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:	Annual report
Previous report	May 2010
Key staff:	Dr Tim O'Neill, ADAS Janet Allen, ADAS Chris Creed, ADAS Harriet Roberts, ADAS Angela Huckle, ADAS Ann Barnes, Fera Charles Lane, Fera
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):	31 March 2012
Key words:	Strawberry, *Verticillium dahliae*, QPCR, soil

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.

Dr Jeff Peters
Fera

Signature ..... Date .....

Dr Tim O'Neill
Principal Research Scientist
ADAS

Signature ..... Date .....

Report authorised by:

Dr Neil Boonham
Fera

Signature ..... Date .....

James Clarke
SCM Science and Business Development Manager
ADAS

Signature ..... Date .....

CONTENTS

GROWER SUMMARY 1

Headline 1

Background and expected deliverables 1

Summary of the project and main conclusions 1

Financial benefits 1

Action points for growers 1

SCIENCE SECTION 6

1. Specificity of *V. dahliae* and *V. albo-atrum* QPCR assays 6

2. Effect of inoculum levels of Verticillium dahliae on development of Verticillium wilt in strawberry plants grown in pots of artificially infested soil 8

3. Effect of inoculum level of *Verticillium dahliae* on development of Verticillium wilt in strawberry plants grown in pots of naturally infested soil 12

4. Effect of soil levels of *Verticillium dahliae* determined by a molecular test on incidence of Verticillium wilt in field crops 17

5. Further validation of QPCR: parallel testing of QPCR and wet sieving methods 23

CONCLUSIONS 24

TECHNOLOGY TRANSFER 24

REFERENCES 24

APPENDIX 1 CROP DIARY FOR POT EXPERIMENT – ADAS 25

APPENDIX 2 CROP DIARIES FOR FIELD SITES 26

APPENDIX 3 INDIVIDUAL PLOT DATA AT SITES A11 AND A12 27

GROWER SUMMARY

Headline

- Good progress is being made in developing and validating a new molecular test for *Verticillium dahliae* and *V. albo-atrum* in field soils.

Background and expected deliverables

The current method for detecting and quantifying *V. dahliae* in soils takes 6-8 weeks from sample receipt to reporting. This method relies on wet sieving soil and plating onto culture medium. Colonies that resemble *V. dahliae* growing from the resting structures, microsclerotia, are counted. These counts are used to provide information on risk of wilt arising from the soil at the intended planting site. However, this method of testing is not able to detect and quantify *V. albo-atrum*. The proposed molecular, QPCR test, will quantify the amount of target pathogen DNA in a few days for around half the price of the conventional test. Additionally, the molecular test is capable of detecting *V. albo-atrum*. In this year's reporting period, the main objective was to determine the relationship between soil levels of *V. dahliae* and *V. albo-atrum*, as measured by QPCR, and the severity of wilt symptoms in strawberry plants grown in pot trials as well as the incidence of verticillium wilt in strawberries grown in the field. The ultimate aim is to provide a rapid, reliable commercial test to growers.

Summary of the project and main conclusions

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

Following further validation work, the assays have been shown to detect only the target pathogen species. So far the assays have not failed to detect *V. dahliae* or *V. albo-atrum* isolated from strawberry. Validation work will continue in year three.

Effect of adding different levels of artificial inoculum to soil on Verticillium wilt – pot experiment

Verticillium dahliae

A pot experiment was set up to investigate the relationship between growing young strawberry plants in soils artificially amended with differing levels of microsclerotia of *V.*

dahliae with wilt symptoms. Six weeks after inoculation, the mean level of wilt as measured by leaf necrosis that developed in cv. Elsanta generally increased with increasing levels of *V. dahliae* inoculum. The assay failed to detect pathogen at the lowest inoculum level (corresponding to 1:160,000 sand maize-meal culture to compost) at this time. This inoculum level was sufficient to cause low levels of leaf necrosis after 6 weeks.

There was no relationship between inoculum level and severity of wilt symptoms in cvs Florence and Symphony at 6 weeks after planting in infested soil.

Verticillium albo-atrum

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 cv. Elsanta generally increased with increasing levels of *V. albo-atrum* inoculum. The assay failed to detect 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 cvs Florence and Symphony at 6 weeks after planting in infested soil.

Effect of natural soil inoculum level on Verticillium wilt – pot experiment

Soil known to be infested with *V. dahliae* was collected from a fruit farm and diluted with sterile soil to create five infestation densities ranging from 0.8 to 7.6 cfu/g (<250 to 766 fg/g). Pots of these soils were planted with young strawberry plants, cv. Elsanta, in May 2010 in a randomized block experiment with four replicates and 10 plants per plot.

No obvious symptoms of Verticillium wilt developed in 2010, although in October the incidence of dead plants was significantly greater (5% of plants) in the only treatment where *V. dahliae* was detected by QPCR than in all other treatments (0-1% plants dead). No *V. dahliae* was isolated from lower leaf petiole or runners sampled from plants in the high infestation soil in August and September. Further checks for Verticillium wilt will be done in 2011.

Effect of soil inoculum level on Verticillium wilt – field experiment

The aim of this experiment was to examine the correlation between soil infestation densities of *V. dahliae* on naturally infested field sites, with Verticillium wilt symptoms that developed

in the first two years after planting. Replicated plots of three varieties differing in susceptibility to Verticillium wilt (Elsanta, Symphony and Florence) were established in spring 2010 in five fields (2 in Cheshire, 1 in Lancashire and 2 in Oxfordshire) that ranged in infestation density from <250 to 560 fg/g DNA (<0.1 to 5.7 cfu/g) of *V. dahliae*. The plants at all sites were from the same supplier and a sample tested before planting was found to be free of *V. dahliae*.

Verticillium wilt symptoms occurred at only two of the sites in 2010 (Table 1). At both of these sites, the incidence of Verticillium wilt in August was significantly greater in cv. Elsanta (10-13% plants affected) than in cvs Symphony and Florence (0.9 – 2.3% plants affected), reflecting the high susceptibility of cv. Elsanta.

When comparing the five sites, wilt only occurred in fields which had microsclerotia levels above 0.5 cfu/g soil when tested in December 2009 by the agar plate (Harris) soil test for *V. dahliae*. The QPCR test (done in December 2009) detected inoculum in one of the two sites where wilt developed and detected inoculum at one site where wilt failed to develop. However, when the sites were re-tested one month prior to planting (in April/May 2010), the QPCR test only detected *V. dahliae* at sites where wilt developed. When comparing individual plot data at the two sites where verticillium wilt developed, QPCR tests detected *V. dahliae* in five plots, and in four of these plants developed symptoms of Verticillium wilt at levels ranging from 2-11% in August. QPCR did not detect inoculum in 18 plots. Plants in 17/18 of those plots developed some degree of wilt. However, the incidence of wilt was generally low: in 14 of the 18 plots, wilt was present in less than 10% of plants. This experiment is continuing in 2011.

