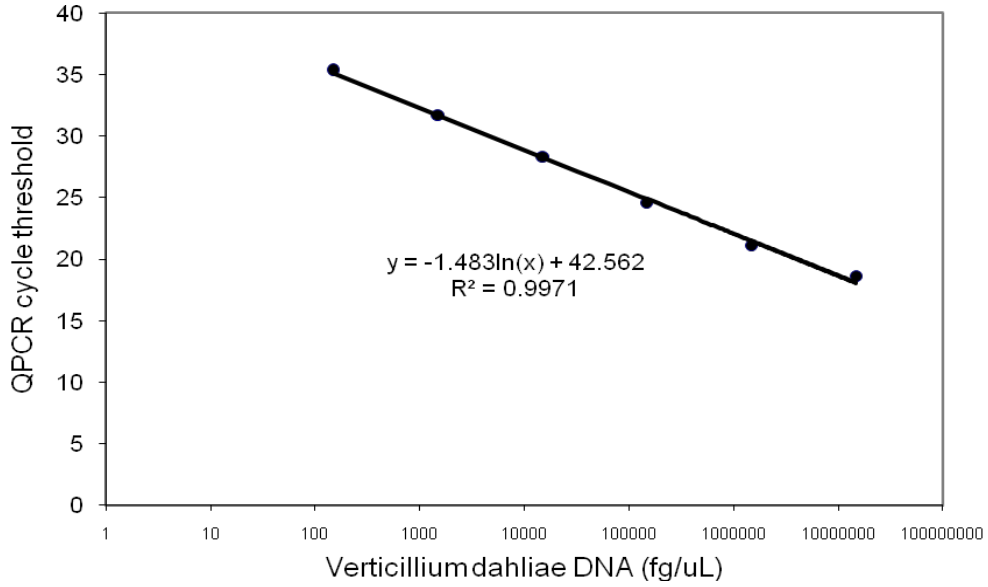


Figure 2.1. Relationship between PCR cycle threshold and amount of DNA extracted from pure culture of *Verticillium dahliae*.

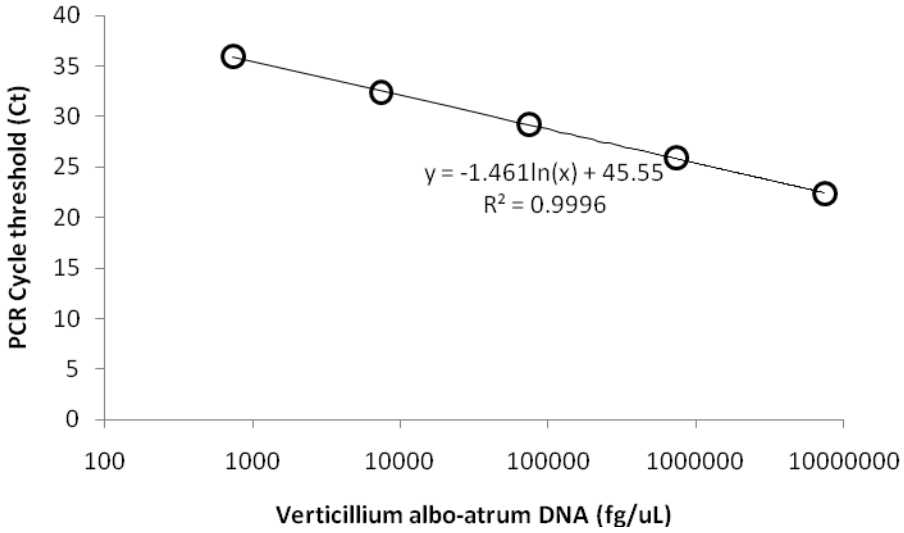


Figure 2.2. Relationship between PCR cycle threshold and amount of DNA extracted from pure culture of *Verticillium albo-atrum*.

DNA extraction from large volumes of soil

Four extraction methods were compared for their effectiveness in extracting total DNA from two agricultural soils (one in Yorkshire, the other in Norfolk) (Table 2.1). The Fera methods (using CTAB or GTC buffers respectively) either performed as well (Yorkshire soil) or better (Lincolnshire soil) than the commercial extraction kits, PowerSoil and PowerMax. The disadvantage with the commercial kits is that they are more expensive than the Fera method, require more hands-on staff time than the Fera method and are not scale-able: the PowerSoil and PowerMax kits can only process soils up to 1 g and 10 g, respectively. For those reasons it was decided to use the Fera methods rather than commercial kits. The CTAB method gave a marginally better yield of DNA in one out of two soils tested, compared with the GTC method. When testing the other soil, both methods gave similar DNA yields. Therefore, it was decided to use the CTAB method for all subsequent soil extractions.

Table 2.1. DNA yield using the Fera extraction method compared with two commercial extraction kits. Different letters following means within the same column are different at *P*<0.05.

Extraction method	DNA Yield (ng/µL	
	East Yorkshire	Norfolk
Fera CTAB (10 g)	6.6[a]	11.9[d]
Fera GTC (10 g)	7.2[a]	9.6[c]
PowerSoil (1 g)	5.7[a]	1.8[a]
PowerMax (10 g)	6.0[a]	4.8[b]
L.S.D$_{(P=0.05)}$	2.53	1.09

A comparison of weight of soil samples processed using the Fera soil total DNA extraction method is given in Table 2.2. The results show that there was a marked drop in the yield of total DNA extracted from samples below 50 g weight (*P*<0.05). In addition, the amount of amplified DNA to total *Strepromyces* spp, common soil inhabiting bacteria, was higher in DNA extracts from soil samples that were above 50 g. There was no obvious benefit to increasing soil sample size above 50 g. Also, the cost of extractions increases as sample weight increases. Therefore, all subsequent extractions were carried out using duplicate 50 g soil samples.

Table 2.2. The effect of soil sample weight processed using a scales extraction method for total DNA yield and Ct from Real-time PCR for a common soil bacterial group, *Streptomyces* spp. Different letters following means within the same column are different at *P*<0.05.

Sample weight (g)	Yield (ng/µl)	*Streptomyces* species	
		Ct	Number. positive/4
1	17.25[b]	26.95[b]	4
5	14.03[ab]	27.27[b]	4
10	11.94[a]	27.54[b]	4
50	37.99[c]	25.33[a]	4
150	37.25[c]	25.29[a]	4
250	46.19[d]	24.73[a]	4
L.S.D$_{(P=0.05)}$	3.83	0.85	

The yield of common soil inhabiting bacteria, *Streptomyces* spp, detected in total nucleic acid extracts from soils obtained from five locations in England are shown in Table 2.3. The extracts were carried out using the preferred (50g) Fera soil extraction method. The results show that absolute levels of surrogate pathogen, *Streptomyces* spp., varied by soil (P<0.001). However, the pathogen was present in all soils. The coefficient of variation (CV%) was low (i.e. <10%) in all soils where there was an appreciable level of target DNA. In the soil where the target pathogen was low (<100 copies/g), the CV% was above 10%, but at 18.5% was still within acceptable limits. All soil extracts for detecting *Verticillium* species in the work presented here were also tested for *Streptomyces* spp. as a means of ensuring that extracts had been successful.

Table 2.3. Yield of DNA (expressed as log copy number/g soil) of a common soil bacterium (*Streptomyces* spp.) detected in total nucleic acid extracts from five soils using the Fera soil extraction method.

Soil	Yield *Streptomyces* spp. copy number (log)	Coefficient of variation (%)
East Yorkshire	1.8	18.5
Lincolnshire	3.8	8.3
Norfolk	3.4	5.4
North Yorkshire	4.8	1.7
Shropshire	4.5	2.6
L.S.D (P=0.05)	0.47	

3. Parallel testing of QPCR and wet sieving methods

Introduction

It was not within the original remit of this project to compare the conventional wet-sieving (Harris) method with the DNA extraction and QPCR method. However, it has become clear through the pot and field trial data that a better understanding of how these methods compare would better inform the results from these trials. With this in mind, 51 commercial and trial soil samples were tested in parallel using both methods.

Materials and methods

Forty eight commercial soil samples, received through Fera's Plant Clinic or ADAS for determination of verticillium inoculum levels, plus three artificially inoculated trial soils, were tested in parallel using the QPCR method (at Fera) and by wet sieving (at ADAS, High Mowthorpe). Testing by both methods is as described previously.

Results

The results presented in Fig 3.1 show that there was a reasonable agreement (R^2=0.67) between the two methods. However, the discrimination by QPCR at levels below 1 microsclerotia/g soil becomes poor. It is likely that the sensitivity of the molecular assay and/or the DNA extraction method is unable to reliably detect below levels below c. 250 fg DNA/g soil (or c. 1 microsclerotia/g soil).

