Project Title	Protected tomato: monitoring rhizosphere micro-organisms to improve understanding and management of root diseases
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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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GROWER SUMMARY

Headline

A molecular fingerprinting method (T-RFLP) based on DNA fragment length is being optimised to investigate the occurrence and relative levels of microbial communities associated with tomato roots to see if root disease can be predicted. A database of fragment lengths is being established and currently contains the theoretical fragment length for 84 fungi and 3 bacteria reported associated with tomato roots. Root samples from soil, rockwool and NFT tomato crops tested using conventional methods and T-RFLP gave similar results for major culturable organisms.

Background and expected deliverables

Root-infecting fungi are commonly found in tomato and occasionally result in severe disease. Non-pathogenic fungi and bacteria are also common in the root environment and the presence and levels of these can influence the occurrence of root disease. Obtaining information on the occurrence and levels of micro-organisms in, on and around tomato roots (the rhizophere) has, until recently, been difficult and time-consuming. As a consequence, the early signs of root disease are often missed. A novel molecular method known as Terminal Restriction Fragment Length Polymorphism (T-RFLP) permits simultaneous relative quantification of micro-organisms. This project aims to use T-RFLP to investigate the occurrence and relative levels of major pathogenic (e.g. species of *Pythium*, *Phytophthora*, *Fusarium*, *Thielaviopsis*) and non-pathogenic micro-organisms (e.g. species of *Penicillium*, *Pseudomonas*, *Trichoderma*) associated with roots of tomato crops in various substrates.

The expected deliverables from this project are:

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1. An increased understanding of the role of rhizosphere micro-organisms in maintenance of root health;
2. Knowledge of whether a molecular test (T-RFLP) that determines occurrence and relative levels of pathogenic and non-pathogenic fungi and bacteria can be used to predict risk of root disease.

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Summary of the project and main conclusions

Fungi and bacteria reported associated with tomato roots

Examination of the scientific literature indicates a world total of at least 66 fungal pathogens (Table 1) and 75 saprotrophs that have been found associated with roots or growing media of tomato plants. A majority of these fungi occur in the UK.

Fungal genera with five or more different species causing disease of tomato roots are *Pythium* (19 species), *Phytophthora* (18), *Fusarium* (7) and *Verticillium* (5). Some fungi that affect aerial parts of tomato plants may also infect roots, including *Botrytis cinerea*, *Didymella lycopersici* and *Phytophthora infestans*. Disease symptoms caused by root-infecting fungi include root rot, foot rot, crown and root rot, damping-off and vascular wilt.

Fungal genera with five or more saprophytic species recorded associated with tomato roots or growing media are *Penicillium* (15 species) and *Aspergillus* (5). For some fungi (e.g. *Fusarium oxysporum*), both pathogenic and non-pathogenic strains are known to occur on tomato roots.

Four bacterial taxa (*Agrobacterium* spp., *Clavibacter michiganensis*, *Pseudomonas syringae* pv. *Tomato* and *Ralstonia solanacearum*) that cause disease in tomato have been recorded in tomato roots. The diseases are all rare apart from root mat disease caused by rhizogenic strains of *Agrobacterium* sp. Bacterial saprotrophs and mycorrhizal fungi and bacteria associated with tomato roots are not well documented.

Review of factors influencing tomato root diseases

Factors that influence tomato root diseases include temperature, moisture and growing medium texture, pH and nutrient levels. Effects are often complex due to interaction of factors and results are sometimes contradictory. Some reported effects on specific diseases are summarised.

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Biotic factors that influence tomato root diseases include microbial amendments and organic matter amendments. Although there are numerous research reports where microbial interventions have influenced development of root disease, no microorganisms have been developed for sale as biocontrol products registered for use on tomato in the UK. For soil-grown tomato, garden waste compost is reported to reduce brown and corky root rot and a lettuce green manure crop reduced fusarium crown and root rot.

Table 1: List of fungal pathogens reported associated with tomato roots

Fungus	Disease	Comment
Alternaria solani	Damping-off	Common
Aphanomyces cladogamus	Root rot, rootlet necrosis	Uncommon
Armillaria mellea	Honey fungus	Rare
Botrytis cinerea	Grey mould	Usually on aerial pathogen
Calyptella campanula	Calyptella root rot	Rare; soil-grown crops only
Colletotrichum coccodes	Black dot	Common
Didymella lycopersici	Didymella stem rot	Uncommon on root
Fusarium species (7*)	Wilt/Crown and root rot /root rot	
Humicola fuscoatra	–	Pathogenicity disputed
Macrophomina phaseolina	Charcoal rot	Rare
Monographella cucumerina	Root rot	Minor pathogen
Phymatotrichopsis omnivora	Root rot	Not present in Europe
Phytophthora species (18*)	Rot rot/foot rot/damping-off	Quite common
Pyrenochaeta lycopersici	Brown and corky root rot	Common in soil crops
Pyrenochaeta terrestris	Root rot	Secondary pathogen
Pythium species (19*)	Root rot/damping-off	Common
Rhizoctonia solani	Rhizoctonia root rot	Common
Sclerotium rolfsii	Southern blight	Not in UK
Spongospora subterranea	Powdery scab	Occasional
Thielaviopsis basicola	Black root rot	Fairly common in NFT crops
Verticillium species (5*)	Verticillium wilt	–

*See Science section for full listing

Monitoring tomato rhizosphere fungi by isolation

Roots samples collected from commercial tomato crops grown in soil, rockwool and by nutrient film technique (NFT) were examined for fungi by plating onto a general nutrient agar and a *Pythium*-selective agar. The aims were: (1) to devise a root sampling procedure for each growing medium; (2) to determine if fungal taxa found associated with roots by culturing on agar were also detected by a T-RFLP test; (3) to provide cultures of a range of fungal isolates identified to genus or species level on morphological features and colony appearance, for use as reference cultures in T-RFLP tests.

For soil-grown tomato, a soil auger was used to collect soil and roots to 20 cm depth and at different locations relative to the stem base. The predominant fungi recovered on agar were *C. coccodes*, *Fusarium* sp., green colonies (probably *Trichoderma* spp.) and pythiaceous spp. One or more fungal colonies grew from almost all root pieces (5-10 mm in length) plated onto agar. *Fusarium* sp. was recovered more frequently from roots mid-way between propagation cubes than adjacent to cubes and vice-versa for *C. coccodes.* Pythiaceous fungi and *Trichoderma* colonies developed more commonly from thick (5-8 mm diameter) than thin (1-1.5 mm) roots. *Fusarium oxysporum*, *Penicillium* sp. and a grey sterile fungus were recovered at a low incidence only from thin roots.

For rockwool-grown tomato, roots were obtained using a 2 cm cork borer inserted to the full depth of the slab and by cutting off roots at the slab corner. The predominant colony types recovered from roots were white (probably pythiaceous and *Fusarium* sp.), pink/red (probably *Fusarium* sp.) and *C. coccodes. C. coccodes* was more common on roots adjacent to the cube than mid-way between cubes or at the slab corner.

For NFT-grown tomato, a wedge of roots was cut from the channel midway between two plants. The predominant fungi recovered were *C. coccodes*, *Fusarium* sp., *Mucor/Rhizopus* and pythiaceous fungi. *Thielaviopsis basicola* was found at a

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low incidence. *Fusarium* sp. was recovered more frequently from brown than white roots.