Table 1. Occurrence of Verticillium wilt in five fields of strawberry differing in soil infestation density of *V. dahliae* – 2010

Site	**County**	**Field soil density of *V. dahliae* in 2009**		**Range of plot soil densities of *V. dahliae* in Apr/May 2010**	**Verticillium wilt symptoms in August 2010**
		cfu/g	*fg/g*	*fg/g*	
A8	Cheshire	<0.1	<250	<250	No
A1	Lancs	0.2	560	<250	No
A7	Cheshire	0.5	<250	<250	No
A11	Oxon	4.6	<250	<250-480	Yes
A12	Oxon	5.7	468	<250-2620	Yes

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 results presented in Fig 1 show that there was a reasonable agreement (R^2=0.76) between the two methods.

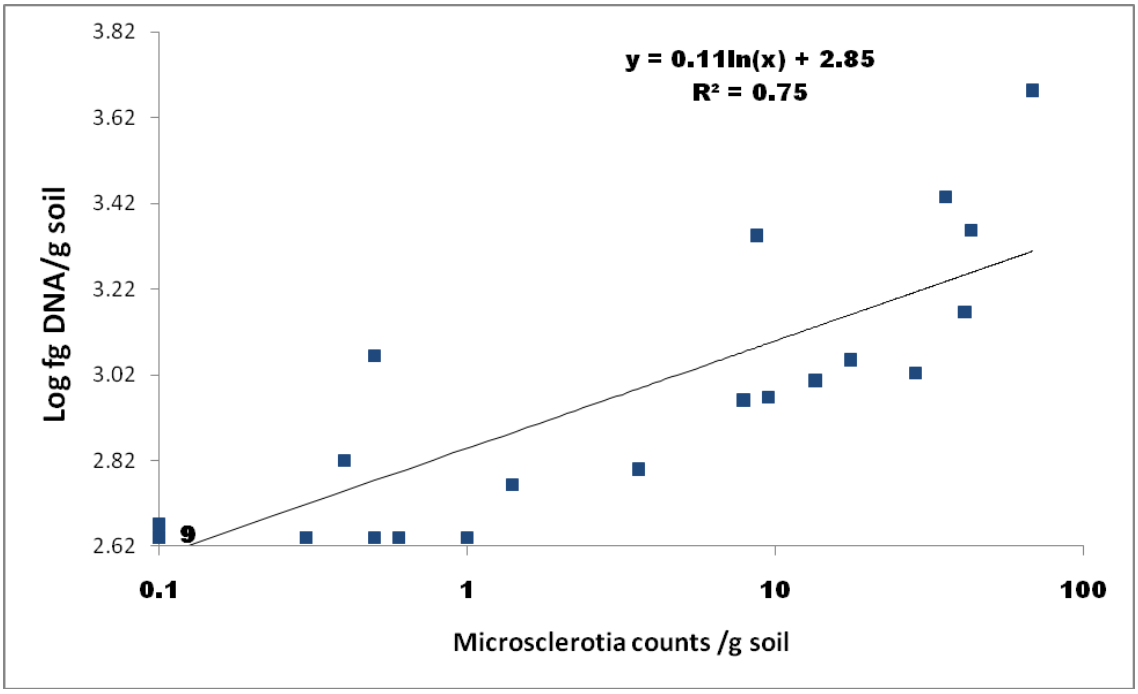


Figure 1. Correlation between microsclerotial counts and DNA levels when commercial samples were tested by QPCR (DNA/g soil)) and wet sieving (microsclerotia counts/g soil, log scale).

Conclusions

- The molecular (QPCR) tests for *Verticillium dahliae* and *V. albo-atrum* continue to detect only target pathogen species after further validation.

- Pot trials at Fera demonstrate that there is a good correlation between inoculum (as measured by QPCR) and wilt in the susceptible cultivar, Elsanta. A poor correlation between inoculum and wilt was found in the more resistant cultivars, Florence and Symphony.

- Work is ongoing to measure the extent to which the QPCR test can be used to predict the risk of wilt in field soils. Results to date show that whilst the test is less sensitive than the conventional wet sieving (Harris) method, QPCR testing one month prior to planting only detected verticillium at sites where wilt developed.

- Results from a direct comparison of *V. dahliae* inoculum levels using wet sieving and QPCR testing of commercial samples show that there is a reasonable agreement between methods.

Financial benefits

When a commercial QPCR test becomes available, it will provide growers with a cheaper and more rapid test method for evaluating levels of *Verticillium dahliae* and *V. albo-atrum* in field soils.

Action points for growers

- No action points have arisen from this project yet.

SCIENCE SECTION

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. The assays were shown to be specific to all of the target species tested and did not detect non-target fungi tested. In year 2, this validation work continued by increasing the number of *V. albo-atrum* and *V. longisporum* isolates tested. In addition, 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

Fifteen isolates identified as *Verticillium* species were selected because there was a high degree of confidence that these had been correctly identified as *V. dahliae*, *V. albo-atrum* or *V. longisporum*. Details of the isolates are given in Table 1.1.

Table 1.1 Information on verticillium isolates used in the phylogenentic investigation

Isolate name	Species	Host	Source
5375	*V. dahliae*	Unknown soil isolate	D. Barbara†
332-1	*V. dahliae*	strawberry	D. Barbara
12070	*V. dahliae*	strawberry	D. Barbara
12080	*V. dahliae*	strawberry	D. Barbara
12701	*V. dahliae*	strawberry	D. Barbara
chrysanthemum	*V. dahliae*	chrysanthemum	Fera
Potato	*V. dahliae*	potato	Fera
OSR-col3	*V. longisporum*	Oil seed rape	Fera
OSR-col7 (clone 1)	*V. longisporum*	Oil seed rape	Fera
OSR-col7 (clone 2)	*V. longisporum*	Oil seed rape	Fera
143	*V. longisporum*	brassica	D Barbara
145	*V. longisporum*	brassica	D Barbara
003	*V. longisporum*	brassica	D Barbara
1871	*V. albo-atrum*	strawberry	D Barbara
Vaa1	*V. albo-atrum*	unknown	Fera

† *D. Barbara, Warwick Crop Centre*

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

mastermix [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 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.

