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The results and conclusions in this report are based on a series of experiments conducted over a two-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.

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AUTHENTICATION

I declare that this work was done under my 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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Headline

Despite significant advances in the development of a rapid and quantifiable DNA-based test to replace the Harris *Verticillium* test, further research is necessary before this approach can be deployed.

Background and expected deliverables

Verticillium wilt of strawberry caused by *Verticillium dahlia* Kleb. is a major risk for strawberry production worldwide. Losses of up to 75% can be experienced. Until recently, infested soils were treated with methyl bromide. However, the chemical has been banned due to its extreme toxicity, its activity as a greenhouse gas and non-salient effects on the environment.

The current standard test for wilt pressure in UK soils is a wet sieving method developed at East Malling Research (EMR), called the Harris test. It has several drawbacks: it is time consuming (6 weeks), labour intensive and known to underestimate populations of *V. dahliae* (Termorshuizen *et al.,* 1998). Its positive aspects are that only infectious propagules are recovered and that extensive research has gone into its calibration for disease pressure in strawberries. As with all methods, it is dependent on correct application of good soil sampling techniques.

Verticillium specific DNA primers, used for molecular detection of the genus, species, or infra-specific groups have been reported (*e.g.* Lievens *et al.,* 2006*;* Mahuku & Platt, 2002; Carder *et al., 1994;* Mercado-Blanco *et al.,* 2003). Several other soil-borne pathogens have been detected from soils and quantified using the Real Time polymerase chain reaction (RT-PCR). The purpose of this project was to determine a reproducible method for quantifiably extracting and detecting *V. dahliae* DNA in soils in order to replace the standard wet-sieve method described by Locke & Harris (1993).

Summary of the project and main conclusions

Extensive work went into evaluating methods for the extraction of fungal DNA from soils. Known quantities of *Verticillium dahliae* microsclerotia (resting structures) were added to uninfested soils and their DNA extracted and compared to quantities recovered by the wet sieve method of Locke & Harris (1993). The most effective of the methods attempted involved use of a specialized cell disruption apparatus and a commercial kit developed for DNA extraction from soils, followed by a two-stage treatment to remove amplification inhibitors.

Primers for RT-PCR were adopted and modified from an existing protocol (Lievens *et al.*, 2006), although primers with greater specificity are still being sought. DNA fragments generated by the RT-PCR method should be small (100-200 base pairs [bp]). Initial experiments were performed with primers that generate a DNA fragment in excess of 400 bp in length, which may explain some of the ambiguities of earlier results.

PCR products have been generated from DNA extracted from soils after filtration through PVP columns. Unfortunately, the RT-PCR products were not quantifiable due to several factors, explained in the science section, below.

Further experiments need to be done to further: validate the extraction method; reproducibly quantify fungal DNA; and correlate DNA quantification with colony forming units of the pathogen.

Financial benefits

At present, there are no financial benefits, but estimates of the cost of testing can be made. To whit: 1. extraction and cleaning of DNA from soil costs approximately £10 per sample (but there should be quadruplicate composite samples per site); amplification of DNA using SYBR green chemistry has consumable costs of approximately £15 (triplicate amplifications) = approximately £120 consumable costs per sampled site and the cost of labour and reporting.

Action points for growers

No action points can be offered to growers as a result of this work.

Science section

Introduction

Verticillium wilt, caused by the fungus *Verticillium dahliae*, remains a major disease risk for UK strawberry growers. If heavily infested soils are not treated, losses of up to 75% may occur (Lovelidge, 2004). A recent survey indicated that up to 84% of sites had some levels of *V. dahliae*, with 24% having high levels as determined by the Harris test (Lovelidge, 2004).

Current soil testing protocols

The presence of *Verticillium dahliae* in soil is currently determined and quantified by a test developed at East Malling Research (Locke & Harris, 1993) and made available as a commercial service. Microsclerotia of *V. dahliae* are retrieved from soil samples, and colonies stimulated to develop on a semi-selective medium. These are counted by skilled microscopists. Results are expressed as colony forming units per gram of soil, and compared to a risk chart based on economic thresholds previously developed for strawberry cultivars (Harris & Yang, 1996). The test takes around 6 weeks to complete, requires a high labour input in both preparation and analysis of samples, and is reliant on specific identification skills possessed by only a few UK scientists.

Potential need for and advantages of a molecular test

The need to accurately detect and quantify *V. dahliae* in soil is likely to increase at least in the short-term with the withdrawal of soil sterilants (*i.e.* methyl bromide), and a lack of equally effective replacements. Although the current soil testing system is reliable, molecular techniques offer the opportunity to increase rapidity (a few days rather than weeks), and reliability (computerised analysis reduces risk of human error). Using the technique of Real Time PCR, accurate quantification of fungal DNA is possible, while the

reduced labour input, and ability to process many samples in a single reaction run (up to 25) allows a cost-effective service.

The actual cost per sample will depend on the number of samples processed at one time (most of the steps can be performed in parallel for a number of samples). At today's prices, the cost of chemical consumables for a molecular test is unlikely to exceed £100-120 per sampled field and in the 'worst' case of a single sample being run, no more than 2-3 hours user input would be required (*i.e.* less than £75 labour costs). The only other real costs relate to use of electricity for running equipment. Therefore, the final charge for such a service should be less than £150 per sampled field, and possibly considerably less for batches of samples. The labour costs of the Harris test remain the same, no matter the number of samples evaluated.