For all growing media, the incidence and diversity of fungi recovered was significantly influenced by type of agar used and whether or not the roots were surface-sterilised before plating. There were also significant differences in isolation frequency between replicate plants sampled along a row, except for the NFT crop, sample position with respect to the stem base (rockwool and soil crops), root thickness (soil crop) and root colour (NFT crop).

Monitoring rhizosphere fungi and bacteria by T-RFLP

The molecular fingerprinting method T-RFLP was chosen for this study because of its simplicity and adaptability and because it detects non-culturable organisms. Using the primers and restriction enzymes detailed in this report, a database of theoretical fragment lengths of fungi and bacteria was created based on previously published data of nucleic acid sequences. So far, the database contains the theoretical fragment length for around 460 bacteria and 150 fungi. This includes 84 fungi and 3 bacteria that have previously been reported associated with tomato roots.

The theoretical length of the Terminal Restriction Fragments (TRFs) of some of these organisms was confirmed by comparison with the actual TRF obtained by T-RFLP of identified cultures (Fig. 1). To date, the TRF length of 15 fungi have been confirmed, including six pathogens (*Colletotrichum coccodes*, *Fusarium oxysporum*, *Phytophthora cryptogea*, *Plectosphaerella cucumerina*, *Pythium diclinum*, *Rhizoctonia solani*) and three saprophytes (*Cladosporium* sp., *Gliocladium* sp. and *Penicillium expansum*) reported associated with tomato roots.

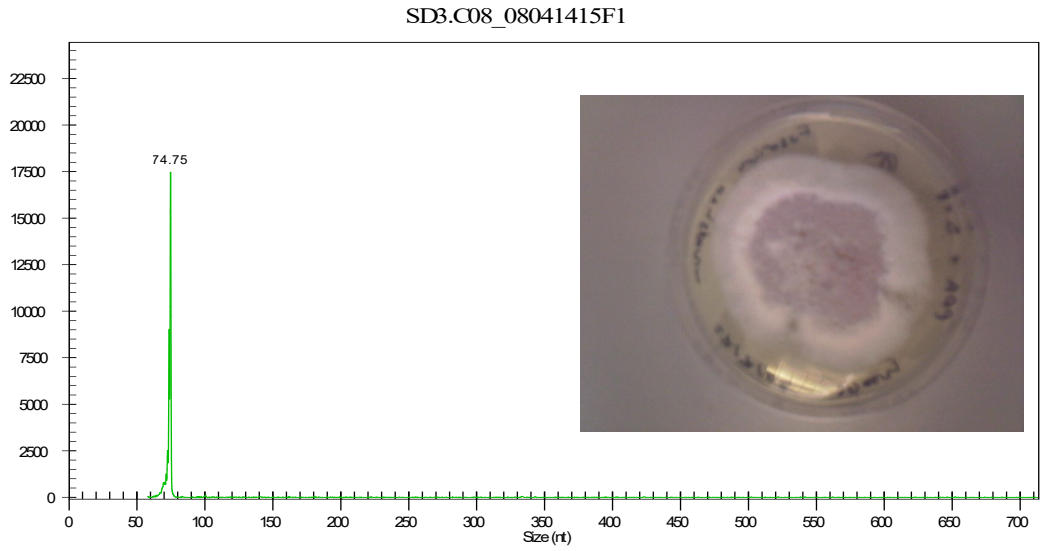

Figure 1: T-RFLP analysis of *a Fusarium oxysporum* culture, confirming the DNA fragment length when cut with a specific restriction enzyme to be 75 base pairs. A culture on agar of the fungus tested is illustrated.

Two DNA extraction methods were compared: direct extraction of c. 100 mg of tomato root and a washing method on larger root samples (<1g). The fungal fingerprints from the two extraction methods were similar; however the wash method failed to detect bacteria, and does not provide an internal plant DNA control. The direct extraction method was chosen for future work as it potentially allows semi-quantitative analysis of both fungal and bacterial genera in a single test.

Several bacterial DNA primers were examined for their consistency and ability to bind to bacterial DNA. There was some variability in the organisms detected using different primer combinations because of variability in the consensus sequences that the primers bind to in different organisms. The pair of primers that gave the most reliable and representative results, and which detected the greatest number of bacteria were selected for future work.

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An examination of the effect of root thickness on microbial diversity showed that there was a greater diversity on young, actively-growing thin roots than older, woody roots of soil-grown tomato. There was also a decrease in the relative level of microorganisms, when compared with tomato DNA control, on the thicker roots (Fig. 2).

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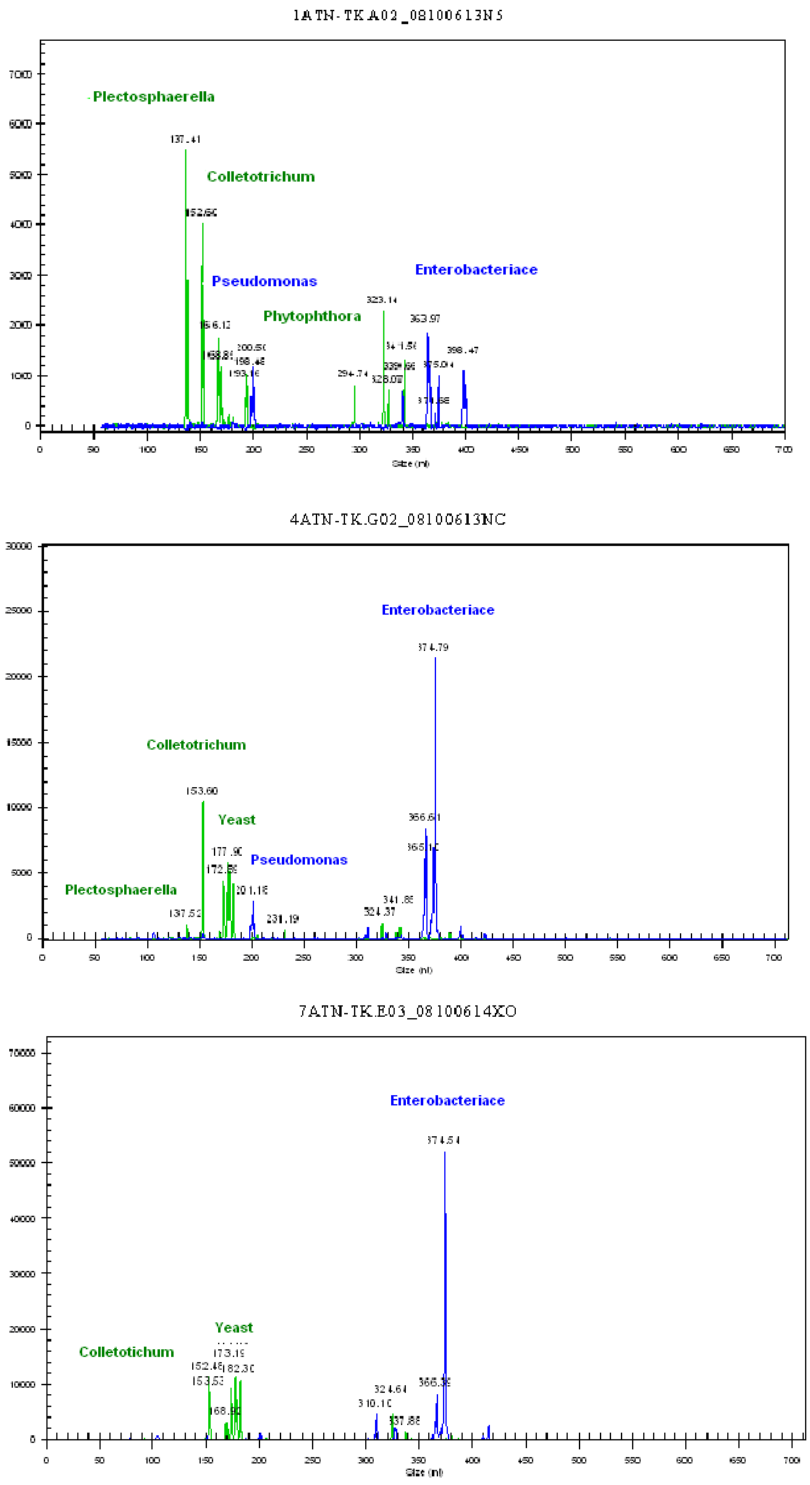