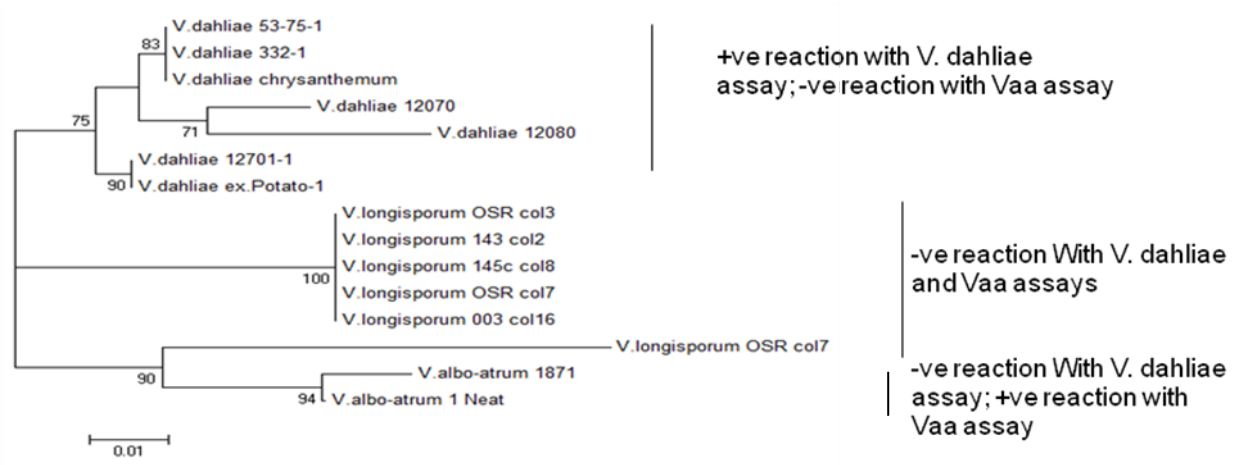


Fig 1.1 Phylogenetic tree showing the relationship between selected isolates of *V. dahliae, V. albo-atrum* and *V. longisporum* and the reaction with the *V. dahliae* (VdC1) and *V. albo-atrum* (VaaC1) QPCR assays.

2. Effect of inoculum levels of Verticillium dahliae on development of Verticillium wilt in strawberry plants grown in pots of artificially infested soil

Introduction

In year 1, a 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, strawberry plants were grown in compost amended with an inoculum dilution series of either *V. dahliae* or V*. albo-atrum.* The objective of this trial was to determine the relationship, if any, between inoculum, as measured by QPCR, and levels of wilt symptoms.

Materials and Methods

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 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) 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 (6 June 2010), the inoculum was thoroughly mixed by hand into 20L volumes of John Innes #3 compost to provide the following dilutions of inoculum and compost (W/V): 1:10,000, 1:20,000, 1: 40,000, 1:80,000, and 1:160,000 (i.e. from 0.1 g inoculum/L compost to 0.0625 g/L compost). A negative control was also done by placing clean sand-maize-meal in compost. The compost mixtures were left overnight to allow any short-lived hyphae or conidia to die. Duplicate DNA extractions were performed on each inoculum treatment prior to placing in pots and real-time PCR assays were performed in duplicate as above.

On 7 June 2010, growing medium (with and without inoculum of isolate 2341) was added to 1L pots then labeled. Three plants of cv. Elsanta, Florence or Symphony were planted in each pot. Each inoculum treatment was done in duplicate. Plants were grown in a temperature regulated glasshouse (temperature range 17 to 25ºC) at Fera until symptoms developed. On 10 June 2010 potting was repeated using compost (with and without inoculum of isolate 1871). Wilt symptoms (leaf necrosis) was visually assessed 6 weeks after planting for each plant. A 5-point scale was used, where 0=no symptoms, 1=1 to 25% necrosis, 2=26 to 50% necrosis, 3=51 to 75% necrosis, 4=76 to 100% necrosis.

The level of wilt for each variety was plotted against soil inoculum as measured by QPCR.

Results

Verticillium dahliae

Leaf necrosis generally developed on lower leaves of plants in infested compost, progressing from the margin. After six weeks, the mean level of leaf necrosis that developed in cv. Elsanta generally increased with increasing levels of *V. dahliae* microsclerotial inoculum (Fig. 2.1 A). The assay failed to detect pathogen at the lowest inoculum level (corresponding to 1:160,000 sand maize-meal culture to compost). This inoculum level was sufficient to cause low levels of leaf necrosis after 6 weeks. Furthermore, *V. dahliae* was detected (by QPCR) in crowns of all plants that had been growing in composts containing inoculum for *7* weeks before testing.

An inoculum of 0.1 g was estimated by the agar plate method to contain around 4.9 cfu/g of *V. dahliae* (2300 fg/g).

The relationship between inoculum level and disease was not clear in cvs Symphony and Florence (Fig. 2.1 B & C). Results for PCR tests on plants to confirm Verticillium infection is ongoing.

Verticillium albo-atrum

After six weeks, the mean level of leaf necrosis that developed in cv. Elsanta generally increased with increasing levels of *V. albo-atrum* inoculum (Fig. 2.2 A). The assay failed to detect the pathogen at the 1:80,000 and 1:160,000 sand maize-meal culture to compost inoculum levels. These inoculum levels caused nil or low levels of leaf necrosis.

The relationship between inoculum level and disease was not clear in cvs Symphony and Florence (Fig. 2.2 B & C). Results for the PCR tests on plants to confirm Verticillium infection is ongoing.