Variants of the plate testing for *Verticillium* by recovery onto semi-selective medium are used world-wide. A number of reviews have highlighted variability of results, particularly with respect to soil type and with the same soil samples analysed in different laboratories (Termorshuizen *et al.,* 1998). Molecular detection offers a much more standardised approach since it is possible to use specific DNA extraction kits and amplification kits, reducing the risk of variability in handling of samples. Real Time PCR probes have been developed and tested for *V. dahliae* as part of a HortLINK project (Hort16/HL0136LSF (DEFRA); CP6 (HDC)) (Krishnamurthy, 2005) as well as elsewhere (Mercado-Blanco *et al.*, 2003). The HortLINK project has identified a reliable approach to isolation of DNA from soil samples, and other research groups have demonstrated this possibility for *V. dahliae* (Mahuku & Platt,2002; Volossiouk *et al.*,1995), *V. tricorpus* (Heinz & Platt, 2000) and *V. chlamydosporum* (Mauchline *et al.,* 2002).

Molecular tools for detection of *Verticillium*

Soil has traditionally been a difficult medium for molecular diagnostics due to the high levels of inhibitory compounds present. The first publications relating

to successful approaches date from around ten years ago (Volossiouk *et al.*,1995).

Specific molecular probes for *Verticillium* species, including *V. dahliae,* have been developed (Mauchline *et al.,* 2002) and quantification of levels in plant tissue is now routinely possible in research laboratories including EMR. Recent advances in equipment mean that very rapid and highly sensitive detection and quantification of DNA are now possible using Real Time PCR. Several quantification techniques exist; they vary in terms of probe design and means of signal analysis. Examples include SYBR Green, TaqMan and molecular beacons. The SYBR Green approach was utilised in project Hort16/HL0136LSF (DEFRA); CP6 (HDC), upon which this project aimed to build.

In this project, *V. dahliae* DNA was obtained from soils by use of commercial kits. The procedure has been developed to handle up to 10g of soil, which is the amount used for the Harris test. By simple attachment of fluorescent dyes to probes specific to *V. dahliae,* these were developed into primers capable of being used in a SYBR Green detection system. In a SYBR Green detection system artificially generated 'new' DNA produced in large quantities by the PCR is detected by the fluorescence of a simple intercalating dye. This simple approach has potentially less specificity than other types of Real Time PCR but has proved entirely adequate in the preliminary work undertaken under Hort16/HL0136LSF (Krishnamurthy, 2005)). The levels of fluorescence can be related to standards run in the same reaction in order to give accurate quantification of DNA in the test samples.

Preliminary work showed that as little as 2 femtograms of DNA could be quantified by this approach. In terms of *V. dahliae* levels this approximates to 1/15 of a genome (i.e. the test is 15 times more sensitive than required to account for one genome of *V. dahliae* in a sample). A complication is that it is difficult to precisely determine the number of genomes in a given *V. dahliae* microsclerotium. An estimate has been made of around 200 genomes for fresh microsclerotia in soil. Therefore a detection limit of 4 picograms of DNA would apply to 1 microsclerotium. However, microsclerotia

vary not only in size but also in age and, over time, their viability in soil will decline. It is necessary to relate numbers of microsclerotia to inoculum potential. The current soil testing service already operates on this basis, and so a clear linear relationship between the PCR approach and plate test method would provide confidence in the reliability of molecular detection. However, the occurrence of a simple linear relationship between amounts of DNA and numbers of microsclerotia over a wide range of soil types and large numbers of samples has yet to be established. Two obstacles have been highlighted.

The first is that assessment of *V. dahliae* microsclerotium levels based on traditional plate testing is highly dependent on the laboratory at which either the test is set-up and/or analysed. Thus, determination of an accurate figure to compare with PCR data was difficult for the student undertaking the work in Hort16/HL0136LSF. EMR is best suited for development of a molecular assay because of the many years of experience in this area and it was anticipated that accurate and consistent counts could be obtained in the proposed research. Even more important, the quantification obtained at EMR would be directly related to the currently established commercial (Harris) test.

Second, there was difficulty in relating PCR results to soil counts in some cases, and this may be due to soil type (*e.g.* inhibitors present), or it may be due to variation in the 'quality' of the microsclerotia (*i.e.* varying in numbers of genomes per microsclerotium; a factor which may also directly affect the 'infectivity' of specific soils).

Good correlation between plating techniques and PCR detection of *V. dahliae* can be obtained from certain soils, as demonstrated in Canada by Mahuku and Platt (2002) who showed an almost perfect linear relationships between microsclerotia detected by a different type of PCR (competitor PCR) and plating methods. Of particular importance for any PCR test will be the performance where levels of infestation are low, and/or close to the economic threshold for soil disinfestations; the test must be proven to be consistent and accurate at these levels. Neither a PCR test nor a plating assay makes any allowance for variations in the inherent pathogenicity of different

isolates but as strawberry is apparently a very susceptible host this probably is not important.