Figure 2: T-RFLP analysis of roots of different size from the same soil grown tomato plant - July 2008. Note that there are more peaks (indicating more micro-organisms) on the thin roots (top) in comparison with the medium (middle) and thick (bottom).

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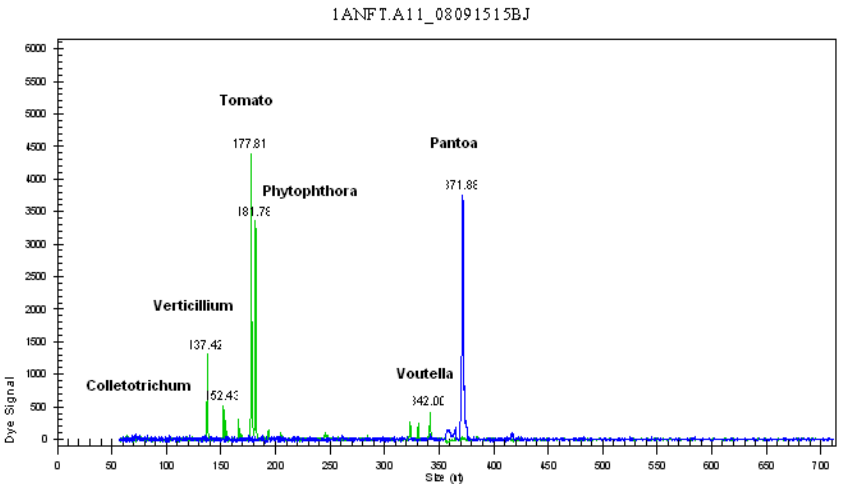
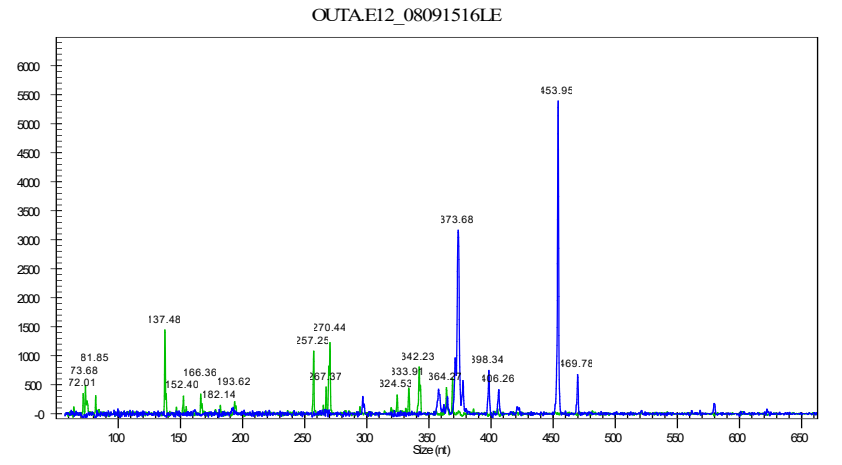
Three sampling methods were compared for tomatoes grown in rockwool slabs: cork-borings adjacent to the propagation cube, cork-borings mid-way between cubes and a slice of roots taken from the slab corner. There was little difference between the samples in DNA recovery or microbial diversity. Results were also very similar between three replicate plants in the same row.

Five root samples taken from one row in an NFT crop were compared. There was little variation among the root samples. There was a far greater diversity of microorganisms in the solution than in root sample (Fig 3), with little difference between inlet and outlet ends.



Root



NFT solution

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Figure 3: T-RFLP analysis of roots and solution from an NFT crop, August 2008. Note the occurrence of *Verticillium* (confirmed in a symptomatic plant in another row) and the greater number of microorganisms present in the nutrient solution.

The range of microorganisms identified by plating onto agar and by T-RFLP was compared. Most microorganisms identified by plating onto agar were also detected by T-RFLP analysis (Table 2). A few fungi detected by plating were not detected by T-RFLP and vice-versa. This result indicates that the T-RFLP protocol being used is appropriate for studying microbial communities on tomato roots from commercial crops.

The results of T-RFLP analysis of numerous root samples show that there are many fungal and bacterial peaks present that do not correspond to TRFs on the database created. Unidentified peaks that occur frequently will be examined to determine DNA sequences; the micro-organisms will be identified by comparison with previously published sequences.

Table 2: Detection of major fungal groups and species, from roots of tomato grown in soil, rockwool and NFT, by conventional and T-RFLP methods

Fungal group or species	Detected in:		
	Soil crop	Rockwool crop	NFT crop
Colletotrichum coccodes	Both	Both	Both
Fusarium sp.	Both	Both	Both
Fusarium oxysporum	Conventional		T-RFLP
Penicillium	Both		T-RFLP
Pythiaceous	Both	Both	T-RFLP
Trichoderma	Both		T-RFLP
Verticillium			T-RFLP
Other	Both	Both	Both

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Financial benefits

Root-infecting fungi are commonly found on tomato plants grown in soil, substrate and NFT crops and occasionally case severe disease. On individual nurseries, root disease may result in widespread plant wilting and necessitate early crop removal. Yield loss due to root dieback associated with minor root pathogens is also possible. Estimates of yield loss to root diseases have not been reported. With 145 ha of protected tomato in the UK in 2007 (Defra Horticultural statistics) and a farm gate value of £150 million (TGA estimate), and assuming 5% of marketable yield is lost due to root disease, this represents lost output valued at £7.5 million If 10% of this loss could be prevented by prediction of the risk of root disease, the annual saving to growers would be around £1.5 million, or £5,172/ha (less the cost of implementing the improved root disease control).

Action points for growers

None at present.