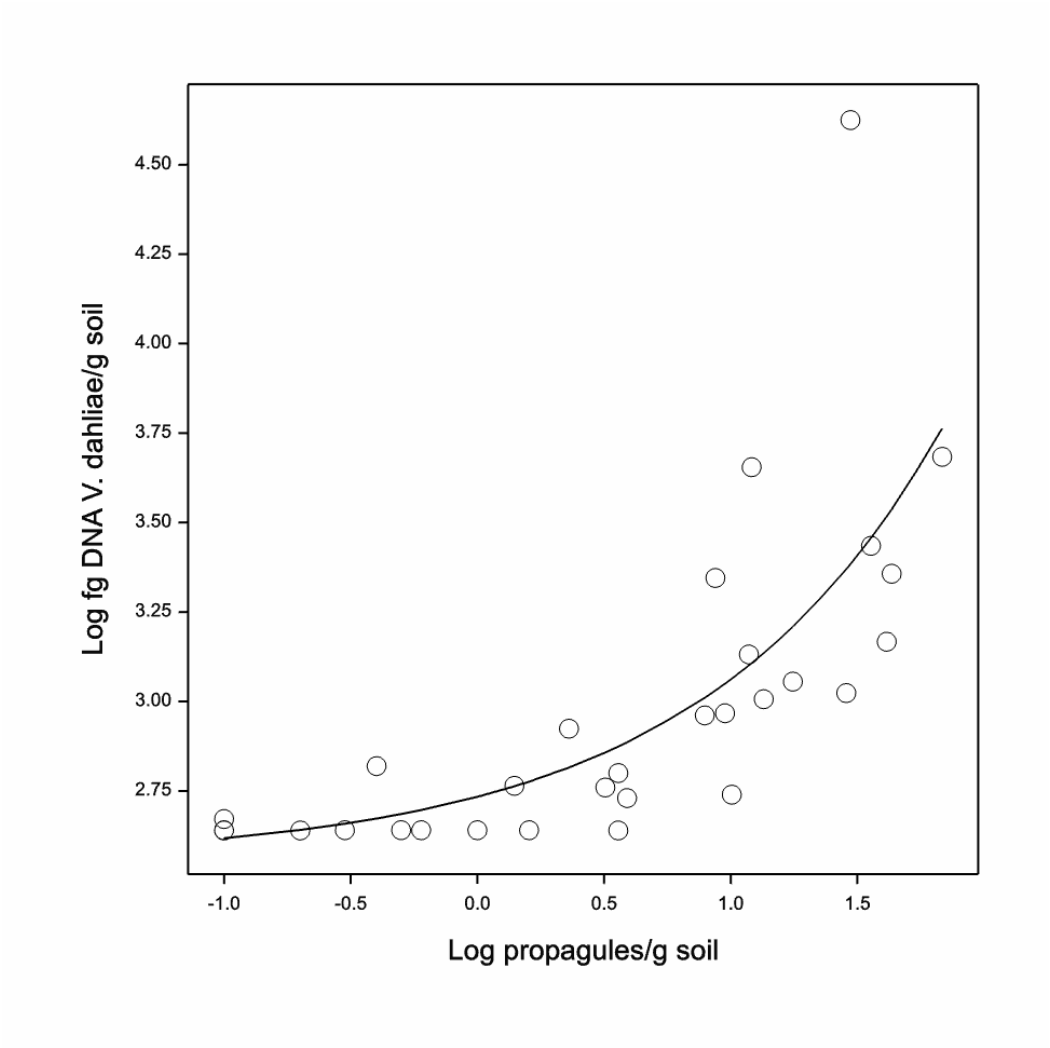


Figure 3.1. Correlation between microsclerotial counts and DNA levels when commercial samples were tested by QPCR (DNA/g soil)) and wet sieving (microsclerotia counts/g soil, log scale). $R^2 = 0.67$.

4. Effect of inoculum levels of *Verticillium dahliae* and *V. albo-atrum* on development of verticillium wilt in strawberry plants grown in pots of artificially infested soil

Introduction

In Year 1 (2009/10), dilution series of *V. dahliae* and *V. albo-atrum* in compost demonstrated that there was a good relationship between the number of microsclerotia added to soil and the level of DNA obtained from soil extracts as measured by QPCR. In year 2 (2010/11), strawberry plants were grown in compost amended with an inoculum dilution series of either *V. dahliae* or V*. albo-atrum.* These trials demonstrated that there was a positive relationship between inoculum of *V. dahliae* and *V. albo-atrum,* as measured by QPCR, and levels of wilt symptoms (see SF 97 Year 2 report).

In Year 3 (2011/12), work continued to further demonstrate the relationship between levels of DNA detected by the quantitative molecular tests (QPCR) in soil artificially inoculated with *V. dahliae* and *V. albo-atrum* and development of wilt symptoms in cv. Elsanta growing in the inoculated compost.

Materials and methods

In 2011 an isolate of *V. dahliae* (2341) and an isolate of *V. albo-atrum* (1871), both supplied by D. Barbara, Warwick Crop Centre, were used as inoculum in pot trials during the growing season. Inoculum, as either sclerotia or melanised hyphae for *V. dahliae* or *V albo-atrum* respectively, were obtained by placing two 1 cm^2 plugs from each 14-day old culture (grown on potato dextrose agar) in 150 g of sand maize-meal in a 250 mL conical flask. Each flask was incubated in a controlled temperature room at 18°C in 12h light/dark cycles. After three weeks (10 June 2011) the inoculum was thoroughly mixed by hand into 20L volumes of John Innes #3 compost to provide the following estimates of microsclerotia/g compost: 0.25, 0.5, 1, 2, 5 and 10 ms/g. Quantification was estimated by QPCR at a high concentration and then diluted with clean soil to achieve the target concentrations. The amount of microsclerotia that was obtained from a sand/maize-meal culture was estimated by counting the number of propagules that had based through a 250 um sieve but retained on a 150 um mesh sieve. A concentration of approximately 8,000 propagules/g was added to 1 L of compost. This propagule/compost mix was adjusted to 10 ms/g soil in 7.5 L clean compost. Then a 1 in 5 dilution serial was done using clean compost to provide the required concentrations. For the control clean compost was used (0 ms/g). The compost mixtures were left overnight to allow short-lived hyphae or conidia to die. Duplicate DNA

extractions were performed for each inoculum level prior to placing in pots. Real-time PCR was performed in duplicate as a check on the levels of pathogen (Fig 2.4). A pathogen-free strawberry plant (either cv. Elsanta or cv. Symphony) was transplanted into each 1 L pot containing compost of each estimated microsclerotia concentration (n = 5 for each concentration). Plants were grown under optimal moisture conditions for wilt development in a glasshouse. After 10 weeks (on 17 August), plants were assessed for wilt symptoms. Symptoms were scored using the following scale:

0 = no symptoms

1 = 1-5%

2 = 6-12%

3 = 13-25%

4 = 26-50%

5 = 51-100%

The yield of strawberry fruits was assessed three weeks later (7 September). Plants were harvested on 9 September. Total nucleic acid was extracted from a proportion of plants and assessed for the presence of pathogen DNA by QPCR.

Tests were also done to determine the sensitivity of the QPCR assay when tested using extracts of soil amended with verticillium inoculum. Isolates of *V. dahliae* (12080 and 2341) and an isolate of *V. albo-atrum* (1871), both supplied by Warwick Crop Centre, were used as inoculum in the pot trials. Inoculum, as either sclerotia or melanised hyphae for *V. dahliae* or *V albo-atrum* respectively, were obtained by placing two 1 cm^2 plugs from each 14-day old culture grown on potato dextrose agar (PDA) in 150 g of sand maize-meal in a 250mL conical flask. Each flask was incubated in a controlled temperature room at 18°C in 12h light/dark cycles.

In 2010, inoculum of *V. dahliae* (isolate 12080) was prepared by mixing clean loam-based compost and sand/maize-meal culture (providing c. 8,000 microsclerotia/g initial concentration as estimated by QPCR). A serial dilution was prepared in fresh compost mix to obtain the following concentrations of microsclerotia: 0.5, 1, 2, 5 and 10 ms/g. Quantification was estimated by QPCR at the higher concentrations. The compost mixtures were left overnight to allow short-lived hyphae or conidia to denature. Total nucleic acid was extracted from each inoculum level using the Fera large scale soil extraction method. Soils were also tested by wet sieving and estimated to be 0.4, 1.4, 4.5, 8.4 and 5.9 ms/g compost, respectively.

In 2011, inoculum of *V. dahliae* (isolate 2341) was prepared by adding known quantities of microsclerotia (counted out by hand from sand/maize-meal culture medium, under a microscope) to loam-based compost to provide the following concentrations: 0.25, 1.0, 2.0, 5.0 and 10.0 ms/g soil.

The compost inoculum mixtures were extracted for total nucleic acid and quantified by real-time PCR.

Results

Effect of soil inoculum levels on wilt symptoms

The relationship between inoculum levels and wilt as measured by leaf necrosis and wilt symptoms that developed in cv. Elsanta was poor for both *V. dahliae* and *V. albo-atrum* (Figs 4.1 and 4.2) although there was a trend for greater leaf necrosis at some of the higher inoculum levels. The relationship between inoculum and disease may have been marred by poor health in the control plants.

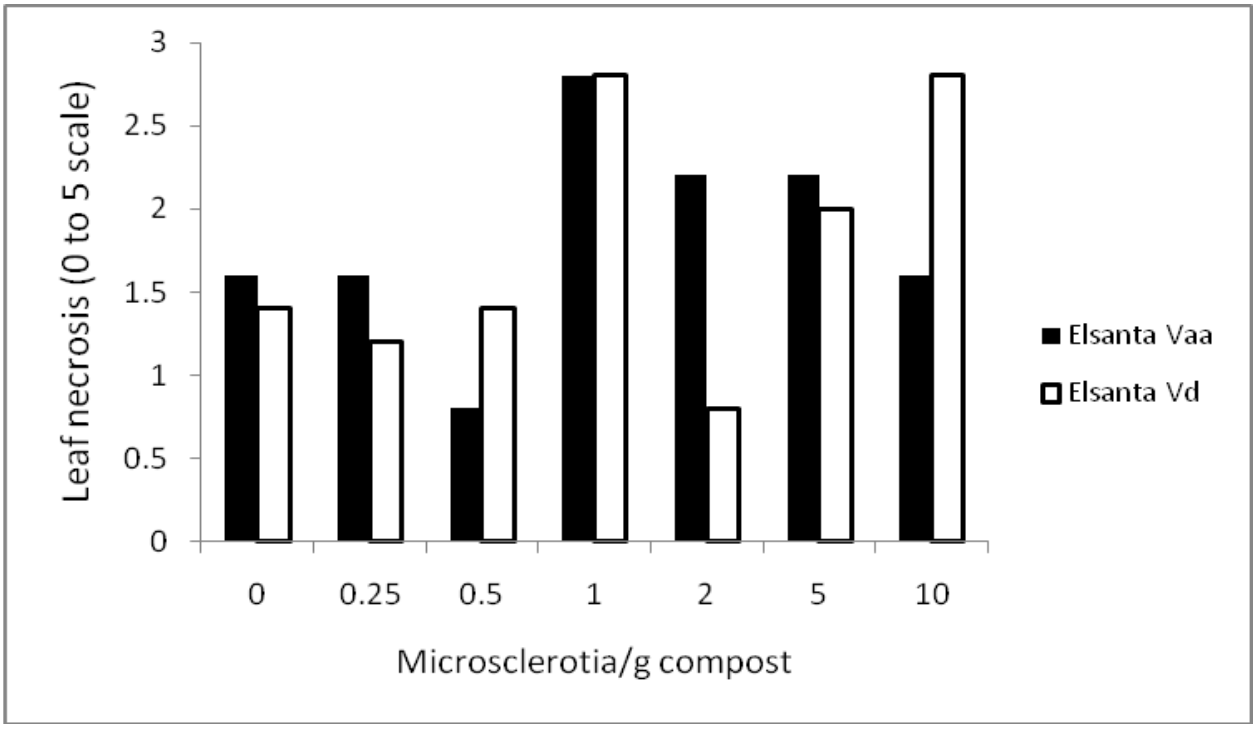


Figure 4.1. Relationship between leaf necrosis severity (% leaf area affected converted to 0 to 5 scale) and inoculum of *V. dahliae* and .*V albo-atrum* in cv. Elsanta. For *V. albo-atrum*, the inoculum was estimated as microsclerotial equivalents – 2011 trials.