Materials and methods

Soil selection

In order to develop and test the DNA-based technique, a range of soils was identified:

a) Soil with no *V. dahliae* present was required in order to establish the relationship between plate testing and PCR when microsclerotia were artificially added. A soil of sandy-loam consistency was selected that had previously been tested within EMR's Plant Clinic system and scored zero colony forming units per gram (cfu/g) for *V. dahliae.* In an attempt to be certain that the score really was zero, the soil was autoclaved for 30 minutes at 121°C.
b) Soils of variable type were identified that had also scored zero for *V. dahliae* presence. These had varying soil type characteristics and were listed as: Peaty, light sandy, red (two of these), sandy loam. These were also zero cfu/g soils, but were not autoclaved prior to use.
c) Nine soils which had been tested as part of a previous project were sampled. These were known to have a wide range of *V. dahliae* content (three had >5 cfu/g, three had 1-5 cfu/g, three had 0.1-1 cfu/g), and varied in terms of soil type and age. The ages, soil type and original number of ms/g are listed in the Results.

Microsclerotia (resting structures) of *V. dahliae* were added to soils from a) and b). These were produced by growing an isolate of *V. dahliae* (EMR ref. 12154) on prune-lactose-yeast agar (per litre: 100ml concentrated prune extract, 5g lactose, 1g yeast extract, 20g agar No. 3, 900ml distilled water), for 6 days, and transferring a segment from the leading edge of the colony to the same medium covered with cellophane. Once visible microsclerotia were being produced, the surface of the cellophane was scraped clean and the

fungal growth added to 500ml water. This was blended at high speed for 1 minute, and then sieved to retain the 20-160µm fraction. The microsclerotia in this fraction were washed with tap water, and re-suspended in 10ml distilled water. Counts were made, and the level adjusted to 1 x 10^4 ms/ml. Microsclerotia were stored at 4°C until required.

It was noted, that the microsclerotia tended to clump on storage, and so prior to any usage they were shaken at 150rpm for 1 hour.

All soils were stored at 4°C, and air-dried. To 200g batches of soil a), microsclerotia were dispensed (by pipetting) to reach a final concentration of 0, 1, 2, 5 or 10ms/g. The soil samples were shaken well to mix.

Five separate 10g samples were taken for analysis by the Harris test. At the same time, five further 10g samples were reserved for molecular analysis.

For soils in b), a fresh batch of microsclerotia was produced, and 200g samples of each soil type mixed with i) 1ms/g and ii) 10ms/g. Again, five batches of 10g were subsampled for analysis by the Harris test, and five identical batches of 10g samples set aside for molecular analysis.
Soils in group c) had already been analysed for *V. dahliae* content in a previous project, so just one 10g sample of each was taken for analysis by the Harris test. The remaining soil was retained for molecular analysis.

Plate testing

As indicated above, Harris tests were carried out on:

- five replicates of soil a) at each of 0, 1, 2, 5, 10 ms/g
- five replicates of each of five soils b) at both 1ms/g and 10ms/g
- one replicate of each of nine soils c) at naturally infested ms levels

The Harris tests were carried out in line with current EMR Plant Clinic protocols.

10g air-dried soil was suspended in 40ml water and shaken at 175rpm for one hour. The suspension was sieved to retain a 20-160µm fraction, which was then re-suspended in 20ml distilled water. One ml of this was spread onto the surface of each of 20 Selective Soil Extract Dox plates, containing antibiotics and biotin. The plates were incubated for 28 days at 22°C, and colonies of *V. dahliae* counted after washing soil off the plates.

Selective Dox soil extract medium (per litre): 24ml soil extract, 12g agar No. 3, 4g K_2HPO_4, 1.5g KH_2PO_4, 2g PGA, 1ml Tergitol. To this is added 2ml of Dox salts. Dox salts (per 20ml): 2g KH_2PO_4, 1g KCl, 1g $MgSO_4.7H_2O$, 0.02g $FeSO_4.7H_2O$, 4g $NaNO_3$. The final medium contains 10ml antibiotic and biotin solution per 100ml. Antibiotic/biotin solution (per 100ml): 0.06g streptomycin, 0.06g chloramphenicol, 0.06g chlortetracycline, and 0.006g biotin.

Optimisation of extraction of DNA

Initially, soil samples (type a) above) were adjusted to either 0.5 or 5 ms/g.

Five protocols were compared in order to judge efficiency of DNA extraction. Duplicate samples of soils at each ms concentration were used.

1. QIAamp® DNA Stool Mini Kit (Qiagen, Crawley, UK), following their protocol for isolation of DNA from Stool for Pathogen Detection – p. 15-17 of Handbook supplied.

2. A modified version of 1), including the following adaptations:
   i) Added 1g soil to 1ml water. Shook for 5 minutes at 300rpm
   ii) Incubated at 95°C for 10 minutes
   iii) Centrifuged at 3000rpm for 5 minutes
   iv) Added 7 volumes of kit buffer ASL (3-3.5ml) to the supernatant from iii) in a fresh tube
   v) Added an Inhibitex tablet and shook manually for 1 minute
   vi) Dispensed suspension into four separate 1.5ml tubes and centrifuged at 7000rpm for 5 minutes

vii) Added supernatant to a new tube and mixed with 1 volume buffer AL and 1 volume ethanol (100%)

viii) Transferred to QIAamp spin columns (10x 600µl)

ix) Spun at 14000rpm for 1 minute and placed column in a new 2ml tube.

x) Followed steps 15- 16 of protocol 1)

xi) Eluted DNA in 10 x 40µl distilled water, then bulked this eluate

3. MoBio Laboratories Inc. (Carlsbad, CA) PowerMax™ DNA kit

4. MoBio Laboratories Inc. (Carlsbad, CA) UltraClean™ DNA kit

5. MoBio Laboratories Inc. (Carlsbad, CA) PowerSoil™ DNA kit

These samples (or fractions of them) were run through a commercial DNA 'clean-up' kit in order to assess the effect of removing any PCR inhibitors.