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Root-infecting fungi are commonly found on the roots of tomato plants grown in nutrient film technique (NFT) and substrate crops, and occasionally cause severe disease. These include *Pythium* root rot, *Phytophthora* root rot and black root rot (*Thielaviopsis basicola*). Other pathogens occur less commonly, or are considered to be weakly pathogenic (e.g. black dot caused by *Colletotrichum coccodes*, root rot caused by some *Fusarium* species). Vascular wilt pathogens (e.g. *Fusarium oxysporum*, *Verticillium albo-atrum*) commonly infect plants via the roots, and verticillium wilt caused by *V. albo-atrum* has been more troublesome since 1996. Additional root diseases have been recorded in soil-grown organic crops (e.g. brown and corky root rot, powdery scab), despite the use of disease-resistant rootstocks. It is difficult to monitor and quantify the development of root diseases due to the difficulty in viewing roots (except for NFT crops) and the need for laboratory tests to determine the precise cause. As a consequence, the early stages of a root disease are often missed.

A complex of fungi and bacteria live in, on and around tomato roots (the rhizosphere). Both plant pathogens and saprophytes, and culturable and non-culturable organisms are likely to be present. There is evidence that the presence of high populations of certain micro-organisms (e.g. *Aspergillus* spp., *Penicillium* spp., *Pseudomonas* spp.) can reduce disease through direct antagonism, the production of antimicrobial compounds, niche exclusion and/or stimulation of systemic plant defence responses. Interactions among pathogenic and non-pathogenic micro-organisms on roots are not well understood because of the difficulty and complexity of studying the root environment. If interactions among rhizosphere micro-organisms were better understood, it might be possible better to predict the risk of damaging root disease.

If it becomes obligatory to recycle waste irrigation solution from substrate crops, as has recently happened in Denmark, the potential for widespread root disease in a crop is significantly increased. Where recycling has been used in the UK

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previously, there were some significant disease problems (e.g. *Phytophthora* root rot). Several UK growers are now planning to recycle irrigation solution because of environmental concerns, increased fertiliser costs and the need to demonstrate sustainable systems.

With the recent move by Government and some retailers towards carbon-neutral sustainable production, there may be increased pressure to grow in organic media (e.g. bark and wood fibre products) rather than media that consume significant energy to produce, are used just once, and create a waste disposal problem (e.g. rockwool). The microbial profile that develops on roots may differ considerably according to the medium in which a crop is grown.

The recently developed molecular technique of Terminal Restriction Fragment Length Polymorphism (T-RFLP), based on Polymerase Chain reaction (PCR) and DNA fragments analysis, allows semi-quantitative determination of fungal and bacterial genera in a single test. All fungi and bacteria associated with roots can be monitored and the relative amounts in different samples can be determined by using the plant DNA as an internal standard. Fungi and bacteria present in a sample are identified by reference to a database of the predicted DNA fragment sizes following PCR and treatment with specific restriction enzymes. This project aims to use T-RFLP to investigate the occurrence and relative levels of major pathogenic and non-pathogenic micro-organisms associated with roots of tomato crops. The information gained will be examined to determine if it can be used to predict the risk of root disease.

In Year 1 of the project the objectives are:

1. To compile a list of fungi and bacteria previously found associated with tomato roots and briefly review factors influencing root diseases;
2. To examine roots from tomato crops grown in soil, rockwool and NFT by culturing on agar and by T-RFLP;

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3. To optimise a T-RFLP protocol for determining fungi and bacteria associated with tomato roots.

Review of fungi and bacteria associated with tomato root and factors influencing root disease

Introduction

The objectives of this brief review were to produce a check-list of fungi and bacteria that have previously been reported associated with tomato roots. The check-list will be used to cross-reference with identifications determined in the project by T-RFLP. At the same time, information on the effect of some abiotic and biotic factors on the development of tomato root diseases is summarised. This will be used in later stages of the project to inform experiments seeking to manipulate tomato rhizosphere microorganisms.

Materials and methods

Records of fungi and bacteria associated with tomato roots and of factors influencing tomato root diseases were obtained from:

Web searches

CABI Bioscience (Index Fungorum database)

CSL check list of fungal plant pathogens recorded in the UK

Fungal records database of Britain and Ireland (Cooper & Kirk, 2007)

Reference books, including:

A colour atlas of tomato diseases: observation, identification and control (Blancard, 1994)

British parasitic fungi (Moore, 1959)

Compendium of soil fungi (Domsch & Gams, 1993)

Compendium of tomato diseases (Jones *et al.*, 1991)

Dematiaceous hyphomycetes (Ellis, 1971)

Diseases of glasshouse crops (Fletcher, 1984)

Fungal wilt diseases of plants (Baker, 1981)

Fungi on plants and plant products in the United States (Farr *et al.*, 1995)

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Pest and Disease Management Handbook (Alford, 2000)

Phytophthora diseases worldwide (Erwin & Ribeiro, 1996)

Report on diseases of cultivated plants in England and Wales for the years 1957–1964 (Baker, 1972)

The genus Fusarium (Booth, 1971)

Vegetable diseases (Koike *et al.*, 2007)

Vegetable crop diseases (Dixon, 1981)

Results

Fungi associated with roots

Fungal pathogens and saprotrophs reported associated with tomato roots are listed in Tables 1.1.1 and 1.1.2 respectively.

Fungal pathogens

A world total of 66 pathogens are listed of which at least 34 occur in the UK. Genera with five or more different species documented are *Pythium* (19), *Phytophthora* (18), *Fusarium* (7) and *Verticillium* (5). Some fungi commonly considered as pathogens of aerial parts also occur on roots, including *Botrytis cinerea*, *Didymella lycopersici* and *Phytophthora infestans*.

Fungal saprotrophs

A world total of 75 saprotrophs are listed associated with tomato roots or growing medium of which at least 68 occur in the UK, though they have not necessarily been recorded on tomato roots or growing mediam. Fungal genera with five or more species recorded in the tomato rhizosphere are *Penicillium* (15) and *Aspergillus* (5). For some species (e.g. *Fusarium oxysporum*) both pathogenic and non-pathogenic strains are known to occur on tomato roots.

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Table 1.1.1: List of fungal pathogens reported associated with tomato roots

Fungus	Disease	Recorded on tomato in UK	Comment	Reference
Alternaria solani	Damping off / leaf spot	?	'A common cause of damping off, causing dark lesions on rootlets' (Ellis)	Ellis (1971)
Aphanomyces cladogamus	Root rot, damping off, rootlet necrosis	?	Few reports	Domsch & Gams (1993), Farr *et al.*, (1995)
Armillaria mellea	Honey fungus	Yes	Rare: on outdoor, soil-grown plants.	Moore (1959)
Botrytis cinerea	Grey mould	Yes	Isolated from root of NFT tomato. Usually a foliar pathogen.	Price (1980)
Calyptella campanula	Calyptella root rot	Yes	Rare: soil-grown crops	Fletcher (1984), Clark (1983)
Colletotrichum coccodes	Black dot	Yes	Common: often considered a weak pathogen.	Blancard (1994), Jones *et al.*, (1991)
Didymella lycopersici	Stem rot	Yes	Common on stems – uncommon on roots	Evans (1979), Watterson (1986)
Fusarium oxysporum	Fusarium root rot	Yes	Quite common	O'Neill & Wedgwood (2006)