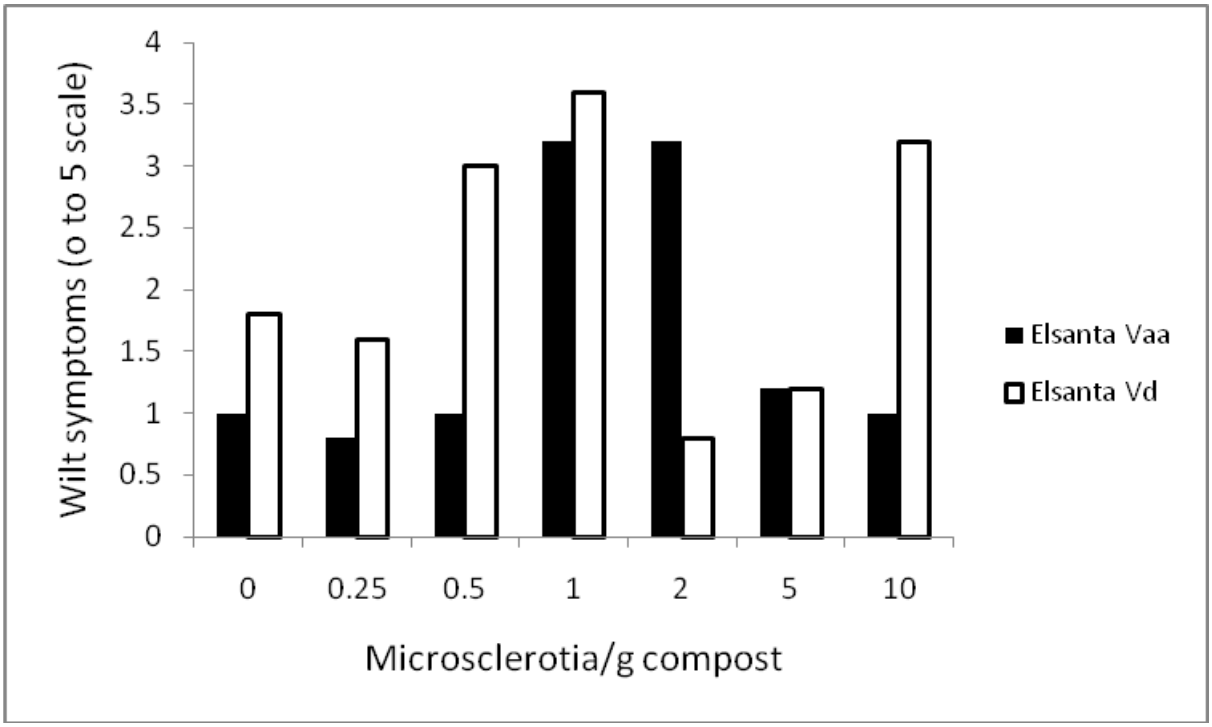


Figure 4.2. Relationship between wilt severity and inoculum of *V. dahlia* and *V albo-atrum*. For *V. albo-atrum*, the inoculum was estimated as microsclerotial equivalents – 2011 trials,.

The yield of strawberry fruits was measured for plants inoculated with *V. dahliae*. Fruit yield was numerically reduced but not significant (*P*=0.082) in plants growing in compost that had been inoculated with *V. dahliae* microsclerotia (mean of all infestation densities). When the interaction between yield and inoculum level is plotted (Fig 4.3) plants grown in compost inoculated with 0.25, 0.5, 1 and 5 ms/g produced greater yield that plants growing in the un-inoculated compost (*P*<0.05). Plant yields in pots that were inoculated with 2 and 10 ms/g were not different from un-inoculated controls.

Trials carried out during spring and summer of 2011 showed that most *V. daliae* microsclerotial dilution ranges from 0.25 to 10 ms/g soil reduced the mean per plant yield of strawberry fruit (Table 4.1). There was no statistical difference in yield between the un-inoculated control pots and concentrations at 2 and 10 ms/g. However, all pots containing microsclerotial inocula produced an arithmetic deduction in fruit yield.

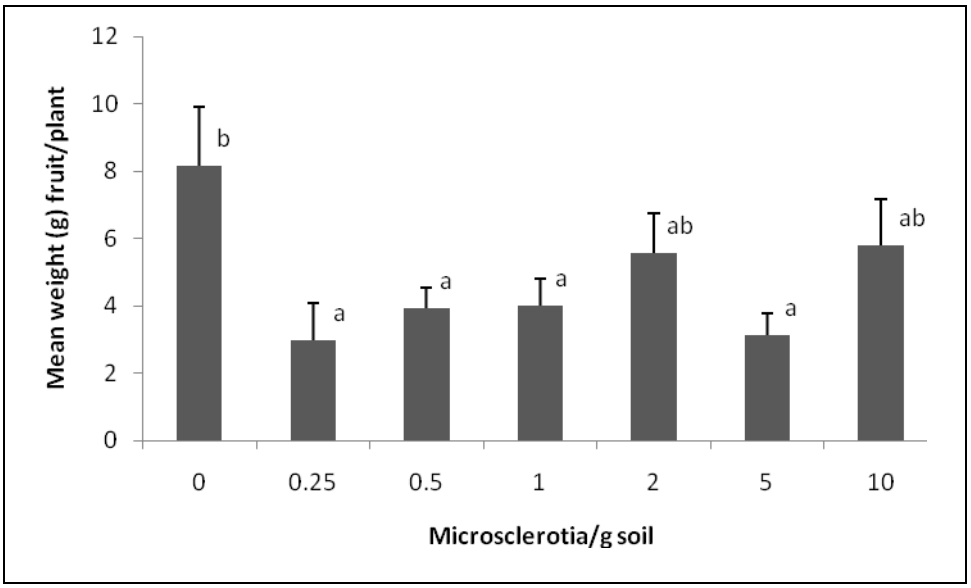


Figure 4.3. Effect of soil inolum levels of *Verticillium dahliae* microsclerotia on yield (mean per plant weight) of strawberry in pot trials (weight to 9 September). Different letters indicate different yields (P<0.05).

Correlation between microsclerotial number and DNA quantity

Under experimental conditions there was a strong and positive logarithmic relationship between the number of microsclerotia determined by counting aliquots of sieved sand-maize meal culture medium under a microscope artificially added to loam-based compost and the amount of *V. dahliae* DNA detected by QPCR (Figs 4.4 and 4.5). QPCR was able to detect from 8 or 10 ms/g soil down to 1.0 or 0.5 ms/g of soil in 2010 and 2011, respectively. The molecular assay failed to detect 0.5 ms/g soil in the 2010 trial and 0.25 ms/g in the 2011 trial.

The relationship between inoculum, DNA levels and disease symptoms was positively correlated in the 2010 trial (see Year 2 report). In the 2011 trial, there was no obvious relationship between inoculum and disease or pathogen DNA levels and disease. However, in general terms disease was higher in inoculated plots than in un-inoculated plots. Also, DNA levels of *V. dahliae* increased with increasing inoculum.

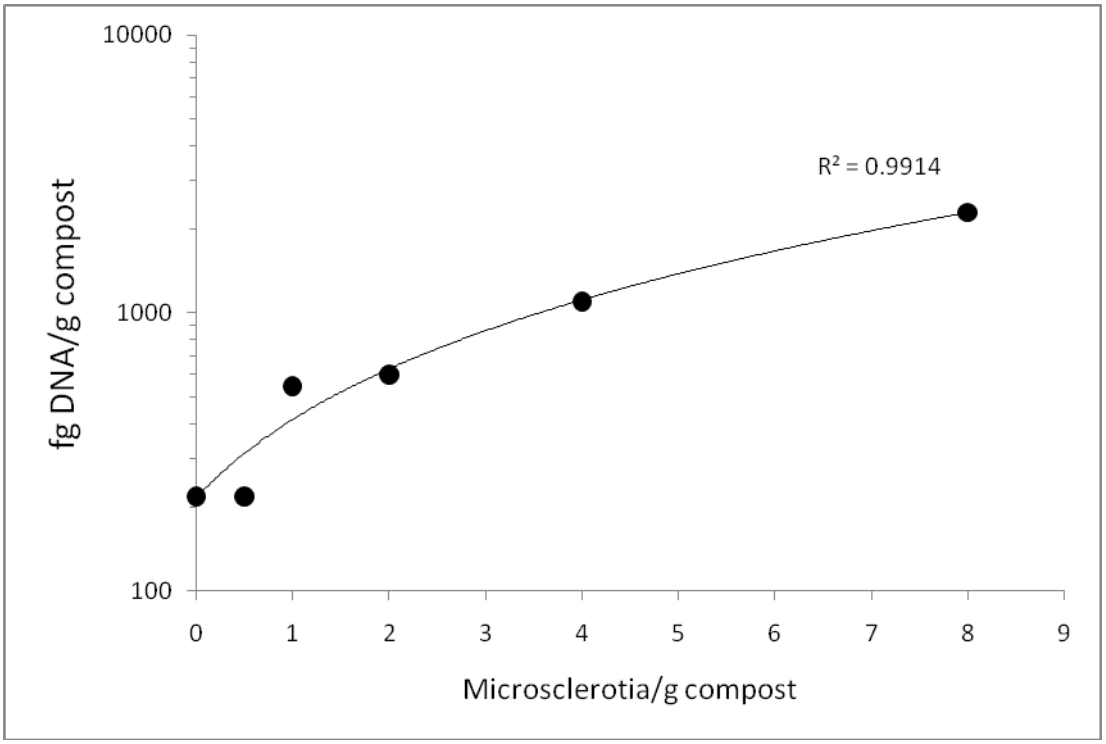


Figure 4.4. 2010 trial showing the relationship between the amount of DNA detected by QPCR when tested against DNA extracts using soil inoculated with microsclerotia of *V. dahliae* (isolate 12080). The assay, VdaC1, was able to detect down to 1.0 microsclerotia (543 fg/g soil) but was unable to detect microsclerotia added at 0.5 ms/g soil.