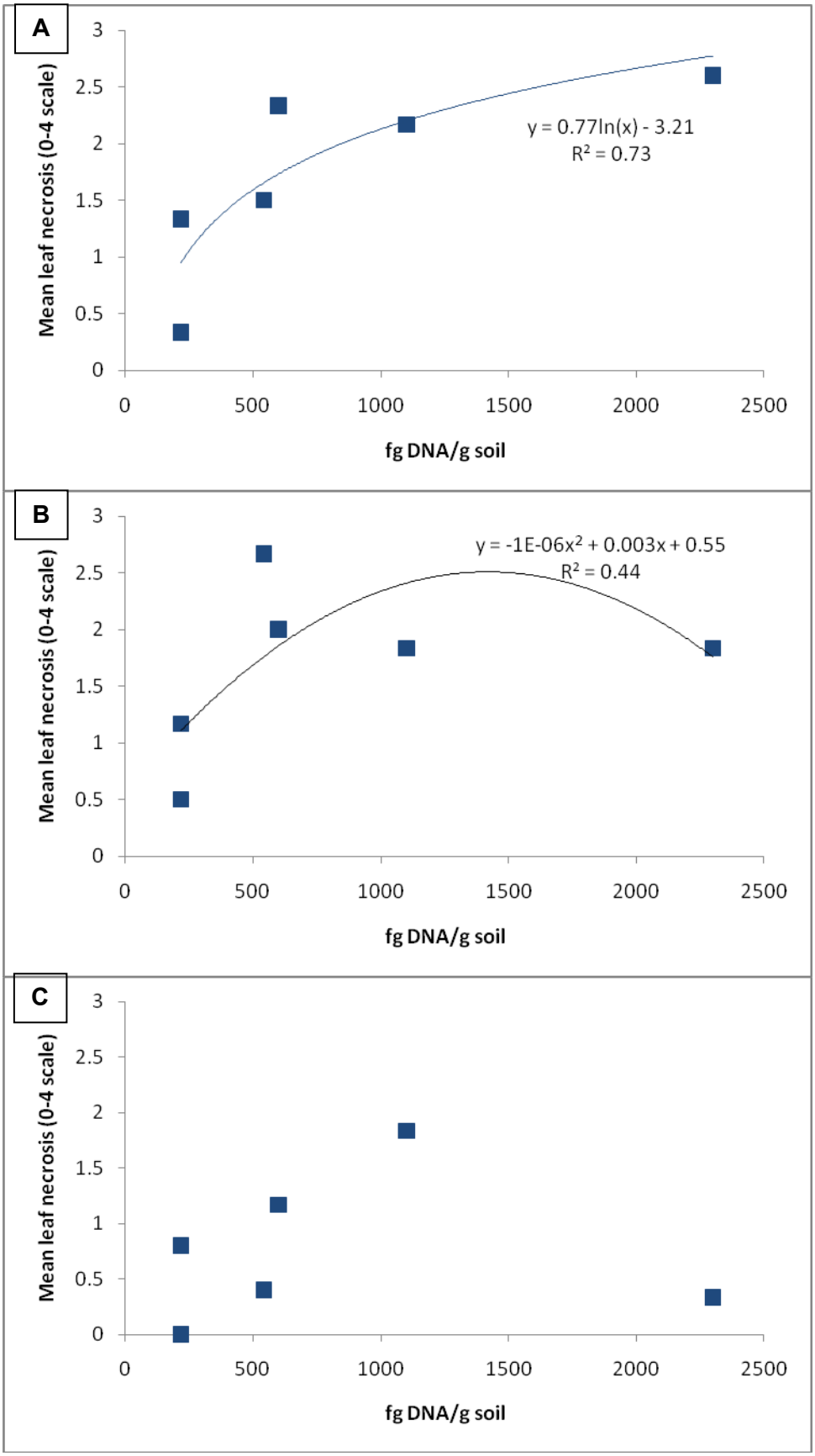
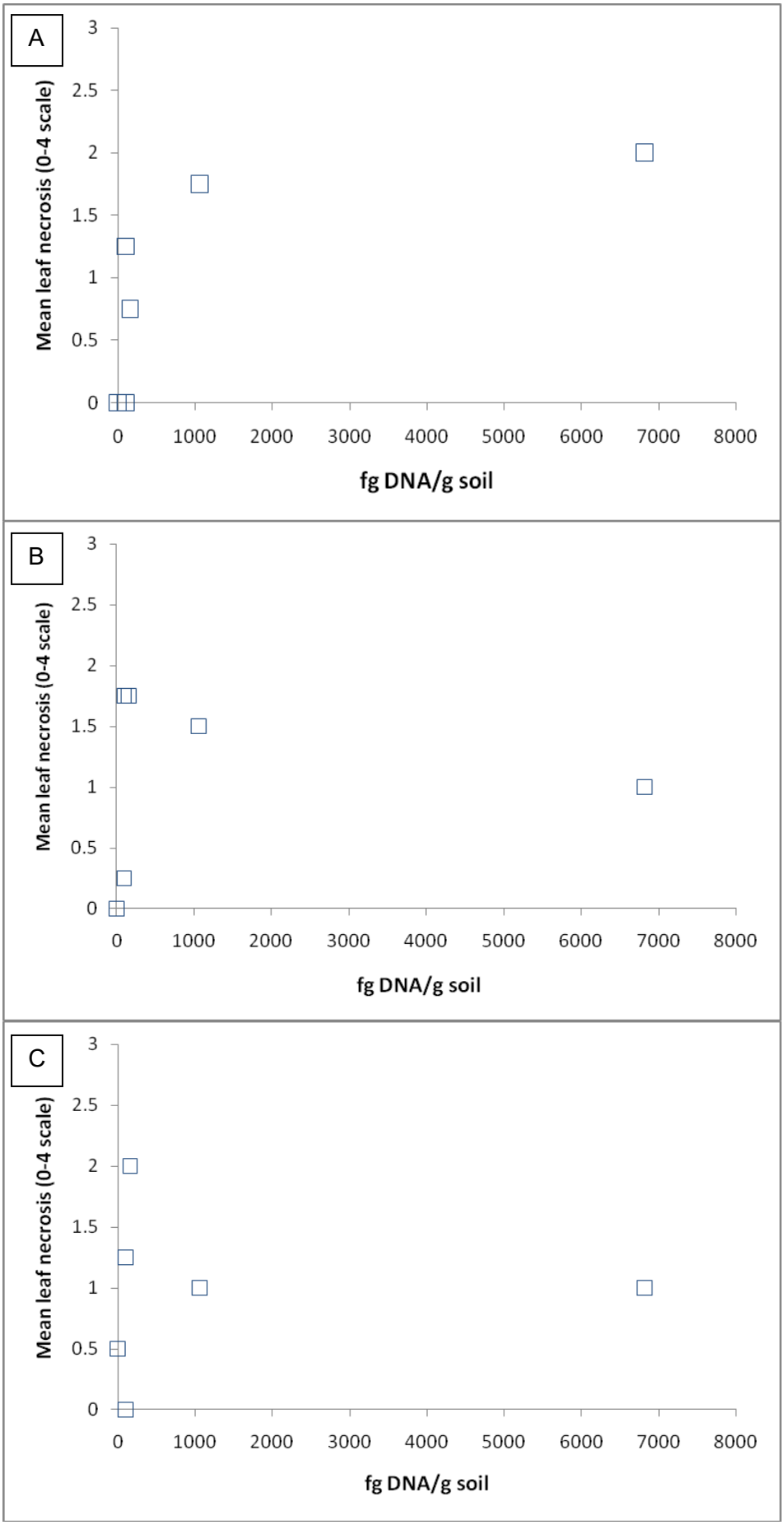


Fig 2.1. Relationship between soil inoculum levels of *Verticillium dahliae* (fg DNA/g soil as measured by QPCR) and leaf necrosis (0-4 scale). A = Elsanta, B = Symphony, and C = Florence

Fig 2.2. Relationship between soil inoculum levels of *Verticillium albo-atrum* (fg DNA/g soil as measured by QPCR) and leaf necrosis (0-4 scale). A = Elsanta, B = Symphony, and C = Florence



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

Introduction

In year 1 a small scale pot trial showed that there was a good linear relationship between the amount of DNA detected by QPCR and the number of microsclerotia of *V. dahliae* added to soil within the range of 1-36 microsclerotia/g soil. Microsclerotia were of a single isolate, 75-150 µm diameter and were mixed into a sandy loam soil. The aim of the current experiment was to examine the correlation between inoculum level of *V. dahliae* and Verticillium wilt symptoms in a pot experiment using soil naturally infested with *V. dahliae*. A range of densities were created by soil dilution of a known infested soil with sterile soil.