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Fungus	Disease	Recorded on tomato in UK	Comment	Reference
Fusarium oxysporum f. sp. *lycopersici*	Fusarium wilt	Yes	Uncommon, unless tomato variety lacks resistance.	Fletcher (1984), Jones *et al.*, (1991), Blancard (1994) and others
Fusarium oxysporum f. sp. *radicis-lycopersici*	Fusarium crown and root rot	Yes	Uncommon	Blancard (1994), Jones *et al.*, (1991)
Fusarium redolens	Fusarium wilt	Yes	Rare	Moore (1959), Leslie & Summerell (2006)
Fusarium semitectum	Damping off	?	Rare	Booth (1971)
Fusarium solani	Root rot	Yes	Uncommon. Associated with decay of already damaged roots. Non-pathogenic strains have potential for control of fusarium wilt (Leslie & Summerell)	Fletcher (1984), Leslie & Summerell (2006)
Fusarium spp.	Root rot	Yes	Quite common. Associated with decay of already damaged roots.	Fletcher (1984)
Humicola fuscoatra		Yes	Uncommon. Associated with corky root symptoms - pathogenicity unproven / doubted.	De Gruyter *et al.*, (1992), Menzies & Ehret (1997), Menzies *et al.*, (1998).

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Fungus	Disease	Recorded on tomato in UK	Comment	Reference
Macrophomina phaseolina	Charcoal rot	No	More important on other hosts, e.g. sunflower, maize, cotton. High temperature pathogen.	Smith *et al.*, (1988)
Monographella cucumerina (syn *Plectosphaerella cucumerina*) (asexual state *Microdochium tabacinum*)	Root rot	No	Minor root pathogen. Very common saprophyte on decaying plant matter in soil.	Smith *et al.*, (1988)
Phymatotrichopsis omnivora	Root rot	No	Reports from USA	Farr *et al.*, (1995)
Phytophthora arecae	Foot rot	No	Found in Netherlands	Erwin & Ribeiro (1996)
Phytophthora capsici	Root rot	No	More important on pepper (recently recorded in UK on this crop)	Watterson (1986), Jones *et al.*, (1991), Smith *et al.*, (1988), and others.
Phytophthora cinnamomi	Root rot	No	Found in Argentina & USA	Farr *et al.*, (1995), Erwin & Ribeiro (1996)
Phytophthora citricola	Seedling wilt	No	Found in Italy	Erwin & Ribeiro (1996)
Phytophthora cryptogea	Foot rot	Yes	Common	Watterson (1986), O'Neill *et al.*, (2000), Smith *et al.*, (1988), and others.
Phytophthora drechsleri	Root rot	No	More common as cause of fruit rot.	Koike *et al.*, (2007)

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Fungus	Disease	Recorded on tomato in UK	Comment	Reference
Phytophthora erythroseptica	Root rot	Yes	Uncommon	Evans (1979), Watterson (1986), Smith *et al.*, (1988)
Phytophthora fragariae var. *Fragariae*	Root rot	No	Found in Canada	Erwin & Ribeiro (1996)
Phytophthora hibernalis	Root rot	No	Found in Israel	Erwin & Ribeiro (1996)
Phytophthora infestans	Tomato late blight	Yes (on foliage and fruit)	Predominantly a foliar pathogen. Found causing root and foot rot in Belgium (see reference)	Lievens *et al.*, (2004)
Phytophthora megasperma var. *megasperma*	Root rot	Yes		CSL checklist of fungal pathogens
Phytophthora mexicana	Root rot, damping off	No	Found in Mexico	Erwin & Ribeiro (1996)
Phytophthora nicotianae var. *nicotianae*	Root rot	Yes	Common. Reclassified as *P. nicotianae* (Erwin & Ribeiro, 1996) as var. *nicotianae* & var. *parasitica* found to be indistinguishable in molecular tests)	Dixon (1981)
Phytophthora nicotianae var.	Root rot	Yes	Common. Reclassified as	Fletcher (1984), Jones *et*

Fungus	Disease	Recorded on tomato in UK	Comment	Reference
parasitica			*P. nicotianae* (see above).	*al.*, (1991), Blancard (1994) and others
Phytophthora palmivora	Root rot	No	Found in USA	Farr *et al.*, (1995)
Phytophthora phaseoli	Seedling blight	No	Found in Philippines	Erwin & Ribeiro (1996)
Phytophthora richardiae	Foot rot	No?	*P. richardiae* listed as present in UK, but doesn't specifically mention on tomato in UK.	Hull (1991)
Phytophthora verrucosa	Toe rot	Yes	Rare	Baker (1972), Erwin & Ribeiro (1996)
Pyrenochaeta lycopersici	Brown and corky root rot	Yes	Common – soil-grown crops	Fletcher (1984), Jones *et al.*, (1991), Blancard (1994) and others
Pyrenochaeta terrestris syn. *Phoma terrestris*	'Secondary' root rot	No		Farr *et al.*, (1995), Westcott (2001)
Pythium arrhenomanes	Pythium root rot / damping off	?	Common?	Jones *et al.*, (1991), Farr *et al.*, (1995)
Pythium butleri	?	Yes	?	CSL checklist of plant pathogens
Pythium debaryanum	Pythium root rot /	Yes	Common?	Jones *et al.*, (1991), Farr

Fungus	Disease	Recorded on tomato in UK	Comment	Reference
	damping off			*et al.*, (1995)
Pythium diclinum	?	?	Isolated from tomato roots – pathogenicity not tested	Rafin & Tirilly (1995)
Pythium echinulatum	?	?	Isolated from tomato roots – pathogenicity not tested	Rafin & Tirilly (1995)
Pythium gracile syn. *P. diclinum*	?	Yes	?	CSL checklist of plant pathogens
Pythium irregulare	Pythium root rot / damping off	Yes	Common?	Domsch & Gams (1993)
Pythium megalacanthum syn. *Pythium buismaniae*	Pythium root rot / damping off	?	Common?	Farr *et al.*, (1995)
Pythium myriotylum	Pythium root rot / damping off	?	Common?	Jones *et al.*, (1991), Farr *et al.*, (1995)
Pythium oligandrum	Damping off / 'soil rot'	Yes	?	Farr *et al.*, (1995), Price (1980)
Pythium paroecandrum	?	Yes	On rockwool grown plant	British Mycological Society database
Pythium periplocum	?	?	Isolated from tomato roots – pathogenicity not tested	Rafin & Tirilly (1995)

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Fungus	Disease	Recorded on tomato in UK	Comment	Reference
Pythium salpingophorum	?	?	One report from USA	Farr *et al.*, (1995)
Pythium torulosum	Pythium root rot / damping off	?	'mildly pathogenic to tomato' – (Domsch & Gams)	Domsch & Gams (1993)
Pythium ultimum	Pythium root rot / damping off	Yes	Common?	Jones *et al.*, (1991), Farr *et al.*, (1995), Rafin & Tirilly (1995)
Pythium vexans	Root rot	?	One report from USA	
Pythium 'group F'	Root rot	?		Rafin & Tirilly (1995)
Pythium 'group G'	?	?	Isolated from tomato roots – pathogenicity not tested	Rafin & Tirilly (1995)
Rhizoctonia solani	Rhizoctonia root rot	Yes	Common	Fletcher (1984), Jones *et al.*, (1991), Blancard (1994) and others
Sclerotium rolfsii	Southern blight	No	Attacks all parts on, in, or close to soil.	Watterson (1986), Jones *et al.*, (1991)
Spongospora subterranea	Powdery scab	Yes	Uncommon – no apparent effect on vigour (Blancard)	Fletcher (1984), Blancard (1994), Farr *et al.*, (1995)
Thielaviopsis basicola	Black root rot	Yes	Fairly common in NFT	Jones *et al.*, (1991),