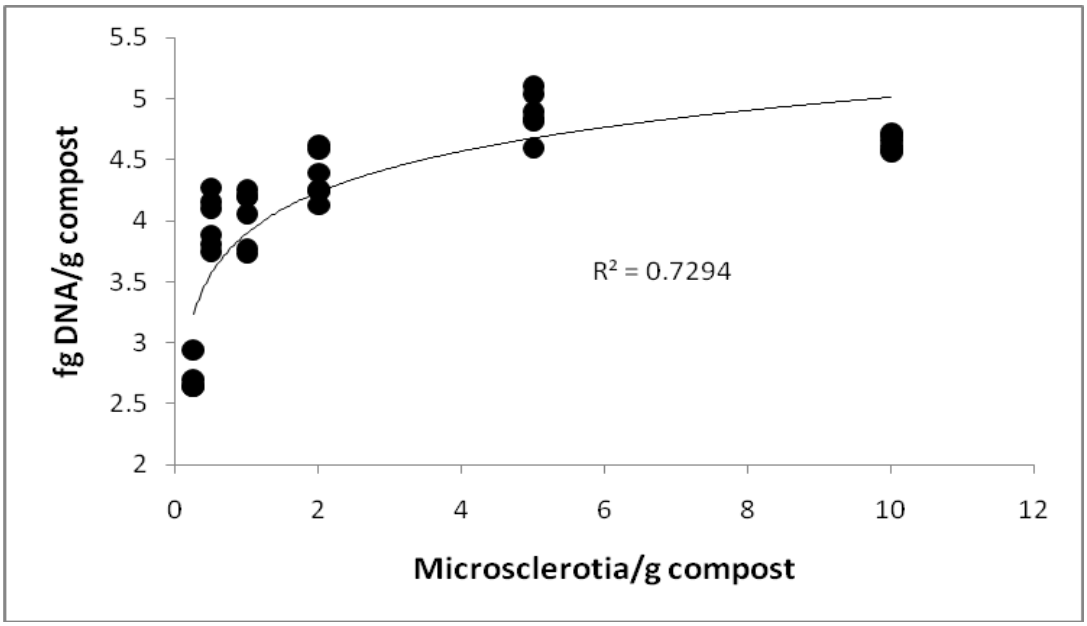


Figure 4.5. 2011 trial showing the relationship between the amount of DNA detected by QPCR when tested against DNA extracts using soil inoculated with microsclerotia of *V. dahliae* (isolate 2341). The assay, VdaC1, was able to detect down to 0.5 microsclerotia (380 fg/g soil) but was unable to detect microsclerotia at 0.25 /g soil.

5. Effect of inoculum level of *Verticillium dahliae* on development of verticillium wilt in strawberry plants grown in pots of naturally infested soil

Introduction

The aim of this experiment was to determine if there was a relationship between soil infestation density of *V. dahliae* and verticillium wilt symptoms using pot grown plants and naturally infested soil. The experiment was established in spring 2010 and results for 2010 are presented in the Year 2 report (March 2011). The results of assessments in the second year of growth are presented below.

Material and methods

These are fully detailed in the year 2 report. Briefly, the experiment consisted of pots of soil at six infestation densities (0 - 7.6 cfu/g; <250 - 977 fg/g; Table 5.1) of *V. dahliae* planted with cv. Elsanta on 14 May 2010. Plants were grown in pots with drip irrigation in a polytunnel (ADAS Boxworth) with side ventilation. The experiment was done in a randomised block design with fourfold replication; each plot contained 20 plants, except for the highest soil infestation density where there were only 10 due to limited infested soil supply.

In 2011 plants were assessed for leaf wilting, necrotic leaves and dead plants on 6 April and 31 May. Assessment data were examined by regression analysis. Twelve dead plants were selected in April from across all treatments and destructively examined for infection by *V. dahliae*. A further 15 plants with severe leaf necrosis were sampled on 31 May and examined by isolation onto agar. Four sections of upper crown tissue (around 2 x 2 mm) were removed, surface sterilised in alcohol (30 s) and plated onto potato dextrose agar. Plates were incubated at 20ºC for three weeks and examined for colonies of *V. dahliae*. In June 2011, 10 plants from T5 and T6, the two highest levels of soil infestation with *V. dahliae*, were sent to Fera to test for the fungus by QPCR. A crop diary is given in Appendix 2.

Results and discussion

Around 15% of plants, largely confined to one block at the tunnel edge, failed to produce new growth in the spring. Examination of roots revealed vine weevil infestation. Dead or dying plants were removed from the trial and a treatment for vine weevil control (Nemasys L drench) was applied twice to the remaining 85% of plants, which grew well. Subsequent assessments for verticillium wilt were done on these plants.

Typical symptoms of verticillium wilt did not develop. A few plants (0-6%) showed wilting or yellowing leaves in April 2011 but there were no significant differences between treatments. When assessed in May 2011 the proportion of plants dead or dying (i.e. plant with predominantly necrotic leaves) was high in three treatments (soil infestation density 1.2 - 7.6 cfu/g) and significantly lower (P = 0.019) in three other treatments (<0.1 - 2.7 cfu/g) (Table 1.1). However, the proportion of plants dead or dying did not relate to soil infestation density of *V. dahliae,* whether measured by the agar plate method or QPCR. It is probable that plant death and leaf necrosis was largely due to root and crown damage caused by earlier vine weevil infestation. The incidence of plants with wilting or yellowing leaves at this time was low, with no significant differences between treatments (P >0.05).

No *V. dahliae* was isolated from the crown tissue of any of 12 dead or dying plants examined in April 2011. Symptoms were indicative of vine weevil damage. No *V. dahliae* was isolated from any of the 15 plants examined at the end of the experiment; *Botrytis cinerea* was recovered from the crowns of two plants and *Fusarium* sp. from two others. It is likely that these infections occurred secondarily to vine weevil damage. From most plants, no pathogenic fungi were recovered.

The lack of symptoms typical of verticillium wilt and the failure to isolate *V. dahliae* from plants grown in soil infested with the fungus indicate that the disease did not develop. These results are consistent with those obtained in 2010 – no symptoms typical of verticillium wilt developed and no *V. dahliae* was isolated from lower leaf petioles or runners in autumn of that year. Consequently, no conclusions can be drawn from this experiment with regard to soil infestation density of *V. dahliae* and occurrence of verticillium wilt.

The reason for verticillium wilt failing to develop is unclear. The levels of soil infestation with *V. dahliae* (0.8 - 7.6 cfu/g; <250 - 977 fg/g) were above those at which one would expect the susceptible cultivar Elsanta to become infected; with field-grown crops, cv. Elsanta is considered to be at low, medium and high risk of developing verticillium wilt at soil levels of 0.2, 0.5 and 1.0 cfu/g respectively. It is highly likely that the strains of *V. dahliae* in the naturally infested soil were pathogenic to strawberry as the soil was collected from a soft fruit farm in a field where strawberries had been affected by verticillium wilt. The infested soil was thoroughly mixed when diluted with sterile soil, by tumbling in a concrete mixture, so an even distribution of *V. dahliae* within soil is likely to have been present. Soils were maintained wet in the first few weeks after planting to favour infection by *V. dahliae*. Perhaps plants grown in pots with drip irrigation are less susceptible to infection by *V. dahliae* for some unknown reason; or possibly plants became infected in the roots but the disease did not progress to cause wilt symptoms due to vigorous plant growth and/or insufficient moisture stress.

Table 5.1. Effect of soil infestation density with *V. dahliae* on appearance of strawberry plants, cv. Elsanta, grown in pots – 2011

Treatment	Soil infestation with *V. dahliae*		Mean % plants with wilting or yellowing leaves		Mean % plant dead or with mostly necrotic leaves
	cfu/g	fg/g	April	May	May
1.	NT[a]	NT[a]	4 (2.7)	0	17 (6.3)
2.	0.8	<250	4 (2.7)	6 (2.7)	18 (6.3)
3.	2.7	<250	6 (3.4)	3 (1.8)	10 (5.0)
4.	1.2	<250	1 (1.6)	6 (2.9)	36 (8.3)
5.	7.6	977	6 (3.4)	5 (2.7)	32 (8.1)
6.	5.1	NT	14 (7.3)	3 (3.1)	59 (11.5)
Significance (15 df)			NS	NS	0.019

[a] NT - not tested; soil in Treatment 1 was John Innes soil and is presumed zero for *V. dahliae*. ( ) – standard error.

6. Effect of soil levels of *Verticillium dahliae* determined by a molecular test on incidence of verticillium wilt in field crops

Introduction

The aim of this experiment was to examine data for any correlation between verticillium wilt symptoms in three strawberry varieties differing in susceptibility to the disease with soil density of *V. dahliae* in naturally infested soil as measured by QPCR.

Materials and methods

Site and crop details

Details of sites are summarised in Table 6.1 below; full details are given in the Year 1 and Year 2 Annual Reports. Crops at sites A8, A1 and A7 were grown as matted beds whereas those at sites A11 and A12 were maintained as individual plants in polythene covered beds. In 2011, for data examination, the total number of plants per plot at sites A8, A1 and A7 was assumed to be 100, the approximate number originally planted, as it was impossible to distinguish original plants from established runners in the matted bed.

At site A11 only, 100 plants of the new cultivar Fenella were planted in guard plots at either end (50 per end) of one of the main beds. These plants were assessed for verticillium wilt symptoms at the same time as the main experiment.

Table 6.1. Details of field sites with differing soil infestation densities of *V. dahliae* planted with strawberry in 2010 and assessed for verticillium wilt in 2010 and 2011

Site code	County	*V. dahliae* (cfu/g)	*V. dahliae* (fg/g)	Date planted
A8	Cheshire	<0.1	<250	9/4/10
A1	Lancs	0.2	<250	14/5/10
A7	Cheshire	0.5	<250	8/4/10
A11	Oxon	4.6	274	3/6/10
A12	Oxon	5.7	468	3/6/10

Assessments

Crops were examined in spring (April) and summer (July/August) 2011 for symptoms of wilt indicative of verticillium wilt; an additional assessment was done in November at sites A11 and A12. Assessments were done by experienced ADAS soft fruit advisers. The number of affected plants in each plot was counted. The number of dead and missing plants was also recorded at sites A11 and A12. Results for these two sites are presented as the proportion of wilted plants out of all live plants (current season wilt); the proportion of wilted + dead plants out of all live + dead plants (current season wilt plus dead); and the proportion wilted, dead or missing out of the original number planted in 2010 (accumulated wilted, dead and missing plants). This procedure gives the maximum levels of verticillium wilt that might have occurred in the trials, assuming that all plant death was due to verticillium wilt and that missing plants had died due to verticillium wilt and then decayed or were blown away following root loss.