A further experiment was performed in order to determine the effect of extremely vigorous shaking of soil prior to DNA extraction (*i.e.* breaking open microsclerotia). In this case, the MoBio PowerSoil extraction approach was followed, except that following the addition of solution C1 from the MoBio kit, a 3mm tungsten carbide bead (Qiagen, Crawley, UK) was added, and the tube shaken for 30 seconds at speed setting 5.5 on a FastPrep machine. The MoBio protocol was then continued as normal.

Subsequently, procedures for bulking up the amount of soil in an extraction were investigated. A typical DNA extraction uses only milligram quantities of DNA.

In order to concentrate the amount of *V. dahliae*, two steps were investigated:

1. The first stage of the current plate testing technique was adopted. This involved shaking the soils in water, and then sieving to retain the 20-160µm fraction. The soil and *V. dahliae* was then washed into weighing

boats, and allowed to settle. The majority of the water was aspirated off using a vacuum pump, and the remainder allowed to evaporate or subjected to treatment 2).

2. The soil/*Verticillium*/water mix was added to 25ml tubes, and lyophilised to total dryness.

Real Time PCR

Several pairs of primers designed to be specific to *V. dahliae* in previous work (Krishnamurthy, 2005; Lievens *et al.,* 2006) were utilised in order to assess amplification efficiency. Where it was necessary to confirm total levels of DNA extracted (i.e. to prove extraction was a success even if PCR is negative for the target organism), 'universal' fungal primers were used.

Primer pair 1 was DB19 (CGG TGACATAATACTGAGAG) and DB22 (GACGATGCGGATTGAACGAA), giving an expected product size of 527bp.

Primer pair 2 was DB19F1 (CGACTTCGTCCCGAGCTCTAG) and DB22R1 (CTCGTGGGACTACTGTTCCA). These were expected to produce a product of size 335bp, and less non-specific background products than DB19/22.

The universal fungal primers were ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC).

PCR conditions had already been determined for use of the DB series primers (Krishnamurthy, 2005) and the ITS-1::ST-VE-1 primer set (Lievens *et al.* 2006)

However, in light of initial results in this project, a few adjustments were made. Attempts included:

- Lowering the primer annealing temperature from 58°C
- Combining primer DB19 with DB22R1 and DB19F1 with DB22
- Increasing the number of PCR amplification cycles from 40 to 45

- Extending annealing, extension and fluorescence detection times from 30s to 45s, 40s to 60s and 40s to 60s respectively
- Setting up a 'nested' PCR with 15 initial cycles using DB19/22, subsampling 1µl from 20µl at this stage, and then performing 40 cycles with DB19F1/22R1

Aside from modifications listed above, all PCRs contained the following components in a 20µl volume: 10µl SYBR Green master mix (Qiagen, Crawley, UK), 450nM each primer and the remainder made up by DNA (usually 1µl) and water.

Each PCR run was performed in 96-well plates in an ABI Prism 7500 Real Time PCR machine. Well A1 always contained a no template control (all components except DNA), the remaining wells either contained *V. dahliae* at standard concentrations, or 'unknown' samples. EMR isolate ref. 12128 was used to provide standards, which were added at 5ng, 500pg, 50pg, 5pg amounts. Three replicates at each amount were always run.

PCR reactions generated a standard curve, against which the amount of *V. dahliae* in 'unknowns' was calculated by the machine.

DNA sequencing

To confirm PCR product identity, aliquots of Real Time PCR samples were visualised using agarose gel electrophoresis (1.5% gels in 0.5x TBE buffer, run at 150V). Bands were excised, DNA purified using GFX PCR purification kit (GE Healthcare, Little Chalfont, UK) and DNA supplied along with an appropriate PCR primer (one used to create the product initially) to the Advanced Biotechnology Centre (London) for sequence analysis.

Results and discussion

Plate tests

Table 1 illustrates the recovery of *V. dahliae* onto agar medium after known amounts of microsclerotia had been added to sterilised soils with no known *Verticillium* present.

Table 1. Recovery of microsclerotia (MS) after addition of known amounts to autoclaved soil

Number of microsclerotia (ms) added to soil (cfu/g)	Number of ms recovered (cfu/g)	Mean number of ms recovered (cfu/g)
0	0, 0, 0, 0, 0	0
1	3.3, 1.3, 1.3, 2.5, 2.4	2.2
2	2.3, 3, 2.4, 2.8, 2.4	2.6
5	12.4, 7.8, 9.9, 8.5, 5.5	8.8
10	11.7, 24.1, 11.2, 17.3, 17.6	16.4

The cfu levels were higher than expected. One explanation may be that the original addition of microsclerotia actually represented clumps of cells which were broken up during the soil sieving process. This could result in greater number of propagules than expected. The absolute amount, however, is not critical. The key experiment was to compare these counts to PCR data with equivalent batches of soil.

Table 2 shows the plate test results for soils of different type to which microsclerotia were added.