Fungus	Disease	Recorded on tomato in UK	Comment	Reference
				O'Neill *et al.*, (2000) and others
Verticillium albo-atrum	Verticillium wilt	Yes	Fairly common – recent outbreaks on varieties with resistance genes	O'Neill (2005, 2006) and others
Verticillium dahliae	Verticillium wilt	Yes	Uncommon	Fletcher (1984), Jones *et al.*, (1991), Blancard (1994) and others
Verticillium nigrescens	Verticillium wilt	Yes	Rare – weakly pathogenic	Isaac (1953)
Verticillium nubilum	Verticillium wilt	Yes	Rare – weakly pathogenic	Isaac (1953)
Verticillium tricorpus	Verticillium wilt	Yes	Uncommon – weakly pathogenic	Isaac (1953), Moore (1959), Jones *et al.*, (1991)

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Table 1.1.2: List of fungal saprotrophs reported associated with tomato roots or growing medium

Fungus	Present in UK	Comment	Reference
Acremonium atricum	Yes	Isolated from root of NFT tomato	Price (1980)
Acremonium sp.	Yes	Isolated from lesion on tomato root	British Mycological Society database
Agaricus arvensis	Yes	From soil of cold glasshouse growing tomatoes	British Mycological Society database
Alternaria humicola (syn. *A. tenuissima?*)	Yes	Isolated from root of NFT tomato	Price (1980)
Alternaria sp.	Yes	Isolated from tomato with brown root rot symptoms – non-pathogenic in inoculation tests	Ebben & Williams (1956)
Aspergillus flavus	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Aspergillus sydowii	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Aspergillus terreus	Yes	In wet soil in whalehide pots	Baker (1972)
Aspergillus ustus	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)

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Fungus	Present in UK	Comment	Reference
Aspergillus sp.	Yes	Isolated from root of NFT tomato	Price (1980)
Aureobasidium pullulans	Yes	Isolated from root of NFT tomato	Price (1980)
Blastomyces sp.	Yes	Isolated from soil and roots	Ebben (1959)
Calyptella capula	Yes	On tomato rootball and dying tomato roots	British Mycological Society database
Cephalosporium acremonium	Yes	Isolated from root of NFT tomato	Price (1980)
Cephalosporium spp.	Yes	Isolated from tomato with brown root rot symptoms – non-pathogenic in inoculation tests	Ebben & Williams (1956)
Chaetomium cochliodes	Yes	Isolated from tomato with brown root rot – could infect seedling radicles but not mature plants in inoculation tests	Ebben & Williams (1956)
Chaetomium elatum	Yes	Found on tomato root	Domsch & Gams (1993)
Chaetomium olivaceum	Yes	Isolated from root of NFT tomato	Price (1980)
Chaetomium spp.	Yes	Isolated from tomato with brown root rot	Ebben & Williams (1956)

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Fungus	Present in UK	Comment	Reference
		symptoms	
Chromalosporium ochraceum	Yes	Isolated from root of NFT tomato	Price (1980)
Conidiobolus coronatus	Yes	Isolated from root of NFT tomato	Price (1980)
Coprinopsis gonophylla	Yes	On soil in pots growing tomatoes	British Mycological Society database
Cryptococcus albidus	Yes	Isolated from root of NFT tomato	Price (1980)
Cunninghamella echinulata	?	Found in tomato rhizosphere	Domsch & Gams (1993)
Cylindrocarpon didymium	Yes	Isolated from root of NFT tomato. Recorded as a cause of root rot & seedling blight on a range of plant species.	Price (1980)
Doratomyces microsporus	Yes	Found on decaying tomato roots	Domsch & Gams (1993), Price (1980)
Epicoccum purpurascens	Yes	Isolated from root of NFT tomato	Price (1980)
Fusarium oxysporum	Yes	Non-pathogenic strain isolated from tomato root mycorrhizae, tested as	Dababat & Sikora (2007)

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Fungus	Present in UK	Comment	Reference
		biocontrol against root-knot nematodes	
Fusarium torulosum (syn. *F. sambucinum* var. *coeruleum*, *F. venanatum*)	?	Recovered from tomato roots	Leslie & Summerell (2006)
Gelasinospora reticulata	Yes	On tomato root	British Mycological Society database
Gilmaniella humicola	Yes	Found on decaying tomato roots.	Domsch & Gams (1993), British Mycological Society database, Price (1980)
Gliocladium roseum	Yes	Found in tomato rhizosphere	Domsch & Gams (1972)
Idriella lunata	?	Found on tomato roots in California. No damage obvious, but same fungus causes strawberry root rot in California.	Domsch & Gams (1993)
Lepiota efibulis	Yes	Growing on mulched soil amongst tomato plants in a cool greenhouse (private garden)	British Mycological Society database
Lycoperdon sp.		Giant puff ball	Baker (1972)
Mortierella polycephala	?	Found in tomato rhizosphere	Domsch & Gams (1993)

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Fungus	Present in UK	Comment	Reference
Mortierella zychae	Yes	On root of rockwool-grown tomato	British Mycological Society database
Mortierella sp.	Yes	Isolated from root of NFT tomato	Price (1980)
Mucor sp.	Yes	Isolated from root of NFT tomato	Price (1980)
Mycotypha microspora	Yes	Isolated from root of NFT tomato	Price (1980)
Myrothecium roridum	Yes	Found on tomato roots (can be pathogenic to aerial parts)	Domsch & Gams (1993), Ebben (1959)
Nectria gliocladioides	Yes	Isolated from root of NFT tomato	Price (1980)
Neurospora crassa	Yes	Isolated from root of NFT tomato	Price (1980)
Oedocephalum sp.	Yes	Isolated from root of NFT tomato	Price (1980)
Olpidium brassicae	Yes	Found in tomato roots	Moore (1959)
Olpidium sp.	Yes	Found in tomato roots	Baker (1972), Blancard (1994)
Paecilomyces lilacinus	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium brevicompactum	Yes	Isolated from root of NFT tomato	Price (1980)

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Fungus	Present in UK	Comment	Reference
Penicillium chrysogenum	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium griseofulvum	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium janthinellum	?	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium jensenii	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium lividum	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium nigricans	Yes	Isolated from root of NFT tomato	Price (1980)
Penicillium purpurogenum	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium stoloniferum	?	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium thomii	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium variabile	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium verrucosum	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium verrucosum var. *corymbiferum*	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)