Samples of plants with suspect symptoms of verticillium wilt were examined for infection by *V. dahliae* or *V. albo-atrum* by isolation tests onto potato dextrose agar (PDA) and by QPCR as described previously. Details of samples tested are given in the crop diary (Appendix 3). The soil infestation density of *V. dahliae* detected by QPCR in each plot is given in Appendix 4.

Results and discussion

Cause of plant wilting

Symptoms of verticillium wilt were observed (Figures 6.1-6.4) and *V. dahliae* was confirmed in sampled plants at four of the five sites in 2011 (Table 6.2). No red core (*Phytophthora fragariae*) or crown rot (*Phytophthora cactorum*) was found at any of the sites. *V. dahliae* was confirmed by isolation (onto agar) from affected plants as the cause of plant wilting at the four sites where wilt symptoms were observed in 2011 (Table 6.2). No *P. cactorum, P. fragariae* or other pathogens likely to cause plant wilting were isolated. It therefore seems probable that verticillium wilt was the predominant cause of plant wilting at these sites. *V. dahliae* was only confirmed in a low proportion of the wilted plants sampled at each site. The failure to confirm *V. dahliae*, either by isolation or by a QPCR test, in a majority of sampled wilted plants is probably due to the general difficulty in confirming this disease.

Similar problems have been reported in raspberry, blackberry, tomato and field-grown trees, even though symptoms were typical of verticillium wilt and other possible causes were excluded. This difficulty is considered due in part to *V. dahliae* being a slow-growing fungus (so that outgrowth from tissue is more likely to be inhibited by bacteria or antifungal compounds in plant tissues than with a fast-growing fungus) and also to the localised distribution of *V. dahliae* within infected plants. The fungus is a vascular pathogen with spores spread in xylem fluid; it is most likely to be present around sieve plates in xylem tissue and it is a matter of chance whether tissue colonised by the fungus is selected when plating onto agar or conducting other tests. It is possible that *V. dahliae* would have been confirmed in a greater proportion of sampled plants that showed wilt symptoms if greater resource had been expended on this aspect of the project. This was not done because the main aim and effort of the project was to relate wilt symptoms occurring in the field to pre-plant soil infestation density of *V. dahliae.*

Table 6.2. Occurrence of verticillium wilt in five fields of strawberry differing in soil infestation density of *V. dahliae* at planting.

Site	Soil density of *V. dahliae*		Verticillium wilt symptoms present		*V. dahliae* confirmed in plants in 2011 (no. positive/no. tested)
	cfu/g	fg/g[2]	2010	2011	
A8	<0.1	<250	No	Yes	Yes (2/2)
A1[1]	0.2	<250	No	No	No
A7	0.5	<250	No	Yes	Yes (1/4)
A11	4.6	274	Yes	Yes	Yes (1/10)
A12	5.7	624	Yes	Yes	Yes (2/19)

[1]High level of plant death over winter 2011 due to cold damage, especially in cv. Elsanta.

[2] DNA levels are means of 12 plots per site.

Figure 6.1: Symptoms of verticillium wilt in 2010 at site A11

Figure 6.2: Symptoms of verticillium wilt in 2010 at site A12.

Figure 6.3: Symptoms of verticillium wilt in 2011 at site A11.

Figure 6.4: Symptoms of verticillium wilt in 2011 at site A12**.**

Effect of soil V. dahliae infestation density on plant wilting

Levels of verticillium wilt recorded in individual plots are shown in Appendix 4, alongside the soil infestation density of *V dahliae* detected by QPCR before planting. Data are shown as

percentage values to account for slight variation in plant number (around 100 – 120 plants) between plots. Results were examined by analysis of variance to determine the overall effect of site (*V. dahliae* soil infestation density) and cultivar on the incidence of verticillium wilt symptoms.

V. dahliae was detected in only five of the 60 plots yet verticillium wilt symptoms were recorded in 35 plots (12 of those at or below 2% incidence).

No verticillium wilt was recorded at site A1 (0.2 cfu/g baseline *V. dahliae*) at any of the assessments. All of cv. Elsanta died at this site during the cold winter of 2010/2011; plant death was considered to be due to cold damage. Data from this site were excluded from a cross-site analysis.

Results are discussed below initially by individual sites, from the lowest to the highest pre-plant soil infestation density with *V. dahliae* as measured by the agar plate test (Table 6.3), and then across sites. The statistical analysis is shown in Appendix 5.

Site A8 (<0.1 cfu/g; <250 fg/g) – Cheshire

The incidence of wilt symptoms was very low (<2%) in both April and August 2011 and there were no significant differences (P >0.05) between varieties (Table 2.3). Around 20-25% of cv. Elsanta and cv. Florence died over the winter period, significantly more than cv. Symphony (10%) (P = 0.002). This difference is probably due to different susceptibilities to cold damage and not to delayed plant death due to infection by *V. dahliae* in 2010 as the incidence of verticillium wilt symptoms was low in both years.

Site A1 (0.2 cfu/g; <250 fg/g) – Lancashire

There was a very high incidence of plant death over the winter period at this site and the surviving plants were struggling at the first assessment in April 2011. The wilt symptoms recorded in April were probably not due to verticillium wilt – no wilting was observed in August and no *V. dahliae* was confirmed in a sample of plants tested for the fungus. As at site A8, the cv. Elsanta and cv. Florence suffered worse over winter, with over 90% and 60% dead respectively by April.

Site A7 (0.5 cfu/g; <250 fg/g) – Cheshire

The results at this site were very similar to those of site A8, which was located on the same farm. A low incidence (<2%) of wilt symptoms was recorded in April and August 2011 and there were no significant differences between cultivars. Plant death was slightly less than at site A8.

Site A11 (4.6 cfu/g; 274 fg/g) – Oxfordshire

A low incidence of verticillium wilt was observed in April 2011 (Table 2.3). By July there was a significantly greater incidence of verticillium wilt symptoms in cv. Elsanta (6.8%) than the other two varieties (2.1% or less) (P = 0.04). Typical symptoms assessed as Verticillium wilt are shown in Figure 6.3. By November this had increased further, with 21.0%, 5.5% and 7.8% in cv. Elsanta, cv. Symphony and cv. Florence respectively (P = 0.02). The incidence of wilting, dead and missing plants was significantly greater in cv. Elsanta than the other two cultivars in April (P = 0.001), August (P <0.001) and November (P <0.001) (Appendix 5). At this site there was a low incidence of plant death over winter and the category wilted + dead + missing is probably a good reflection of the accumulated plant losses to verticillium wilt over the two years of the trial. *V. dahliae* was confirmed in one, though not the majority of dead or wilting plants which were removed from the trial and tested for infection by isolation onto agar from crown tissue.

The incidence of cv. Fenella plants recorded either with wilt symptoms or dead was 0, 5 and 11% in April, July and November 2011 respectively. These values are less than those recorded in cv. Elsanta (1, 8 and 22%) and similar to cv. Symphony (0, 2 and 6%) and cv. Florence (1, 2 and 8%). Two cv. Fenella plants with wilt symptoms were sampled in November 2011 and *V. dahliae* was confirmed in one of them. The pre-planting soil infestation densities of *V. dahliae* was not determined for these two plots; the overall value for the field trial site was 4.6 cfu/g (<250 fg/g). These results suggest that cv. Fenella is more resistant to verticillium wilt than cv. Elsanta, and similar to cv. Symphony and cv. Florence.

Site A12 (5.7 cfu/g; 468 fg/g) – Oxfordshire

This site, which had the highest level of *V. dahliae* detected in soil pre-planting (from the conventional agar plate test), developed the highest incidence of plants with verticillium wilt symptoms. The incidence of verticillium wilt symptoms was 3.1 - 9.1% in April, 10.6 - 20.7% in July and 21.2 - 41.6% in November (Appendix 2). Symptoms observed at this site are shown in Figure 6.4. There was no significant difference (P >0.05) between cultivars at any of the assessment dates. There was also no significant difference between cultivars in the incidence of wilting plus dead plants (taken as a measure of current season verticillium wilt) or of wilting plus dead plus missing plants (taken as a measure of accumulated verticillium wilt). Although cv. Elsanta is widely recognised as being highly susceptible to verticillium wilt when compared with other cultivars it is probable that this was not demonstrated at this site due to the limited plot replication and varying infestation densities of *V. dahliae* in the soil. At the final assessment in November, the incidence of wilting plus dead plus missing

plants was 54.9, 29.8 and 25.6% respectively for cv. Elsanta, cv. Symphony and cv. Florence, reflecting their rank order of susceptibility to verticillium wilt; this difference was not quite significant at the 95% level (P = 0.097).

Cross site analyses

The combined data were examined to determine the overall effect of site, cultivar and assessment date on occurrence of verticillium wilt symptoms. Data for site A1 (winter kill) and November assessments (done at sites A11 and A12 only) were excluded in order to maintain a balanced data set.

The specific site, as expected, had a significant effect (P<0.001) on levels of verticillium wilt recorded for all three assessment categories (wilt only, wilt plus dead plants, wilt plus dead plus missing plants) (Table 6.4). For the categories wilt only and wilt plus dead plants, site A12, which had the highest overall soil infestation of *V. dahliae* pre-planting (5.7 cfu/g), showed a much greater incidence of symptoms than the other three sites. The high incidence of wilt plus dead plus missing plants at site A8 reflects plant death over winter in cv. Elsanta and cv. Florence at this site, probably due to the very cold winter temperatures. Site A11, which had the second highest level of *V. dahliae* soil infestation pre-planting, showed the second highest levels of wilted and wilted plus dead plants. The fact that sites A8 and A7 showed similar levels of wilt, despite differing levels of *V. dahliae* soil infestation pre-planting (as determined by the conventional agar plate test), may be due to the low level of replication at individual sites or a patchy distribution of soil infestation. Alternatively, it may indicate that the agar plate test was incorrect and that the QPCR test, which reported *V. dahliae* was below limit of detection at these sites, was the better predictor of verticillium wilt risk (i.e. the low level of wilt symptoms recorded at these two sites was not due to verticillium wilt).