Table 2. Recovery of microsclerotia (ms) after addition of known amounts to soils of different composition

Number of ms added / Soil type	Recovery from 1 ms/g	Mean	Recovery from 10 ms/g	Mean
Sandy loam	0 0.5 0.7 0.9 0.2	0.5	1.6 4.7 2.5 3.0 1.9	2.7
Sandy	0.6 0.3 1.4 1.0 0.1	0.7	2.3 1.6 3.1 1.5 1.8	2.1
Red	0.7 0.2 0.1 0.1	0.2	3.1 5.3 1.4 0.7	2.6

Number of ms added / Soil type	Recovery from 1 ms/g	Mean	Recovery from 10 ms/g	Mean
	0		2.6	
Red (lighter)	0.3 0.7 0.1 0.1 0.4	0.3	3.5 1.1 2.2 4.7 1.3	2.6
Peaty	0 0.1 0.3 0.2 0.1	0.1	0.8 1.7 0.4 0.4 0	0.7

On this occasion the cfu counts were consistently lower than expected. Further checks were carried out to try and establish why this might be so.

Results suggested that:

- microsclerotia were added at the correct starting concentration (this was a different microsclerotia preparation to that used to obtain results in Table 1).
- the consistently low scores do not result from sampling variability
- incubation facilities were functioning correctly
- a viability test indicated that the microsclerotia were 96% viable, so there was not a problem with the isolate.
- unlike the results in Table 1, the results in Table 2 were obtained by adding ms to non-sterile soil. It is possible that the presence of other microbes has contributed to the low counts due to antagonism, parasitism or inhibition.

As long as the colony forming units recovered reflect true disease risk more than the number of microsclerotia added, then this is adequate for risk assessment. If not, then the reliability of plate testing might be questioned.

Table 3 shows the data when plate tests were performed on a series of soils previously analysed by a range of non-molecular methods including the current Clinic protocol.

These soils vary slightly in terms of age, and in terms of soil type and *V. dahliae* content.

The general trend is that viability appears to have declined since these samples were analysed 30 months earlier in 2003. In a few cases counts have gone up. This could be due to sampling variability or to break up of large clumps of microsclerotia into smaller ones over time.

Table 3. Recovery of *V. dahliae* from soils of variable soil type, age and estimated microsclerotia content (cfu = colony forming units)

EMR ID number	Age of soil	Soil type	Counts (cfu/g) in 2003	Counts (cfu/g) in current work
PC 324/01	2001	Light, sandy	18.3	0.9
PC 471/02	2002	Red	12.9	8.1
PC 495/02	2002	Sandy	25.4	47.9
PC 478/02	2002	Red	3.4	2.2
PC 485/02	2002	Sandy	2.7	0
PC 488/02	2002	Clay-loam	8.5	0.3
PC 489/02	2002	Loamy	0.6	0.8
PC 497/02	2002	Sandy-loam	0.1	0
PC 506/03	2003	Red	0.2	0.3

Evaluation of DNA extraction from *V. dahliae* in soil

Table 4 illustrates the results of PCR amplifications after using five different protocols to extract total DNA from soil. Two different levels of *V. dahliae* (0.5ms/g and 5ms/g) had been added to these soils. A portion of the extract was put through a DNA clean-up kit prior to PCR (not Real-Time), which was performed using ITS1/4 primers and the following conditions:

2.5µl NH_4^+ reaction buffer, 0.2mM each dNTP, 1.5 units *Taq* polymerase (Qiagen), 0.2mg/ml final concentration BSA, 5µl Q-solution, 15pmol primers and 1µl DNA in a final volume of 25µl.

Cycling parameters were:

One cycle of {94°C for 3 minutes, 54°C for 40 seconds, 72°C for 1 minute}
34 cycles of {94°C for 1 minute, 54°C for 40 seconds, 72°C for 1 minute}

A final step at 72°C for 5 minutes

Table 4 shows that fungal DNA could be isolated and amplified using commercial kits. However, no amplification was detected using the *Verticillium* primers DB19/ DB 22 nor DB19F1/DB22R1 (data not shown).

Table 4. Results of PCR amplifications using primers ITS1/4 following extraction of DNA from soil samples by various methods (ms = microsclerotina)

Extraction kit used	0.5 ms/g *V. dahliae* added	0.5 ms/g added, and DNA cleaned before PCR	5 ms/g *V. dahliae* added	5 ms/g added, and DNA cleaned before PCR
DNA Stool kit – method 1	-	-	-	-
DNA Stool kit, adapted – method 2	-	-	-	(+)
PowerMax – method 3	-	-	+	(+)
UltraClean – method 4	-	+	-	++
PowerSoil – method 5	+	(+)	++	+

An interesting observation was that greater intensity of product was seen with the same soil when 5 ms/g was added, as opposed to 0.5 ms/g. This would imply that the difference must be due to *V. dahliae* and that some of the ITS1/4 product might well be *V. dahliae.* The fact that DNA could be amplified from some of these extracts implies that PCR inhibitors do not explain why the *V. dahliae* specific primers have not worked. However, as previously noted, the length of the PCR product was supra-optimal for qPCR.

After these experiments, the most effective extraction kits appeared to be the MoBio PowerSoil and UltraClean. The PowerSoil appeared effective to some extent with or without clean-up and seemed to offer the best prospects of the five extraction methods tested.

Enhanced soil preparation for DNA extraction

In order to attempt to increase the amount of soil mass in each extraction, soils containing 5ms/g *V. dahliae* were sieved to remove fractions outside the range 20-160µm. The wet soil remaining was either used directly for extraction, or freeze-dried before use. The results are shown in Table 5.

PCR conditions for DB19F1/22R1 and DB19/22 were as listed previously for ITS1/4, except that 10 pmol of the primers were used.