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Fungus	Present in UK	Comment	Reference
Penicillium verrucosum var. *cyclopium*	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Penicillium verrucosum var. *melanochlorum*	Yes	Found in tomato rhizosphere	Domsch & Gams (1993)
Petriella asymmetrica	Yes	Isolated from tomato with brown root rot – could infect seedling radicles but not mature plants in inoculation tests	Ebben & Williams (1956)
Peziza ostracoderma	Yes	From sterilised compost in glasshouse growing tomatoes	British Mycological Society database
Pyronema amphalodes	Yes	Coral pink or scarlet fruit bodies on soil whalehide pots	Baker, 1972
Rhizopus nigricans	Yes	Isolated from root of NFT tomato	Price (1980)
Rhizopus oryzae	Yes	Found in tomato rhizosphere	Domsch & Gams (1993), Price (1980)
Rhodotorula glutinis	Yes	Isolated from root of NFT tomato	Price (1980)
Sporobolomyces roseus	Yes	Isolated from root of NFT tomato	Price (1980)

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Fungus	Present in UK	Comment	Reference
Torulopsis famata	Yes	Isolated from root of NFT tomato	Price (1980)
Tricocladium adspersum	Yes	On decaying tomato roots	British Mycological Society database
Trichoderma koningii	Yes	Isolated from root of NFT tomato	Price (1980)
Trichoderma viride	Yes	Found in tomato rhizosphere – also used as a biological control agent in some crops. Also found as successor to *Aspergillus* on whalehide pots.	Domsch & Gams (1993), Baker (1972)
Trichoderma sp.	Yes	Isolated from roots and soil	Ebben (1959)
Trichurus spiralis	?	Found in tomato rhizosphere	Domsch & Gams (1993)
Volutella ciliata	Yes	Isolated from tomato roots	Domsch & Gams (1993), Ebben (1959)

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Bacteria associated with roots

Bacterial pathogens

Bacterial pathogens reported associated with tomato roots are listed in Table 1.2.1. The number is few and those which do occur are generally rare or uncommon.

Bacterial saprotrophs and mycorrhizal fungi and bacteria

In many of the experiments in which the isolation of saprophytic bacteria has been attempted from either tomato roots or the rhizosphere, the aim has been to recover bacteria that are potential biological control agents for diseases or pests of the crop. Large numbers of bacteria are usually recovered in these experiments, and in most cases individual species of bacteria are not reported, but the organisms are grouped into types (e.g. rhizoplane, rhizosphere, internal colonisers) or strains. To search through large numbers of scientific papers for mentions of individual saprophytic species is unlikely to be cost-effective in terms of information of value to this project.

A similar situation occurs with mycorrhizal fungi and bacteria. Once again there are large numbers of scientific papers dealing with mycorrhizae on tomato roots. However, the vast majority of these experiments involve the inoculation of tomato roots with various mycorrhizal preparations, to improve plant growth or disease control, rather than the isolation of mycorrhizal organisms naturally associated with the roots.

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Table 1.2.1: List of bacterial pathogens reported associated with tomato roots

Bacterium	Disease	Recorded on tomato in UK	Comment	Reference
Agrobacterium radiobacter	Crown gall	Yes	Rare	Weller & O'Neill (2006)
Agrobacterium rhizogenes	Root mat	Yes	Uncommon	Weller *et al.*, 2000
Agrobacterium tumefaciens	Crown gall	Yes	Rare	Blancard (1994)
Clavibacter michiganensis	Bacterial canker	Yes	Rare – notifiable when on propagation sites.	Blancard (1994), Jones *et al.*, (1991), O'Neill *et al.*, (2000)
Pseudomonas syringae pv.	Bacterial speck	Yes	Rare. Primarily a disease of the fruit, but the causal bacteria can survive in association with roots.	Watterson (1986), Blancard (1994), Jones *et al.*, (1991), Schneider & Grogan (1977)
Ralstonia solanacearum	Bacterial wilt	Yes	Rare – notifiable	Blancard (1994), Jones *et al.*, (1991), O'Neill *et al.*, (2000)

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Factors influencing tomato root diseases

Abiotic

The effects of some abiotic factors on development of tomato root diseases are summarised in Table 1.3.1 and discussed below.

Table 1.3.1: Reported effect of some abiotic factors on some root diseases of tomato

Disease	Pathogen	Reported effect
Temperature		
Fusarium wilt	*F. oxysporum* f. sp. *lycopersici*	Favoured by soil temperature of 28°C.
Fusarium crown and root rot (FCRR)	*F. oxysporum* f. sp. *radicis – lycopersici* (FORL)	Favoured by cool soil temperature (20-22°C).
Verticillium wilt	*Verticillium albo-atrum*	Inhibited by warm temperatures; worse early and late season.
	V. dahliae	Causes wilt over a wide temperature range.
Brown and corky root rot (BCRR)	*Pyrenochaeta lycopersici*	Optimal at 15-20°C (i.e. worse in cool soils).
Rhizoctonia root rot	*R. solani*	Varies with strain of *R. solani.* Disease more severe at temperature adverse to crop growth (15-18°C).
Black dot	*Colletotrichum coccodes*	Optimum at 22-24°C.
Bacterial wilt	*Ralstonia solanacearum*	Favoured by high temperatures (30-35°C)
Phytophthora root rot	*P. nicotianae*	Favoured by temperatures too low for optimal crop growth
Moisture		
Phytophthora root rot	*P. nicotianae*	Favoured by soil saturation > 5h and alternate cycles of low and high moisture.
Pythium root rot	*Pythium* spp.	Favoured by soil near to

Disease	Pathogen	Reported effect
		saturation
Rhizoctonia root rot	*R. solani*	Active over wide range of soil moisture.
Black dot	*C. coccodes*	Worse in well-drained soils
FCRR	*FORL*	Worse where excess water.
Verticillium wilt	*V. albo-atrum*	Worse when insufficient moisture.
Calyptella root rot	*C. campanula*	Worse on very wet soils.
BCRR	*P. lycopersici*	Worse if soil overwatered
<u>Substrate, pH, nutrition</u>		
Fusarium wilt	*F. oxysporum* f. sp. *lycopersici*	Favoured by low soil pH. and ammoniacal nitrogen. Prefers sandy soils. Contradictory reports on phosphorus.
Pythium root rot/ damping-off	*Pythium* spp.	Favoured by high nitrogen.
Black dot	*C. coccodes*	Salt injury may predispose roots to infection.
Verticillium wilt	*Verticillium* sp.	Contradictory reports on effect of soil pH.
Bacterial canker	*Corynebacterium michiganense*	Increased severity at high nitrogen levels

<u>Temperature</u>

Fusarium wilt is favoured by soil (and air) temperatures of 28°C (Jones *et al.*, 1991). Blancard (1994) also gives the optimum temperature for this fungus as 28°C. However, Davies (1983), whilst quoting the optimum temperature for *F. oxysporum* as 25-27°C, also comments that 'in recent years experience has shown that the optimum temperature for *F. oxysporum* may be lower (than this)'.

According to Jones *et al.*, (1991), Fusarium crown and root rot (*Fusarium oxysporum* f.sp. *radicis-lycopersici*) (FORL) is favoured by cool soil temperatures of 20-22°C. Blancard (1994) states that FORL appears able to attack plants irrespective of soil

temperature, with outbreaks occurring at temperatures in excess of 26ºC. However, he also mentions that the severity of the disease can be increased when roots have been subjected to low temperatures at any time.