Cultivar had a significant effect on the incidence of verticillium wilt, wilt plus dead plants, and wilt plus dead plus missing plants (P <0.001) (Table 6.4). Cv. Elsanta was significantly more susceptible than cv. Florence, which was itself more susceptible than cv. Symphony. The identification of cv. Elsanta as the most susceptible variety of the three is consistent with previous field observations, but the greater susceptibility of cv. Florence compared with cv. Symphony is contrary to previous field observations. This difference between cv. Florence and cv. Symphony was not statistically significant at any of the four individual sites where verticillium wilt was confirmed, although the trend was in this direction at all four sites. It would be useful to compare the susceptibility of the two cultivars using a standard inoculum of *V. dahliae* to determine more definitively if and how they differ.

Assessment date, as expected, had a significant effect on incidence of verticillium wilt symptoms, with higher levels, of all assessment categories, in July/August than April (Table 6.4), reflecting the greater time for disease development within plants to a point where water supply to leaves is restricted and wilt symptoms develop.

Table 6.3. Effect of variety and pre-planting soil infestation density with *V. dahliae* on occurrence of verticillium wilt symptoms and plant death in strawberry at five field sites – 2011

Cultivar	April			July/August		
	Mean % plants:			Mean % plants:		
	Wilt	Wilt + Dead	W+D+M	Wilt	Wilt + Dead	W+D+M
Site A8						
Elsanta	0	0	23.4	1.3	1.3	24.5
Symphony	0.3	0.3	9.8	0.8	0.8	10.3
Florence	0.4	0.4	27.0	1.0	1.0	27.5
Significance (72 df)	NS	NS	0.002	NS	NS	0.004
Site A1						
Elsanta	76.6	76.6	91.0	-	-	100
Symphony	4.5	4.5	18.1	0	-	13.8
Florence	34.9	34.9	61.8	0	-	43.5
Significance (72 df)	0.002	0.002	0.006	-	-	<0.001
Site A7						
Elsanta	0.3	0.3	14.0	1.1	1.4	14.9
Symphony	0	0	3.8	0.2	0.3	4.0
Florence	0.3	0.3	10.2	0.6	0.6	10.5
Significance (72 df)	NS	NS	NS	NS	NS	NS
Site A11						
Elsanta	1.0	1.1	15.1	6.8	8.0	21.3
Symphony	0	0	2.2	1.6	1.6	3.8
Florence	1.3	1.3	3.2	2.1	2.1	4.1
Significance (72 df)	NS	NS	0.001	0.04	<0.03	<0.001
A12						
Elsanta	5.8	5.8	20.7	33.7	42.1	54.0
Symphony	3.1	3.1	10.6	16.3	16.9	24.3
Florence	9.1	9.1	13.5	46.9	46.9	50.8
Significance (72 df)	NS	NS	NS	NS	NS	NS

W-wilted; D-dead; M-missing.

Table 6.4. Overall effect of site (pre-planting soil infestation density with *V. dahliae*), cultivar and assessment date on occurrence of verticillium wilt symptoms and plant death in strawberries at four field sites – 2011

Factor and initial *V.dahliae* level (cfu/g)	Mean % plants		
	Wilt	Wilt + Dead	Wilt + Dead + Missing
Site			
A8 (<0.1)	0.6 (0.4)	0.6 (0.4)	20.3 (1.9)
A7 (0.5)	0.4 (0.3)	0.4 (0.3)	9.6 (1.4)
A11 (4.6)	2.0 (0.5)	2.2 (0.6)	8.2 (1.2)
A12 (5.7)	18.2 (1.6)	19.6 (1.7)	28.3 (2.1)
Significance (72 df)	<0.001	<0.001	<0.001
Cultivar			
Elsanta	6.0 (0.9)	7.2 (0.9)	23.2 (1.7)
Symphony	2.7 (0.6)	2.8 (0.6)	8.4 (1.1)
Florence	7.0 (0.8)	7.1 (0.9)	17.6 (1.5)
Significance (72 df)	0.001	<0.001	<0.001
Assessment date			
April	1.7 (0.4)	1.8 (0.4)	12.6 (1.1)
August	8.9 (0.8)	9.8 (0.8)	20.4 (1.3)
Significance (72 df)	<0.001	<0.001	<0.001

Standard errors are shown in parentheses.

Effect of soil inoculum density in individual plots (measured in late 2009/early 2010) on verticillium wilt symptoms in 2011

The occurrence of verticillium wilt symptoms plus dead plants (i.e. current season wilt) in 2011 for individual plots is given in Appendix 4. Data for site A1 is excluded due to the high incidence of winter kill at this site.

At sites A8 and A7, no *V. dahliae* was detected pre-planting by QPCR in any of the 24 plots. Although verticillium wilt symptoms were recorded in 14 of the plots at one or both assessment dates, only 1-2% of plants were affected. It is possible that this wilting was largely due to a factor other than verticillium wilt; if so the QPCR test pre-planting was a good measure of verticillium wilt risk within two years of planting.

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

pre-planting, verticillium wilt symptoms were recorded in all plots at levels ranging from 2% to 55% of plants. Considering only levels of wilt above 10% in July (on the possibility that low levels of wilt symptoms may have been caused by a factor other than *V. dahliae* infection), then the QPCR test accurately identified the single plot in 12 at site A11 with a high risk of wilt. However, using the same criterion at site A12, the QPCR accurately predicted risk in four plots but failed to predict the high level of wilt in seven of the 12 plots.

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

7. General discussion

The basic hypothesis underlying this project is that there is a relationship between *V. dahliae* soil infestation density and verticillium wilt in strawberry sufficiently close to be useful to growers making planting decisions. The Harris test, based on wet sieving of soil followed by plating onto a selective agar medium to determine the number of colony forming units (cfu) per gram of soil, has been used for over 30 years by strawberry growers in the UK. Observations by growers and ADAS consultants indicate that the incidence of verticillium wilt in strawberry crops planted on land where *V. dahliae* infestation density was determined pre-planting by the Harris test is generally consistent with the predicted risk. However, these observation data correlating soil infestation density and wilt incidence are skewed to low soil infestation densities as growers do not plant, or sterilise the soil pre-planting, or grow a more tolerant variety where a high infestation density (and high risk of verticillium wilt) is found.

Worldwide there has been work on a number of verticillium wilt susceptible crops (e.g. Acer, olive, potato) seeking to make use of information on *V. dahliae* soil infestation density for better management of verticillium wilt diseases (Goud *et al*, 2011; Jiménez-Díaz *et al*, 2012). Conclusions on the ability to correlate soil infestation density with wilt incidence have varied; no single test procedure has been widely adopted worldwide. There is now increasing interest in the use of molecular methods to quantify fungal pathogens in soil, including *V. dahliae*. Potentially, quantification of *V. dahliae* in soil by PCR has some distinct advantages over quantification by wet sieving and soil plating: a high level of specificity; a high level of sensitivity; ability to detect non-sclerotia-forming *Verticillium* spp., a rapid test and reduced cost.

There are numerous factors that influence the degree of reliability that can be achieved when seeking to predict verticillium wilt incidence in a crop from soil infestation density of *V. dahliae*. Some of these factors are unique to one test method; others are common to different test methods. Some of these factors can be allowed for, to a greater or lesser extent, when interpreting test results; other factors are unknown in their effect and cannot (at present) be allowed for. There may also be factors we are unaware of that influence the relationship between soil infestation density of *V. dahliae* and the risk of verticillium wilt. Table 7 lists the major factors we consider to influence the relationship between *V. dahliae* soil infestation density and verticillium wilt, identifies those that apply to the Harris test and those to a PCR test, and notes where mitigation measures can be applied to reduce variation when seeking to predict verticillium wilt risk.

From Table 7, it can be seen that most variables apply equally to the Harris test and the PCR test. Particular advantages of the PCR test are its specificity, speed and ability to run a concurrent test for *V. albo-atrum*. Current disadvantages, compared with the Harris test, are reduced sensitivity and detection of non-viable pathogen. Work is in progress elsewhere to increase test sensitivity (e.g. EU project 246140 on olive wilt); detection of non-viable pathogen is considered to introduce little variation due to its rapid degradation in soil. It is not know whether measurement of total DNA content, rather than the number of colony forming units, is an advantage or a disadvantage. If infectivity is closely related to microsclerotial size, then potentially measurement of total *V. dahliae* DNA could relate well to infection risk. However, this is offset by the inability of a PCR test to provide information on the number of infection points (colony forming units) in a soil. One large microsclerotium may result in the same measured quantity (fg/g) of *V. dahliae* in a soil as 10 small microsclerotia occurring at separate points in the soil. The latter might result in a tenfold greater infection risk than the former.

Further information is required on the profile of microsclerotial size distribution in soils and the infectivity of microsclerotia of different sizes to help assess the influence of the variables when relating *V. dahliae* infestation density to risk of verticillium wilt. If the range of microsclerotial sizes in soils naturally infested is relatively narrow (e.g. less than two-fold), then quantification of *V. dahliae* DNA may still relate well to risk. But if the range of microsclerotial size is quite large, and size has little influence on infectivity, then it is probable that measurement of *V. dahliae* DNA content may, in some soils, relate poorly to infection risk (e.g. a few large microsclerotia may result in an overestimate of risk). One possible way to compensate for risk of overestimation would be to divide the total *V. dahliae* DNA content measured in a soil by the mean DNA content of a small microsclerotium in order to get an estimate of the maximum number of (small) microsclerotia; and by the mean

DNA content of a large microsclerotium to get an estimate of the minimum number of (large) microsclerotia, and express the result as a range (eg A – B cfu/g).

The current project has developed a highly specific, reasonably sensitive PCR assay for *V. dahliae* and a soil test to extract and quantify *V. dahliae* DNA. It has shown that there is a broad relationship between *V. dahliae* soil infestation density measured by PCR and that measured by the Harris test; also, that there is a broad relationship between soil infestation density measured by the PCR test and occurrence of verticillium wilt in strawberry. However, there were a sufficient number of unexplained instances of verticillium wilt symptoms occurring at quite high incidences (>10% of plants) in our plots where no *V. dahliae* was detected pre-planting, to be able to offer the test as a chargeable service, at present, as a reliable alternative to the Harris test. The PCR test is currently useful as a rapid method for testing soils and excluding as planting sites those fields where a high quantity of *V. dahliae* is recorded. In order for the molecular test to be more generally useful, it is likely to be necessary: i) to increase the test sensitivity by a factor of x5-10; ii) to examine the relationship between soil *V. dahliae* infestation, density and verticillium wilt symptoms in strawberry on different soil types; iii) to determine if *V. dahliae* DNA content in a soil relates sufficiently well to occurrence of verticillium wilt when allowance is made for any influence of microsclerotial size. The latter could be investigated by specific experiments and/or by testing a large number of field soils by the PCR test with assessment of the verticillium wilt incidence that develops in associated commercial crops of susceptible strawberry varieties planted on these sites.