Cycling parameters were:

One cycle of {94°C for 3 minutes, 52°C for 1 minute and 72°C for 1 minute}
34 cycles of {94°C for 1 minute, 52°C for 1 minute and 72°C for 1 minute}
A final step of 72°C for 5 minutes

Some of the kits (Stool and Plant DNEasy) appeared to successfully yield DNA from soil only after sieving, freeze-drying and 100x dilution, implying that inhibitors were a problem.

DB19F1/22R1 were shown capable of amplifying *V. dahliae*, but not after extraction from soil under these PCR conditions.

DB19/22 showed promise following sieving and drying steps and a PowerSoil extraction.

At the commencement of this research, a tentative protocol was already in place (Krishnamurthy, 2005) for amplification of *V. dahliae* DNA using Real Time PCR.

Table 5. Pre- and post-DNA extraction manipulation of soil samples in order to successfully amplify *V. dahliae* DNA

Extraction method	PCR with DB19F1/22R1	PCR with DB19/22	PCR with ITS1/4
(1) Sieved → Stool kit	-	-	-
(2) Sieved and freeze-dried → Stool kit	-	-	-
(3) Sieved and freeze-dried → DNEasy Plant kit (Qiagen)	-	-	-

(4) Sieved →PowerSoil kit	-	-	++
(5) Sieved and freeze-dried → PowerSoil kit	-	+	++
As (1), then diluted 100x for PCR	-	-	-
As (2), then diluted 100x for PCR	-	-	+
As (3), then diluted 100x for PCR	-	-	+
As (4), then diluted 100x for PCR	-	-	-
As (5), then diluted 100x for PCR	-	-	-
V. dahliae pure DNA	+	++	++

For a 20µl reaction this included:

Water, 10µl SYBR Green master mix, 300nM each primer (DB19F1/22R1) and the DNA sample (5µl).

The cycling parameters were:
1 step at 50°C for 2 minutes
1 step at 95°C for 15 minutes
40 cycles of {95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds and 79°C for 30 seconds}
The reaction was completed by generating a dissociation profile according to the Real Time PCR machine software.

In the current study, the most successful amplification of PCR products has been obtained by making the following adjustments, highlighted in bold below.

Water, 10µl SYBR Green master mix, **500**nM each primer **(DB19/22)** and the DNA sample (**1**µl undiluted).

The cycling parameters were:
1 step at 50°C for 2 minutes
1 step at 95°C for 15 minutes
40 cycles of {95°C for 30 seconds, **56**°C for **45** seconds*****, 72°C for **60** seconds and 79°C for **60** seconds}

The reaction was completed by generating a dissociation profile according to the Real Time PCR machine software.

***** - this temperature was lowered by 0.1°C on each consecutive cycle

Combining primer DB19 with DB22R1 and DB19F1 with DB22 did not improve amplification efficiency – the latter combination was entirely unsuccessful.

Increasing the number of PCR amplification cycles from 40 to 45 did lead to slightly greater levels of PCR product and may be useful in amplification of samples with very low *V. dahliae* amounts.
Setting up a 'nested' PCR with 15 initial cycles using DB19/22, subsampling 1µl from 20µl at this stage, and then performing 40 cycles with DB19F1/22R1 resulted in greater difficulty in maintaining a standard curve, and so has not been pursued further. The aim of this was to 'kick-start' amplification, and then boost this for low copy number templates, by greater overall number of cycles.

DNA sequencing identified that this had a sequence similarity of 97-99% with known sequences of *V. dahliae*. In order to confirm that this was not a PCR product from the closely related *V. tricorpus*, DNA was extracted from a culture of such, and amplified using both ITS1/4 and DB19/22. Whereas DB19/22 could amplify *V. dahliae*, they could not amplify *V. tricorpus.* ITS1/4 was successful in amplifying *V. tricorpus,* confirming that its DNA was viable for PCR.

This means that amplification of *V. dahliae* from a soil with zero cfu/g plate counts has occurred. Validation of the PCR against plate testing will determine how common this effect is, and host testing may be needed to interpret its significance.

Further investigations indicate that the amplicon size for the DB19/DB22 primer pair was too large for effective quantitative PCR. That is, an amplicon of between 150 and 200 bp is ideal for RT-PCR, where the product for DB19/DB22

is greater than 400 bp. Other primer pairs have, as a consequence, been chosen in preference to the DB19/DB22 primer pair: ITS1F and ST-VE1R, which yield a product of near-optimal size (Lievens et al., 2006) were evaluated. However, the ST-VE1R primer was found to anneal to its own product, thus rendering it unsuitable for qPCR. A modification of this primer was designed (ST-VE1mR) with an oligonucleotide sequence of: 5'-GTTTTAATAATGGTTCGCTAAGA-3' and is presently being evaluated in the context of a HortLink project on biofumigation.

Extraction efficiency: the efficiency of the MoBio PowerSoil extraction kit was compared to that of the FastPrep cell dispruptor (MPBiomedicals LLC, Ohio, USA) and associated chemistry (FastDNA Spin for Soil Kit [MPBiomedicals])(Fig. 1). The FastPrep was found to yield more DNA and of higher quality than the other methods. The use of tungsten carbide beads (Qiagen, Crawley, UK) did not improve extraction efficiency (data not shown).