Materials and methods

Site and crop details

The experiment was done in a polytunnel at ADAS Boxworth. Strawberry plants cv. Elsanta, supplied as cold-stored 60-day plants, were grown in 1 litre pots of soil for two years. Before potting, a sample of 50 plants from the batch was destructively examined by isolation from crown tissue onto agar and by QPCR to check for freedom from *V. dahliae* and *Phytophthora cactorum*. Plants were graded by crown diameter and extremes discarded. Plants were potted on 13 and 14 May 2010, and pots were placed on capillary matting on upturned plastic chitting trays to prevent contact with the tunnel floor, and irrigated by dripline irrigation.

Powdery mildew was controlled by high volume sprays of Fortress (quinoxyfen), Systhane 20EW (mycobutanil) and potassium bicarbonate. Pests were controlled by introduction of predators and parasites. Fruits were harvested from plants as they ripened. Runners were left on plants until autumn. A crop diary is given in Appendix 1.

Treatments

Soil known to be infested with *V. dahliae* was collected from a fruit farm in Surrey and the infestation level determined by an agar plate test at ADAS High Mowthorpe. This soil was then diluted with John Innes No 2 soil to create infestation densities of approximately 5, 1, 0.5, 0.2 and 0.1 colony forming units per gram of soil (cfu/g). John Innes soil was used as an uninfested control soil. The infested soil was thoroughly mixed with the John Innes soil by tumbling in a concrete mixer, starting with the lowest infestation density. The range of soil *V. dahliae* densities used was chosen to give verticillium wilt risk levels that ranged from very low to very high for the susceptible variety Elsanta. The actual densities of *V. dahliae*

achieved in each soil were determined by QPCR and by the wet sieving and agar plate test (see Table 3.1).

Disease assessments

Plants were examined for symptoms of Verticillium wilt each week from potting (mid-May) until early October. Additionally, in August 2010, one lower leaf and one runner were removed from 20 plants in Treatment 1 (uninfested control) and Treatment 6 (target infestation density of 5 cfu/g) and tested for *V. dahliae* by plating lower petiole and runner crown tissue onto potato dextrose agar after surface sterilisation in sodium hypochlorite (1% for 1 minute). Plates were incubated at 20ºC and examined for colonies typical of *V. dahliae* after 14 and 21 days. Petiole bases were also examined for *V. dahliae* by incubation at high humidity in the laboratory for 21 days.

On 10 October, individual plants were assessed according to predominant leaf colour as green, yellowing, necrotic or yellowing + necrotic. The numbers of plants in each category was determined.

Experiment design and statistical analysis

The experiment was a randomised block design with fourfold replication. Each plot consisted of 20 plants except for the highest inoculum level (5 cfu/g), where there were only 10 plants due to the limited supply of infested soil. Results were examined by analysis of variance

Effect on growth

The numbers of stolons and runners per plant was assessed on 26 August 2010. Previous studies on verticillium wilt of strawberry have shown that the disease can reduce stolon and runner production (Jordan, 1974).

Results and discussion

Soil infestation densities of *V. dahliae*

The soil densities of *V. dahliae* in each treatment as determined by the two test methods are shown in Table 3.1.

Using the agar plate test, *V. dahliae* was detected in treatments 2-6 at levels ranging from 0.8 to 7.6 cfu/g. However, none of the actual levels of the diluted soils were close to the target level. When sub-samples of the same soils were tested by the QPCR test, *V. dahliae* was detected only in the soil with the highest density (T5), as determined by the agar plate method. This soil had 7.6 cfu/g or 997 fg/g of *V. dahliae.* No *V. dahliae* was detected by

isolation or the QPCR test in the sample of 50 plants taken from the batch used in the experiment.

Development of Verticillium wilt

No symptoms typical of Verticillium wilt (i.e. yellowing, wilting, necrosis and collapse of the outer whorl of leaves, followed by collapse of the whole plant) were observed in the plants in 2010. No *V. dahliae* was isolated from the lower leaf petiole or runners tested by isolation onto agar and damp incubation.

There was no difference in the mean number of stolons produced per plant, which ranged from 6.0 to 7.1 (Table 3.2). The mean number of runners (new strawberry plantlets) was greatest on untreated plants (5.6/plant) and least on plants grown in the highest inoculum density of *V. dahliae* (2.6/plant) although this difference was not statistically significant at p=0.05.

When plants were assessed on leaf appearance in October 2010, the incidence of dead plants was significantly greater in T5 (5%) than all other treatments (0-1%); T5 was the only soil in which *V. dahliae* was detected by QPCR (at 977 fg/g). This treatment also had the lowest proportion of plants with predominantly green leaves compared with untreated control plants (P = 0.073). Soil infestation density of *V. dahliae* had no significant effect on the proportion of plants with yellowing, necrotic or yellowing + necrotic leaves (Table 3.3).

Observations on occurrence of Verticillium wilt symptoms in plants will continue in 2011.

A summary of correspondence between agar plate tests and QPCR tests for *V. dahliae* on the same soil samples is given in Table 3.4.

Table 3.1: Infestation densities of *V. dahliae* in a naturally infested soil used for growing strawberry cv. Elsanta in pots – 2010

Treatment	Target	Measured *V. dahliae*		Predicted wilt risk
	V. dahliae (cfu/g)	cfu/g	fg DNA/g	in cv. Elsanta from target level
1.	Nil	NT	NT	Nil
2.	0.1	0.8	<250	Very low
3.	0.2	2.7	<250	Low
4.	0.5	1.2	<250	Medium
5.	1.0	7.6	977	High
6.	5.0	5.1*	NT	Very high

* Mean of tests on 2 sub-samples (4.6 and 5.6 cfu/g). NT – not tested.