Table 7. Factors influencing prediction of verticillium wilt risk in strawberry based on quantification of *V. dahliae* (Vd) soil infestation density by two different methods

Variation introduced by:	Risk (✓) applies to:		Mitigation measures
	Harris test	PCR test	
<u>Soil sampling</u>			
1. Non-uniform distribution of Vd across a field	✓	✓	Sample many points in a suitable pattern.
2. Non-uniform distribution of Vd with soil depth	✓	✓	Sample to root depth of intended crop after last soil cultivation
3. Decay of Vd infested crop debris with time	✓	✓	Repeat test over time.
4. Spread of Vd by soil cultivation	✓	✓	Minimise cultivations between sampling and planting
5. Spread of Vd in soil water	✓	✓	

Pathogen variation

6. Quantification of non-viable DNA	-	✓	Run a rRNA test to check?
7. Differences in age/infectivity of microsclerotia	✓	✓	
8. Vd strains differ in pathogenicity to strawberry	✓	✓	
9. Size of microsclerotia influences infectivity	✓	(✓)	PCR test would take account of this if good relationship
10. Microconidia of Vd are quantified (overestimate of risk?)	✓	✓	
11. Infection is by *V. albo-atrum* not *V. dahliae*	-	(✓)	Run a PCR test for Vaa in parallel

Test procedure

12. Soil sieving breaks apart large microsclerotia	✓	-	Use a standard method at one lab.
13. Test provides no information on number of infective units	-	✓	Estimate equivalent minimum and maximum of cfu/g soil (see discussion)
14. Variation in DNA extraction efficiency between soils	-	✓	Use an internal control
15. Variation in copy number of DNA target sequence between cells	-	✓	
16. Test sensitivity is too low	-	✓	Improve DNA extraction from soil?
17. Change in soil Vd population between sampling and result	(✓)	-	Probably small
18. Growth of Vd is inhibited by other microorganisms	(✓)	-	Use selective agar
19. Mistaken identification of Vd by assessor	(✓)	-	Use trained staff and a control (known infestation density) soil at each run

Infection

20. Soil physical characters (pH, EC, texture, minerals)	✓	✓	
21. Soil climate (temperature, moisture)	✓	✓	
22. Soil microbiology	✓	✓	

23. Soil nematodes	✓	✓	Measure soil population of pathogenic nematodes
Host			
24. Cultivars differ in susceptibility	✓	✓	Interpret results according to varietal susceptibility

✓- identified risk of variation; (✓) - possible risk of v

Conclusions

PCR assays

1. PCR assays specific for *V. dahliae* and *V. albo-atrum* are now available.
2. The PCR assay for *V. dahliae* can detect down to 147 fg DNA of the fungus; the assay for *V. albo-atrum* can detect down to 740 fg of the fungus.

Extraction of DNA from soil

3. A soil DNA extraction method was devised that can extract total DNA from soil samples of up to 50 g; the quantity of DNA extracted did not increase with soil samples greater than 50 g (up to 250 g).
4. The use of an internal control based on the quantification of ubiquitous soil inhabiting bacteria, *Streptomyces* spp, provided a measure of the DNA extraction efficiency.

Testing V. dahliae soil infestation density by QPCR

5. Under experimental conditions there was a strong and positive logarithmic relationship between the number of cultured microsclerotia added to a loam-based soil (estimated at a high concentration and prepared by soil dilution) and the amount of *V. dahliae* DNA detected by QPCR.
6. The assay was able to detect down to around 1 microsclerotia/g soil (543 fg/g) with one isolate of *V. dahliae* in 2010 and to 0.5 microsclerotia/g soil (380 fg/g) with another isolate in 2011. The test did not detect *V. dahliae* at 0.5 microsclerotia/g in 2010 or 0.25 in 2011.

Correlation of QPCR and Harris soil tests for V. dahliae

7. QPCR and Harris tests on 51 soil samples showed reasonably good agreement (R^2 = 67%).

8. Detection of pathogen DNA by QPCR falls off sharply below 1 microsclerotia/g soil; the Harris test limit of detection is 0.1 microsclerotia/g soil.

Correlation of V. dahliae soil infestation density and wilt symptoms

9. Correlation of *V. dahliae* soil infestation density measured by QPCR and verticillium wilt symptoms in pot grown strawberries was poor. A significant positive correlation was found between *V. dahliae* soil infestation density measured by QPCR and verticillium wilt symptoms in a pot test with cv. Elsanta in 2010 but not in 2011, and not with other varieties in either 2010 or 2011.

10. Correlation of *V. dahliae* soil infestation measured by QPCR and verticillium wilt symptoms in field-grown strawberry was moderately good. When five fields were tested by multiple (12) QPCR tests to obtain a mean infestation density of *V. dahliae*, the test correctly identified two fields which subsequently developed a high incidence of verticillium wilt (>10% of plants) and did not detect the fungus at two sites which developed a low incidence of wilt (<2% of plants); results at a fifth site were unclear.

11. The above results indicate that multiple tests of a field soil by QPCR are required to get a reasonably accurate estimate of *V. dahliae* soil infestation density; and that the sensitivity of the current test needs to be increased (x5 to 10) in order for the test to be of use in informing decisions on planting highly susceptible varieties, such as cv. Elsanta.

12. Further work is needed to determine the sampling strategy needed to inform the relationship between level of *V. dahliae* inoculum (as determined by the QPCR test) in soil and strawberry wilt risk across a range of soil types.

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Appendix 1. Details of *Verticillium dahliae* and *V. albo-atrum* PCR assay sequences.

Target/Name	Description	Sequence (5′–3′)
V. dahliae		
Vda-CF	Forward primer	GTCTTTGTCGGAGACGGAGAAC
Vda-CR	Reverse primer	CAGAGGGTCGGATAGCCATGT
Vda-CP	FAM-TAMRA labelled Probe	CGAGTTTCGAATCTTGTTGCCCATCG
V. albo-atrum		
Vaa-CF	Forward primer	CATCGCCAATCGACAACATG
Vaa-CR	Reverse primer	CATCGTCCAGGTGACCTGAA
Vaa-CP	FAM-TAMRA labelled Probe	CCACCCGAACCTCTGTCTCGCTTTT

Appendix 2. Crop diary for pot experiment – ADAS

Trial Task	Date completed
Soil collected from Liss, Hampshire	01/12/2009
Soil samples sent to Jeff Peters (FERA) for testing verticillium levels	07/12/2009
Soil samples sent to ADAS High Mowthorpe to confirm low levels verticillium	18/03/2010
Soil samples taken at Stanton St John	04/05/2010
Strawberry runners collected from supplier	10/05/2010
Results obtained from FERA showed high levels of verticillium for Stanton St john samples	12/05/2010
Soil concentrations mixed and T1 and T2 plants potted	13/05/2010
Plants potted and trial set up in polytunnel	14/05/2010
Systhane spray applied to control powdery mildew infection	23/06/2010
Dosatron set up to feed 1kg per 10L Sangral 3:2:6 at 1:100 dilution	23/06/2010
Potassium bicarbonate applied to control powdery mildew	28/06/2010
Picking commenced	30/06/2010
Systhane spray applied to control powdery mildew	05/07/2010
Stunting observed in plants with high verticillium treatments	05/07/2010
Potassium bicarbonate applied to control powdery mildew	13/07/2010
Systhane applied to control powdery mildew	16/07/2010
Nimrod applied for powdery mildew and Dipel DF for caterpillars	23/07/2010
Final picking and Fortress spray applied for powdery mildew	30/07/2010
Dipel DF spray applied for caterpillars	05/08/2010
Feed reduced to 0.8kg/10L	12/08/2010
5 plants sampled from each rep of treatment 1 and 6 and samples plated	17/08/2010

Potassium bicarbonate spray applied to control powdery mildew	24/08/2010
Plated samples examined – no verticillium	25/08/2010
Runner and stolon counts carried out	27/08/2010
Feed rate increased to 2kg in 10L	03/09/2010
Plated samples examined – no verticillium	24/09/2010
Runners removed from plants	30/09/2010
Plants assessed for yellowing and necrotic leaves	06/10/2010
Feeding reduced to 0.8kg/10L. Die-back observed on some plants	11/10/2010
Feeding stopped and watering reduced. Plants showing die-back for winter	01/11/2010
Plants trimmed back.	21/2/2011
Noticed vine weevil infestation.	
Soil sample taken – high levels of nutrients left from last year, EC also at a high level.	
Plants cut back.	25/2/2011
Vine weevils found; some crowns very badly damaged	
Plots 1, 2, 4, 5 14, 21 have one dead plant per plot. Plot 6 has two dead plants and plot 9 has three dead plants.	28/2/2011
Drained down and flushed slow drip irrigation.	2/3/2011
Treated for slugs with Draza.	
Soil drench for vine weevils with Nemasys L (62ml per pot)	11/3/2011
Re-treated all pots with Nemasys L after frost at the weekend.	15/3/2011
Cut back plants.	18/3/2011
Soil sample taken.	