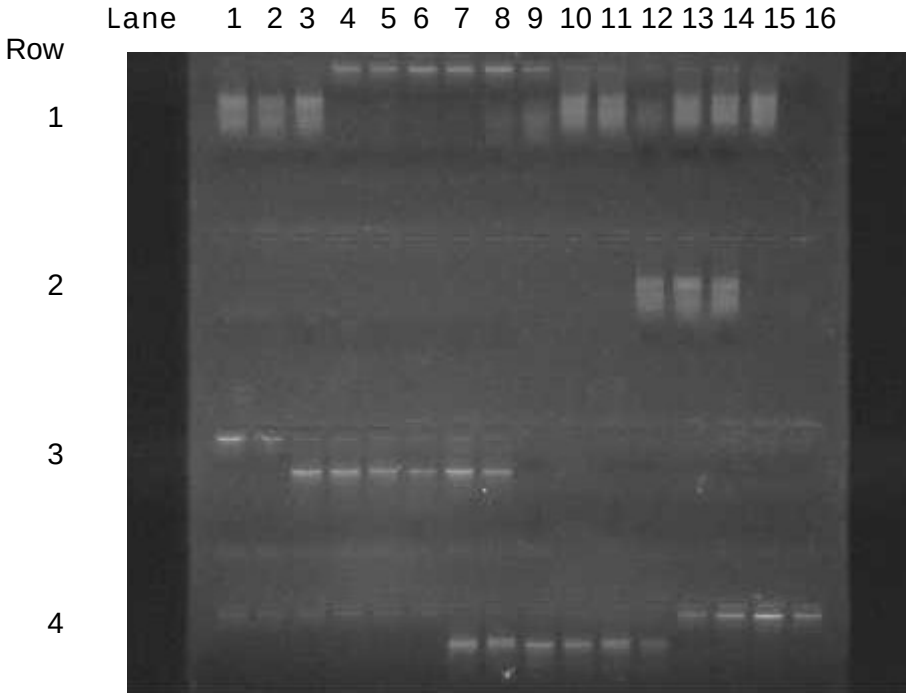

Figure 1. **Comparison of FastPrep Cell Disruptor to a rotary bead beater with and without subsequent clean-up of DNA products with and without polyvinylpyrrolidone spin column for amplification with universal prokaryotic (top two rows) and eukaryotic (bottom two rows) primers.**

Top row: lanes 1-3: Bead beater + PVP; lanes 4-9: FastPrep without PVP; lanes 10-15: FastPrep with PVP; lane 16: no template control. Second row: lanes 12-14: bead beater + PVP. Third row: lanes 1-2: Fast Prep + PVP; lanes 3-8: FastPrep + PVP; lane 9: no template control; lanes 11-16: bead beater –PVP; row 4: lanes 1-6: bead beater –PVP; lanes 7-12: bead beater + PVP; lanes 13-16: FastPrep – PVP

The GeneClean (MPBiomedicals, Ohio, USA) system, which utilises guanidine for the removal of humic PCR inhibitors was also evaluated and found to enhance both the yield of genomic DNA and amplifiability of the extracts. The GeneClean system was compared to the GFX DNA Cleaning system and found superior (Fig. 2.).

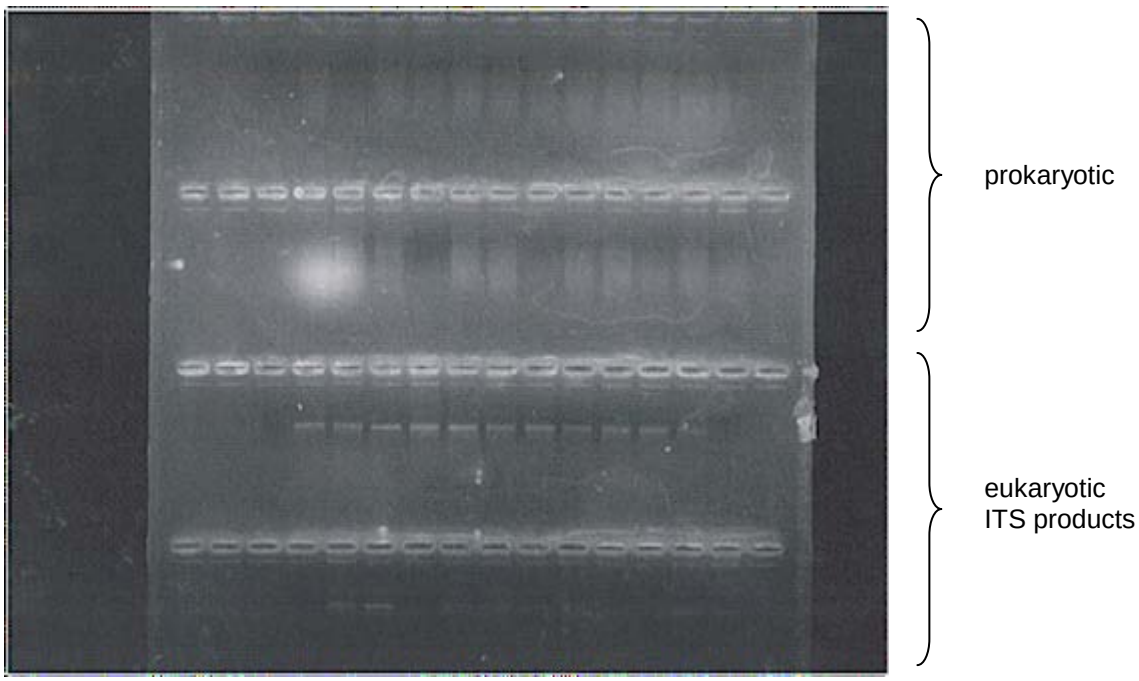

Figure 2. **Comparison of GFX PCR and GeneClean Spin kits. Upper two rows: prokaryote ITS amplification products: upper row cleaned by GeneClean and lower by GFX. Lower two rows: eukaryote ITS amplification products: upper row GeneClean and lower row GFX**