Table 3.2: Effect of soil infestation density with *V. dahliae* on stolon and runner production of pot-grown strawberry cv. Elsanta – September 2010

Treatment	Soil infestation with *V. dahliae* (cfu/g and fg/g)		Mean number per plant: Stolons	Runners
1.	NT	NT	7.1	5.6
2.	0.8	<250	6.8	4.2
3.	2.7	<250	7.2	4.2
4.	1.2	<250	6.0	3.7
5.	7.6	977	6.9	4.1
6.	5.1	NT	6.1	2.6
Significance (15 df)			NS	NS
LSD			-	-

Table 3.3: Effect of soil infestation density with *V. dahliae* on appearance of strawberry plants – October 2010

Treatment	Soil infestation density with *V. dahliae* (cfu/g and fg/g)		Mean % plants with predominantly: Green leaves	Yellowing leaves	Necrotic leaves	Yellowing + necrotic leaves	% dead plants
1.	NT	NT	56.2	23.8	11.2	8.7	0.0
2.	0.8	<250	48.8	32.5	7.5	11.2	0.0
3.	2.7	<250	42.5	40.0	8.8	8.8	0.0
4.	1.2	<250	51.2	36.2	1.2	10.0	1.3
5.	7.6	977	32.5	35.0	11.2	16.2	5.0
6.	5.1	NT	55.8	23.3	13.3	7.5	0.0
	Significance		0.073	NS	NS	NS	0.014
	LSD		17.10	-	-	-	2.95

Table 3.4: Correspondence of agar plate and QPCR tests for *V. dahliae* on various soil samples tested by both methods

Soil source and method of preparation	*V. dahliae* density		
	Target level (cfu/g)	**Agar plate (cfu/g)**	**PCR (fg/g)**
1. Sterile soil inoculated with flask-grown microsclerotia (Fera, 2009)	0.5	0.4	<250
	1	1.5	540
	2	4.6	600
	4	8.4	1110
	8	4.9	2300
2. Naturally infested soil from a strawberry crop diluted with sterile soil (ADAS, 2010)	0.1	0.8	<250
	0.2	2.7	<250
	0.5	1.2	<250
	1.0	7.6	977

4. 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 the 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 summarized below; full details are in the year 1 Annual Report. Initially a soil sample was taken from the full trial area and tested by QPCR and the agar plate method in order to select fields with a spread of soil infestation densities of *V. dahliae.* Subsequently, soil samples were taken from individual plots at each site and tested by QPCR. Repeat soil samples were taken from plots 1 and 4 at site A12 on 16 August 2011; soil samples were taken using a hand augur from close to each of around 50 plants per plot and bulked to make a plot soil sample. Individual plots were also tested for *V. albo-atrum* by a QPCR test and none was found. The sites were planted in April and May 2010 with cvs Elsanta, Symphony and Florence (Table 4.1). Plants were planted at normal spacing on prepared beds with around 100 plants per plot. Additionally at site A11 and an additional site in Buckinghamshire, around 100 plants of the new variety Fenella were planted as observation plots.

Table 4.1: Details of field sites with differing soil infestation densities of *V. dahliae* planted with strawberry in 2010

Site Code	County	*V. dahliae* (cfu/g)	*V. dahliae* (fg/g)	Date soil sampled	Date planted
A8	Cheshire	<0.1	<250	2/12/09	9/4/10
A1	Lancs	0.2	560	16/4/10	14/5/10
A7	Cheshire	0.5	<250	2/12/09	8/4/10
A11	Oxon	4.6	<250	4/5/10	3/6/10
A12	Oxon	5.7	468	4/5/10	3/6/10

Results and discussion

Health of planting material

No *V. dahliae* or *V. albo-atrum* was isolated from the plant samples tested before planting and no *Verticillium* spp. were detected in crowns using PCR tests.

Field assessments

In 2010, Verticillium wilt symptoms were observed and *V. dahliae* was confirmed in plants at the two sites (A11 and A12) with the two highest infestation densities of *V. dahliae* and not at the other sites (Table 4.2). The incidence of plants with Verticillium wilt symptoms at sites A11 and A12 was assessed on 4 August and 18 October. At site A12, when plants were examined around 8 weeks after planting, some plants had failed to establish following disturbance by birds and hares. Numbers of dead or dying plants per plot due to failure to establish were recorded and these were excluded when calculating Verticillium wilt incidence data.

At the assessment on 4 August there were significant differences between varieties with a high incidence of Verticillium wilt in cv. Elsanta and low levels of the disease in cvs Symphony and Florence (Table 4.3). At both sites the rank order of Verticillium wilt incidence in the three varieties corresponded with their known susceptibility to verticillium wilt.

At the assessment on 18 October, there were no significant differences between varieties. It should be noted however, that the degrees of freedom for individual sites is low, necessitating relatively large and consistent differences between varieties to record a significant effect.

The incidence of affected plants was greater in October than in August for all varieties at site A12 and for 2 of the 3 varieties at site A11. The apparent fall in incidence of affected cv. Elsanta plants at site A11 may have been due to: an increase in dead or missing plants, which were not recorded as Verticillium wilt (especially plots 1, 4 and 12); some plants recovering from Verticillium wilt; wilting at the first assessment was not caused by *Verticillium* spp. and plants subsequently recovered. It is considered that the first explanation is the most likely.

The incidence on cv. Florence plants showed the greatest increase between the August and October assessments, from 1.1 to 5.1% at site A11 and from 0.9 to 16.7% at site A12. Possibly the later expression of Verticillium wilt in this variety is a reflection of its greater resistance than the other two varieties. Also, the weather was hot and dry from planting onwards in SE England and in many plantations plants growth ceased for a while. Generally

Verticillium wilt first appeared in crops planted in spring and summer in 2009, and those planted very early in 2010. Crops planted later in 2010, where on infested ground, tended to show symptoms in autumn 2010.

The correlation between Verticillium wilt incidence with *V. dahliae* soil infestation density (fg/g) for individual plots is shown in Table 4.4.

Over the two sites, *V. dahliae* was detected before planting in 5 of the 24 plots by the QPCR test. All of these plots developed Verticillium wilt when assessed in August (14.4, 0, 1.9, 11.4 and 8.2% plants affected) and/or October (8.6, 29.6, 8.7, 13.3 and 5.1% plants affected).

Of the 19 plots when *V. dahliae* was not detected before planting by the QPCR test, 14 of them showed <5% plants with symptoms of Verticillium wilt in August, including four with no symptoms. In October, 18 of the 19 plots had plants with symptoms of Verticillium wilt, including 4 with over 10% of plants affected.

No symptoms of Verticillium wilt were observed in the non-replicated plot of Fenella at site A11.

Further assessment of Verticillium wilt will be done at all five sites in 2011.

Table 4.2: Occurrence of Verticillium wilt in five fields of strawberry differing in soil infestation density of *V. dahliae* – 2010

Site Code	Soil density of *V. dahliae*		Verticillium wilt symptoms present in 2010	Dates crops examined for Verticillium wilt
	cfu/g	fg/g (Plot range†)		
A8	<0.1	<250 (<250)	No	02/12/09
A1	0.2	560 (<250)	No	16/04/10
A7	0.5	<250 (<250)	No	02/12/09
A11	4.6	<250 (<250 - 480)	Yes	04/05/10
A12	5.7	468 (<250 - 2620)	Yes	04/05/10

† *Results from samples collected from individual plots before planting in April and May 2010*

Table 4.3: Effect of variety and soil infestation density with *V. dahliae* on incidence of Verticillium wilt in strawberry – 2010

Site and Variety	*V. dahliae* infestation in soil		Predicted risk of wilt	Mean % plants affected	
	Agar plate test (cfu/g)	PCR test[a] (fg/g)		4 Aug	18 Oct
A11	4.6	274			
Elsanta	-	323	Very high	12.8 (1.7)	7.5 (2.4)
Symphony	-	218	High	2.3 (0.2)	2.6 (1.3)
Florence	-	218	Medium	1.1 (0.5)	5.1 (1.8)
Significance (6 df)				<0.001	NS
A12	5.7	624			
Elsanta	-	910	Very High	10.3 (1.5)	12.0 (4.1)
Symphony	-	600	Very high	1.9 (0.7)	4.7 (2.6)
Florence	-	363	High	0.9 (0.4)	16.7 (4.5)
Significance (6 df)	-			0.001	NS

[a] Value for overall site sample before planting, and means of 4 plot values for each variety.