Removed dead plants. Destructive assessment of dead plants to find the cause. Samples taken from12 plants plated out onto PDA + S. Most rot appears to be associated with vine weevil damage and frequently caused a firm black rot to spread throughout the entire crown.	6/4/2011
Assessed plants for necrotic leaves, yellowing and wilting. Plants were well watered before assessment but were wilting at the end of the day which had been warm and sunny.	
Agar plates checked and no verticillium present. Other fungi probably secondary to vine weevil damage.	18/4/2011
Assessed plants for necrotic leaves, yellowing and wilting. Fifteen plants with leaf necrosis examined for *V. dahliae* by plating onto agar.	31/5/2011
Five plants from each of T5 and T6 sent to Fera for *V. dahliae* test by QPCR.	2/6/2011
Nil *V. dahliae* recovered from crowns of plants sampled: Plots 1 (x2), 2, 4 (x2), 5 (x2), 6, 8, 12, 13, 14, 18, 21 and 24.	4/7/2011

Appendix 3. Crop diaries for field sites – ADAS

Trial Task	Date completed
Soil samples taken from fields and sent to ADAS High Mowthorpe for testing	03/08/2009
Trial sites marked and additional samples taken from, Cheshire	02/12/2009
Sites A and B planted, Cheshire	09/04/2010
Damp chambers and plate tests for 150 samples of strawberry runners	14/04/2010
All samples in damp chambers and on plates assessed for verticillium and phytophthora	20/04/2010
Soil samples taken in two fields at Stanton St. John. Samples sent to Jeff Peters at FERA for molecular testing.	04/05/2010
Sites planted at Warrington	14/05/2010
Trials planted at Stanton St. John	03/06/2010
Cv. Fenella plants planted in Bucks and soil samples taken	16/06/2010
Plants counted at Stanton St. John to assess extent of bird damage and plant loss due to heat	25/06/2010
Cheshire and Warrington sites assessed – no verticillium observed	28/07/2010
Trials assessed at Stanton St. John and plant samples collected	04/08/2010
Samples from Stanton St John assessed on PDA – no verticillium observed	27/09/2010
September assessments at Cheshire and Warrington sites – only one plant with possible wilt symptoms but typical wilt observed in plants adjacent to trials.	27/09/2010
Stanton St John sites assessed – some possible wilt symptoms and plant samples taken	18/10/2010
Samples plated on PDA and some sent to Jeff Peters at FERA	22/10/2010
Samples from Stanton St. John assessed – clear colonies of *Verticillium dahliae* on samples from plots 3,4,6,8 and 12	03/11/2010
Assessment of sites A11 and A12 (JA). Plants growing away well, a few flowering. No dead plants found. Plant recorded as 'wilt' where there is weak, pale green growth, uneven growth or actual wilting.	22/4/2011
Sites A1, A7 and A8 assessed (CC).	22/4/2011
Sites A11 and A12 visited. Cv. Elsanta in full pick and fruit ripening on cv. Symphony and cv. Florence.	29/5/2011
Site A12 assessed (JA). Photographs of plants with wilting symptoms. Cv. Florence still being harvested.	13/7/2011
Site A11 assessed (JA). All plants had been topped; healthy	22/7/2011

ones were growing actively. Photographs of plants with wilt symptoms.	
Site A1 assessed (CC). Cv. Elsanta all died but not with wilt.	18/8/2011
Site A7 and A8 assessed (CC).	28/8/2011
Four plants from site A7 and two plants from site A8 sent to Fera for QPCR test for *V. dahliae*.	12/9/2011
V. dahliae confirmed in plants from site A8 (2/2 plants), not at site A7 (0/4 plants) by Fera.	7/10/2011
Site A12 assessed (JA). Wilted plants collected from plots 1, 2, 4, 5, 7 and 11.	16/11/ 2011
Site A11 assessed (JA). Wilted plants collected from plot 4, 6, 9, 10 and cv. Fenella (extra plot).	25/11/2011
Sites A8 and A7 visited (CC). One plant only, plot 2 site A7 (cv. Florence), with wilt symptoms.	29/11/2011
Isolation plates from sites A12 samples (16 Nov) and A11 samples (25 Nov) examined.	14/12/2011
V. dahliae confirmed at site A12, plot 1, cv. Elsanta (nil in 12 other plants) and at site A11 in cv. Fenella (extra plot at end of trial bed). No phytophthora found.	

Summary of plants tested for *V. dahliae* in 2011

Site	Date plants sampled	Number plants tested	Number plants positive:	
			Isolation	QPCR
A8 (<0.1 cfu/g)	Aug	2	0	2
A7 (0.5 cfu/g)	Aug	4	1	0
A11 (4.6 cfu/g)	Nov	10[a]	1	NT
A12 (5.7 cfu/g)	Aug	6	1	NT
A12 (5.7 cfu/g)	Nov	13[a]	1	NT

[a] *Botrytis cinerea* and *Cylindrocarpon destructans* isolated quite frequently from crown tissue.

NT – not tested.

Appendix 4. Individual plot V. dahliae *soil infestation density and wilt*

Verticillium inoculum densities in soil sampled from field plots and corresponding losses to presumptive verticillium wilt – 2011 (two sites with low incidence of wilt symptoms)

Plot and Cultivar	*V. dahliae* (fg/g)	Mean % plants affected (wilt + dead) [a]	
		22 April	24 Aug
Site A8 (<0.1 cfu/g)			
1 Florence	<250	0	2
2 Elsanta	<250	0	1
3 Symphony	<250	1	0
4 Elsanta	<250	0	0
5 Florence	<250	0	1
6 Symphony	<250	0	2
7 Symphony	<250	0	1
8 Elsanta	<250	0	2
9 Florence	<250	1	0
10 Florence	<250	0	0
11 Symphony	<250	0	0
12 Elsanta	<250	0	1
Site A7 (0.5 cfu/g)			
1 Elsanta	<250	0	4
2 Florence	<250	1	2
3 Symphony	<250	0	0
4 Florence	<250	0	0
5 Symphony	<250	0	1
6 Elsanta	<250	1	0
7 Symphony	<250	0	0
8 Elsanta	<250	0	1
9 Florence	<250	0	0
10 Symphony	<250	0	0
11 Florence	<250	0	0
12 Elsanta	<250	0	0

[a] Estimate based on a planting of around 100 plants per plot, now grown into a matted bed. No wilt symptoms were observed in 2010 at either site. Data for site A1 are not presented;

this site suffered a high incidence of plant death over winter and the number of surviving plants was too few for an accurate assessment of wilt symptoms.

Verticillium inoculum densities in soil samples from field plots and corresponding losses to presumptive verticillium wilt in 2010 and 2011 (two sites with a high incidence of wilt symptoms)

Plot and Cultivar	*V. dahliae* (f/g)	Mean % plants affected (wilt + dead)				
		4 Aug 10	18 Oct 10	22 Apr 11	13/22 Jul 11	16/25 Nov 11
A11						
1. Elsanta	<250	17.8	3.0	0	3.7	6.4
2. Symphony	<250	0	4.6	0	3.7	8.4
3. Florence	<250	0	2.7	0.9	0.9	2.8
4. Elsanta	480	14.4	8.6	0	21.6	48.2
5. Florence	<250	1.8	6.3	4.2	7.6	22.2
6. Elsanta	310[†]	13.8	10.1	2.0	5.2	21.4
7. Symphony	<250	1.0	2.0	0	0	3.9
8. Florence	<250	1.9	1.9	0	0	2.8
9. Symphony	<250	0	0	0	2.7	6.4
10. Florence	<250	0.9	10.1	0	0	4.5
11. Symphony	<250	0	3.7	0	0	3.6
12. Elsanta	<250	5.5	5.5	1.8	2.1	10.1
A12						
1. Elsanta	<250	2.0	28.3	11.9	49.4	54.7
2. Symphony	<250	6.0	6.0	3.1	22.2	34.0
3. Florence	700	0	29.6	15.1	48.5	37.5
4. Elsanta	<250	20.5	7.2	8.8	32.9	52.9
5. Symphony	<250	1.9	2.9	0.9	5.0	6.8
6. Florence	<250	1.0	13.3	9.6	29.8	23.3
7. Symphony	1650	1.9	8.7	4.9	17.3	23.6
8. Florence	<250	1.0	19.0	6.7	38.3	15.0
9. Elsanta	520	11.4	13.3	1.8	31.0	32.7
10. Symphony	<250	2.9	6.9	3.9	15.0	26.2
11. Elsanta	2620	8.2	5.1	2.1	19.8	17.2
12. Florence	<250	1.8	6.1	5.2	11.7	10.9

[†] *Verticillium* sp, not *V. dahliae.*

Appendix 5. Effect of variety and site on verticillium wilt - 2011

Effect of variety and pre-planting soil infestation density with *V. dahliae* on occurrence of verticillium wilt symptoms and plant death in strawberry at five field sites – 2011

Cultivar	April			July/August		
	Mean % plants:			Mean % plants:		
	Wilt	Wilt + Dead	W+D+M	Wilt	Wilt + Dead	W+D+M
Site A8						
Elsanta	0	0	23.4 (2.2)	1.3 (0.9)	1.3 (0.8)	24.5 (2.6)
Symphony	0.3 (0.2)	0.3 (0.2)	9.8 (1.5)	0.8 (0.6)	0.8 (0.6)	10.3 (1.8)
Florence	0.4 (0.2)	0.4 (0.2)	27.0 (2.2)	1.0 (0.8)	1.0 (0.8)	27.5 (2.7)
Significance (72 df)	NS	NS	0.002	NS	NS	0.004
Site A1						
Elsanta	76.6 (8.6)	76.6	91.0 (5.9)	-	-	100
Symphony	4.5 (2.8)	4.5	18.1 (8.1)	0	-	13.8 (4.4)
Florence	34.9 (7.9)	34.9	61.8 (10.1)	0	-	43.5 (6.3)
Significance (72 df)	0.002	0.002	0.006	-	-	<0.001
Site A7						
Elsanta	0.3 (0.2)	0.3 (0.2)	14.0 (4.7)	1.1 (0.6)	1.4 (0.6)	14.9 (4.7)
Symphony	0	0	3.8 (2.6)	0.2 (2.8)	0.3 (0.2)	4.0 (2.6)
Florence	0.3 (0.2)	0.3 (0.2)	10.2 (4.1)	0.6 (0.4)	0.6 (0.4)	10.5 (4.1)
Significance (72 df)	NS	NS	NS	NS	NS	NS
Site A11						
Elsanta	1.0 (0.7)	1.1 (0.7)	15.1 (2.0)	6.8 (1.6)	8.0 (1.9)	21.3 (2.2)
Symphony	0	0	2.2 (0.8)	1.6 (0.7)	1.6 (0.8)	3.8 (1.1)
Florence	1.3 (0.7)	1.3 (0.7)	3.2 (0.9)	2.1 (0.8)	2.1 (0.9)	4.1 (1.1)
Significance (72 df)	NS	NS	0.001	0.04	<0.03	<0.001
A12						
Elsanta	5.8 (2.1)	5.8 (2.1)	20.7 (3.4)	33.7 (11.0)	42.1 (11.6)	54.0 (10.6)
Symphony	3.1 (1.5)	3.1 (1.5)	10.6 (2.6)	16.3 (7.2)	16.9 (7.9)	(24.3 (8.7)
Florence	9.1 (2.4)	9.1 (2.4)	13.5 (2.9)	46.9 (10.8)	46.9 (11.7)	50.8 (11.4)
Significance (72 df)	NS	NS	NS	NS	NS	NS

W-wilted; D-dead; M-missing. ( ) – standard error.