Preparation of polyvinylpyrrolidone (AppliChem GmbH, Darmstadt, Germany) spin columns (BioRad, Hemel Hempstead, UK) for removal of putative PCR inhibitors was evaluated (Fig. 1). The quality of extracted DNA improved markedly through use of the spin column, although some DNA was

(inevitably) lost during the cleaning. PCR detection improved, but quantitation became even more difficult.

The 'best' method for extraction would appear to be: (1) use of a FastPrep cell disruptor apparatus with the FastDNA Spin for Soil Kit extraction kit; subsequent cleaning of the extract with the GeneClean kit; followed by purification by centrifugation through a PVP column.

Development of an internal standard for estimating extraction efficiency has yet to be performed. However, a strategy for accomplishing this is outlined: the addition of known quantities of endospore forming bacteria that have been transformed with an 'exotic' gene not likely to be found in soil, such as the green fluorescent protein, to soil can be compared to the amplification efficiency of the bacterial gene, alone. That is, spiking the soil with a known quantity of foreign DNA and amplification of the material will yield a procedure calibration standard.

Conclusions

1. Recovery of *V. dahliae* by plate testing varies depending on a number of factors, including soil type, age of soils, and probably, the presence of other microbes.

2. Extraction and amplification of *V. dahliae* DNA from soil is possible using protocols developed prior to, and during this project.

3. *V. dahliae* DNA may be recovered from soil where plate testing gives results of 0 cfu/g implying a lower detection threshold than that of the Harris test.

4. Primers appropriate for RT-PCR need to be developed for quantification of *V. dahliae* inoculum in soils. Detection of *V. dahliae* has been shown.

Technology Transfer

None

Glossary

- amplicon: the DNA product that is abundantly reproduced by PCR
- PCR: polymerase chain reaction
- cfu: colony forming unit
- ms: microsclerotia (resting structures)
- Real Time (or quantitative) PCR: a polymerase chain reaction in which the product is determined by the fluorescence of a probe or intercalating dye after each amplification cycle yielding data as to the initial quantity of DNA in the sample
- PVP: polyvinylpyrrolidone
- SYBR green: a fluorescent dye that intercalates between the double strands of DNA. Fluorescence increases as the strands denature. Hence, the fluorescence is a measure of the amount of DNA produced during an amplification cycle

References

Carder J.H., Morton, A., Tabrett, A.M. & Barbara, D.J. (1994). Detection and differentiation by PCR of sub-specific groups within two *Verticillium* species causing vascular wilt in herbaceous hosts. In '*Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification*'. Eds A. Schots, F.M. Dewey & R. Oliver. CAB International, Wallingford pp 91-97

Harris, D.C. & Yang, J.R. (1996). The relationship between the amount of *Verticillium dahliae* in soil and the incidence of strawberry wilt as a basis for disease risk prediction. *Plant Pathology* **45**:106-114

Heinz, R.A. & Platt, H.W. (2000). A competitive PCR-based assay to quantify *Verticillium tricorpus* propagules in soil. *Canadian Journal of Plant Pathology* **22**:122-130

Krishnamurthy, V. (2005). Quantification of *Verticillium dahliae* in soil. PhD Thesis, University of Aberdeen

Lievens, B., Brouwer, M., Vanachter, A.C.R.C., Cammue, B.P.A., Thomma B.P.H.J. (2006) Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. *Plant Science* **171**: 155-165

Locke, T & Harris, D. (1993). Testing time for berry wilt. *Grower* **120(4)**: 28-29

Lovelidge, B. Strawberry wilt tests confirm widespread infection. *The Fruit Grower* April 2004, 18-19.

Mahuku, G.S. & Platt, H.W. (2002). Quantifying *Verticillium dahliae* in soils collected from potato fields using a competitive PCR assay. *American Journal of Potato Research* **79**: 107-117

Mauchline, T.H., Kerry, B.R. & Hirsch, P.R. (2002). Quantification in soil and the rhizosphere of the nematophagous fungus *Verticillium chlamydosporium* by competitive PCR and comparison to selective plating. *Applied and Environmental Microbiology* **68**: 1846-1853

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodriguez-Jurado, D. & Jimenez-Diaz, R.M. (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with

real-time polymerase chain reaction. *Physiological and Molecular Plant Pathology* **63**: 91-105

Termorshuizen, A.J., Davis, J.R., Gort, G., Harris, D.C., Huisman, O.C., Lazarovits, G., Locke. T., Vara, J.M.M., Mol, L., Paplomatas, E.J., Platt, H.W., Powelson, M., Rouse, D.I., Rowe, R.C., & Tsror, L. (1998). Interlaboratory comparison of methods to quantify microsclerotia of *Verticillium dahliae* in soil. *Applied and Environmental Microbiology* **64**: 3846-3853

Volossiouk, T., Robb, E.J., Nazar, R.N. (1995). Direct extraction of DNA for PCR-mediated assays of soil organisms. *Applied and Environmental Microbiology* **61**: 3972-3976