Table 4.4: Verticillium inoculum densities in soil sampled from field plots and corresponding cumulative losses to presumptive Verticillium wilt in 2010

Fera Number	Site name	Plot number	Variety	*V. dahliae* Inoculum (fg DNA/g soil)	% plants affected	
					4 Aug	18 Oct
YA60	A11	Plot 1	Elsanta	<250	17.8	3.0
YA61		Plot 2	Symphony	<250	0	4.6
YA62		Plot 3	Florence	<250	0	2.7
YA63		Plot 4	Elsanta	480	14.4	8.6
YA64		Plot 5	Florence	<250	1.8	6.3
YA65		Plot 6	Elsanta	310[†]	13.8	10.1
YA66		Plot 7	Symphony	<250	1.0	2.0
YA67		Plot 8	Florence	<250	1.9	1.9
YA68		Plot 9	Symphony	<250	0	0
YA69		Plot 10	Florence	<250	0.9	10.1
YA70		Plot 11	Symphony	<250	0	3.7
YA71		Plot 12	Elsanta	<250	5.5	5.5
YA72	A12	Plot 1	Elsanta	<250	2.0	28.3
YA73		Plot 2	Symphony	<250	6.0	6.0
YA74		Plot 3	Florence	700	0	29.6[b]
YA75		Plot 4	Elsanta	<250	20.5	7.2[b]
YA76		Plot 5	Symphony	<250	1.9	2.9
YA77		Plot 6	Florence	<250	1.0	13.3[b]
YA78		Plot 7	Symphony	1650	1.9	8.7
YA79		Plot 8	Florence	<250	1.0	19.0[b]
YA80		Plot 9	Elsanta	520	11.4	13.3
YA81		Plot 10	Symphony	<250	2.9[a]	6.9
YA82		Plot 11	Elsanta	2620	8.2	5.1
YA83		Plot 12	Florence	<250	1.8	6.1[b]

Elsanta – very susceptible; Symphony – susceptible; Florence – moderately resistant.

[†] *Verticillium* spp. (not *dahliae*)
[a] *V. dahliae* confirmed in sampled plant by PCR; [b] *V. dahliae* confirmed in sampled wilting plant by isolation from crown tissue.

Confirmation of *V. dahliae*

Samples of eight plants of cv. Elsanta and two of cv. Symphony showing symptoms of verticillium wilt were taken on 17 July 2010 from site A12. *V. dahliae* was confirmed in one

of the Symphony plants and not in cv. Elsanta. No *Phytophthora cactorum* or other pathogens were found. The crowns of all plants appeared healthy.

A further 10 plants with suspect symptoms of Verticillium wilt were collected from site A12 on 22 September; these comprised 4 plants of cv. Elsanta, and one each of Symphony and Florence. *V. dahliae* was confirmed by isolation from the Symphony and Florence plants, not from the Elsanta plants. Isolates were unusual and did not produce microsclerotia in culture but they tested positive for *V. dahliae* by QPCR.

A further eight plants (one plant from each of 8 plots) with suspect symptoms of Verticillium wilt were collected on 18 October. These plants were examined for *V. dahliae* by isolation onto PDA following either hypochlorite or ethanol disinfection; roots were examined as well as crowns. *V. dahliae* was confirmed in five of the eight plants (Table 2.5). The fungus was recovered from the crown tissue and not roots; and mostly from ethanol disinfected tissue.

These results indicate that a majority of the plants assessed as affected by Verticillium wilt probably were affected by the disease. However, some plots may have wilted for other reasons. Such assessments would particularly affect conclusions on correlation of soil test results with subsequent wilt development when the incidence of wilting plants is low (e.g. <5% or 10%). This may partly explain why the correlation between pre-planting soil levels of *V. dahliae,* as determined by QPCR, with levels of Verticillium wilt were poor when taken at the per-plot level. There was better agreement between PCR levels of soil inoculum and wilt when mean levels of DNA at the field scale are used to assess risk of wilt.

Further plants will be tested in 2011 to determine cause of wilting.

Table 4.5: Recovery of *V. dahliae* from strawberry plants with suspect symptoms of Verticillium wilt sampled on 18 October 2010 (site A12)

Plot	Variety	Number of tissue pieces (of 5) from which *V. dahliae* was isolated after disinfection with:			
		Ethanol		Hypochlorite	
		Crown	Roots	Crown	Roots
1	Elsanta	0	0	0	0
3	Florence	5	0	0	0
4	Elsanta	3	0	0	0
6	Florence	5	0	1	0
7	Symphony	0	0	0	0
8	Florence	1	0	0	0
9	Elsanta	0	0	0	0
12	Florence	2	0	0	0

5. Further validation of QPCR: 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, 29 commercial soil samples were tested in parallel using both methods.

Materials and Methods

Twenty nine commercial soil samples, received through Fera's Plant Clinic for Verticillium inoculum levels, 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 5.1 show that there was a reasonable agreement (R^2=0.76) between the two methods. However, the discrimination at levels below 1 microsclerotia/g soil becomes poor. It is likely that at levels below 1 ms/g variability between sub-samples becomes so great that it becomes unrealistic to expect replicate assessments to provide consistent results.

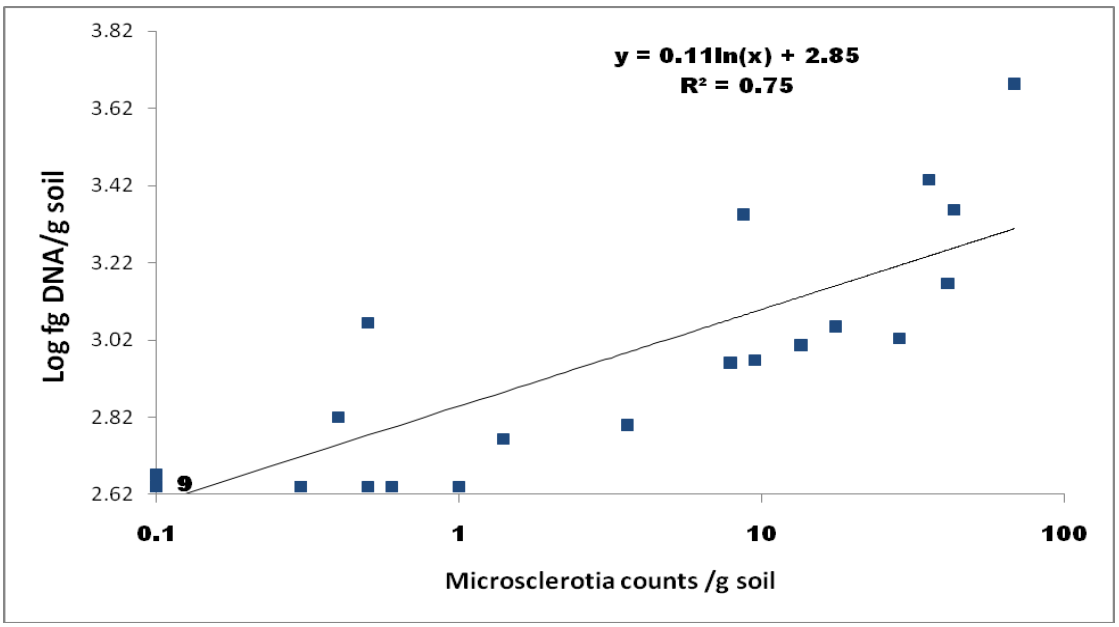


Figure 5.1. Correlation between microsclerotial counts and DNA levels when commercial samples were tested by QPCR (DNA/g soil)) and wet sieving (microsclerotia counts/g soil, log scale).

Conclusions

1. The molecular (QPCR) tests for *Verticillium dahliae* and *V. albo-atrum* continue to detect only target pathogen species after further validation.

2. Pot trials at Fera demonstrate that there is a good correlation between inoculum (as measured by QPCR) and wilt in the susceptible cultivar Elsanta. A poor correlation between inoculum and wilt was found in the more resistant cultivars Florence and Symphony.

3. Work is ongoing to measure the extent to which the QPCR test can be used to predict the risk of wilt in field soils. Results to date show that whilst the test is less sensitive than the conventional wet sieving (Harris) method, QPCR testing one month prior to planting only detected Verticillium at sites where wilt developed.

4. Results from a direct comparison of *V. dahliae* inoculum levels using wet sieving and QPCR testing of commercial samples show that there is a reasonable agreement between methods.

Technology transfer

Peters J & O'Neill TM (2010). New Verticillium wilt test helps manage risk. *HDC News* **166**, p19.

O'Neill T M (2010). New developments in soil disinfestation and soil testing for Verticillium wilt control. IPPS Conference, Ipswich, 8 October 2010.

References

Jordan V W L (1974). Verticillium wilt disease of strawberry: cultivar reaction and effect on runner health and production. *Plant Pathology* **23**, 8-13.

Appendix 1 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

Appendix 2 Crop diaries for field sites

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
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

Appendix 3 Individual plot data at sites A11 and A12

Site: A11 4.6 cfu/g

Plot and Variety		Max no. plants	15 July		4 August		18 October			
			Dead or missing	Wilt	Cumulative wilt losses	Dead or missing	Total planting spaces assessed	No. live	No. missing	No. wilt
1	El	106	5	16	18	5	105	91	14	3
2	Sy	112	4	0	0	4	112	109	3	5
3	F	114	4	0	0	3	112	110	2	3
4	El	112	5	14	15	8	110	93	17	9
5	F	112	0	0	2	0	114	114	0	7
6	El	113	4	8	15	4	110	102	8	11
7	Sy	106	5	0	1	4	107	103	4	2
8	F	112	4	0	2	4	111	107	4	2
9	Sy	112	4	0	0	4	112	110	2	0
10	F	111	0	1	1	0	118	118	0	11
11	Sy	112	4	0	0	3	110	110	0	4
12	El	113	6	5	6	4	111	101	10	6

Site A11 – 2011

Plot and variety	No. plants established		Cumulative no. plants with wilt		% plants with wilt[a]	
	15 July	4 Aug	4 Aug	18 Oct	4 Aug	18 Oct
1. El	101	101	18	3	17.8	3.0
2. Sy	108	108	0	5	0	4.6
3. F	110	111	0	3	0	2.7
4. El	107	104	15	9	14.4	8.6
5. F	112	112	2	7	1.8	6.3
6. El	109	109	15	11	13.8	10.1
7. Sy	99	102	1	2	1.0	2.0
8. F	108	108	2	2	1.9	1.9
9. Sy	108	108	0	0	0	0
10. F	110	111	1	11	0.9	10.1
11. Sy	108	109	0	4	0	3.7
12. El	107	109	6	6	5.5	5.5

[a] Based on the number of live plants on 4 August.

Site: A12 (5.7 cfu/g)

Plot and Variety		Max no. Plants	14 July		4 August		23 Sep	18 October			
			Dead or missing	Removed as wilt	Cumulative wilt losses	Dead or missing	Removed as wilt	Total planting spaces assessed	No. live	No. missing	No. wilt
1	El	112	5	2	2	13	2	112	97	15	24
2	Sy	111	10	0	1	11	0	110	100	10	6
3	F	109	1	0	0	1	0	108	107	1	32
4	El	109	5	2	17	26	0	110	82	26	4
5	Sy	111	4	2	2	7	0	111	104	7	1
6	F	115	9	0	1	10	0	115	105	10	14
7	Sy	111	4	0	2	8	0	110	104	6	7
8	F	110	5	0	1	5	1	111	105	6	18
9	El	110	2	0	12	5	2	110	105	5	12
10	Sy	109	4	2*	3	7	1	109	102	7	4
11	El	112	3	2	8	14	0	112	98	14	3
12	F	115	2	0	2	1	0	115	115	0	7

Site A12 – 2010

Plot and variety		No. plants established[a]		Cumulative no. plants[b] with wilt		% plants with wilt[c]	
		14 July	4 Aug	4 Aug	18 Oct	4 Aug	19 Oct
1	El	107	99	2	28	2.0	28.3
2	Sy	101	100	1	6	6.0	6.0
3	F	108	108	0	32	0	29.6
4	El	104	83	17	6	20.5	7.2
5	Sy	107	104	2	3	1.9	2.9
6	F	106	105	1	14	1.0	13.3
7	Sy	107	103	2	9	1.9	8.7
8	F	105	105	1	20	1.0	19.0
9	El	108	105	12	14	11.4	13.3
10	Sy	105	102	3	7	2.9	6.9
11	El	109	98	8	5	8.2	5.1
12	F	113	114	2	7	1.8	6.1

[a] Number planted less losses due to hare or bird damage, or drought.
[b] Includes plants removed with suspect wilt symptoms and sent for testing.
[c] Based on the number of live plants on 4 August.