Blancard (1994) states that verticillium wilt is favoured by cool temperatures of 20-23ºC. According to Fletcher (1984) there is a seasonal pattern with verticillium wilt. *Verticillium albo-atrum* causes wilt early in the growing season and again towards the end of the crop, as this species is favoured by temperatures of around 25ºC and may be inhibited by warm conditions. *Verticillium dahliae* is not so temperature-dependant, and causes wilt over a wide temperature range. Davies (1983) states that the optimum temperature for *Verticillium dahliae* is 25-27ºC, whereas an average of 20ºC is favourable for the spread of *V. albo-atrum*.

Jones *et al.* (1991) state that the development of brown root rot and corky root (*Pyrenochaeta lycopersici*) is optimal at 15-20ºC, and declines above these temperatures. Jones (1983) also states that cold soils will make the disease worse. According to Blancard (1994) strains of the pathogen adapted to cold, temperate and warm conditions can survive in the same soil, and cause disease at different times of the year. Northern European strains have a temperature optimum of 15-20ºC, whereas strains originating from the Mediterranean basin (Tunisia, Libya) are still active at temperatures of 26-30ºC.

Jones *et al.*, (1991) state that the optimum temperature at which *Rhizoctonia solani* causes damage varies according to the strain of the pathogen. Disease is often more severe at temperatures that are adverse to the growth of the plant, such as unusually low temperatures (15-18ºC). Blancard (1994) gives the temperature range for the development of *R. solani* as 15-26ºC.

Blancard (1994) states that the optimum temperature for black dot (*Colletotrichum coccodes*) is 22-24ºC.

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Infection by bacterial wilt (*Ralstonia solanacearum*) is favoured by high temperatures, with an optimum of 30-35ºC (Jones *et al.*, 1991).

Blancard (1994) states that *Phytophthora nicotianae* is favoured by soil or substrate temperatures that are too low for optimum growth by the plant, and is capable of developing at temperatures of 15-26ºC.

Macrophomina phaseolina is favoured by soil temperatures in excess of 28ºC (Smith *et al.*, 1988). *Sclerotium rolfsii* is favoured by even higher soil temperatures (30-35ºC) (Jones *et al.*, 1991). Neither of these pathogens is found in the UK.

Moisture

Pathogens such as *Pythium* and *Phytophthora* are favoured by high moisture levels within the soil or growing medium. These organisms have a microscopic zoospore as one of their primary means of infection. Zoospores require water in order to move towards the roots of the host plant, often attracted by chemical exudates produced from the roots. Soil saturation as a result of compaction or poor drainage increases the severity of disease caused by these pathogens.

Soil saturation of five hours or longer favours infection by *Phytophthora* species such as *P. nicotianae* (Jones *et al.*, 1991). Watterson (1986) states that phytophthora root rot appears to be favoured by alternating cycles of low and high moisture levels, which correspond to the production of sporangia by the fungus and the release of infectious zoospores. Soil moisture near to saturation stimulates vegetative mycelial growth and asexual reproduction (zoospores) in *Pythium* spp. (Jones *et al.*, 1991).

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Rhizoctonia solani is active over a range of soil moisture levels, although at the extremes (very dry or waterlogged soils) activity of the fungus may be inhibited (Jones *et al.*, 1991).

Colletotrichum coccodes (black dot) prefers light soil, as heavy soil tends to retain water, decreasing the longevity of the resting structures (microsclerotia) of the fungus (Blancard, 1994).

The severity of fusarium crown and root rot (*Fusarium oxysporum* f.sp. *radicis-lycopersici*) (FORL) appears worse when roots have been subjected to excess water (Blancard, 1994).

O'Neill (2005) found that root infection by *Verticillium* was less in a 'dry' (plants grown under water stress) than a 'wet' (plants supplied with adequate water) growing medium.

Calyptella root rot (*Calyptella campanula*) appears to be associated with very wet soils (Clark, 1983; Fletcher, 1984). Where the soil is sufficiently moist to support the growth of mosses, conditions are usually suitable for the disease (O'Neill *et al.*, 2000).

Calyptella root rot (*Calyptella campanula*) can be controlled by reducing the quantity and frequency of watering, and by moving the drip outlet from the plant base to about 20 cm away (O'Neill *et al.*, 2000).

Brown root rot & corky root (*Pyrenochaeta lycopersici*) is worse where there is poor soil structure, a soil pan, poor drainage or where the crop has been overwatered (Jones, 1983).

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Infection by the bacterial wilt pathogen *Ralstonia solanacearum* is favoured by high moisture levels (Jones *et al.*, 1991).

Substrate type, pH and nutrient levels

Jones *et al.*, (1991) state that fusarium wilt (*Fusarium oxysporum* f.sp. *lycopersici*) is favoured by low soil pH. The virulence of the pathogen is also enhanced by micronutrients, phosphorus and the use ammoniacal nitrogen rather than nitrate nitrogen.

Blancard (1994) states that *F. oxysporum* f.sp. *lycopersici* prefers sandy and acidic soils. The fungus colonises soil at great depth (below 80 cm). Plants are particularly sensitive when suffering from a deficiency of nitrogen, phosphorus or calcium. According to Watterson (1986), increasing nitrogen levels can result in increased severity of fusarium wilt, although he makes no comment on the source of nitrogen.

Addition of calcium to the soil in the form of hydrated lime, ground limestone or gypsum raises the pH and decreases the severity of fusarium wilt (Watterson, 1986).

Raising the soil pH to 6.5-7.0, and using nitrate nitrogen rather than ammoniacal nitrogen reduces the development of fusarium wilt (*Fusarium oxysporum* f.sp. *lycopersici*). This can give yield increases equivalent to those obtained by injecting fumigants into pH 5.5 soil (Jones *et al.*, 1991).

Excessive nitrogen may favour infection by *Pythium* species (Jones *et al.*, 1991).
Jones *et al.*, (1991) state that soluble salt injury may predispose plants to infection by black dot (*Colletotrichum coccodes*). Their recommendations for control include the avoidance of closed-recirculation irrigation-fertilization systems and the prevention of root injury.

According to Jones *et al.*, (1991), damage from verticillium wilt seems more severe on neutral to alkaline soil. This contrasts with work described by Watterson (1986), in which low soil pH and low calcium levels resulted in an increase in verticillium wilt. He also states that increasing nitrogen levels can result in an increase in verticillium wilt, but that the use of nitrate-nitrogen reduces wilt caused by *Verticillium albo-atrum*.

Goodenough & Maw (1973) advocate the application of potassium and phosphorous (but not nitrogen) to plants affected by brown root rot & corky root (*Pyrenochaeta lycopersici*), since there are losses of these elements in plants affected by the disease.

Increasing nitrogen levels can also increase the severity of bacterial canker (*Clavibacter michiganensis*), bacterial wilt (*Ralstonia* solanacearum) and fusarium crown and root rot (*Fusarium oxysporum* f.sp. *radicis-lycopersici*) (Watterson, 1986).

Introduction of the bacterial wilt pathogen *Ralstonia solanacearum* into a hydroponic system resulted in 100% disease after 10 days. Even a slower developing pathogen such as *Colletotrichum coccodes* (black dot) required only 36 days to cause disease in all tomato plants in the system (Jenkins & Averre, 1983).

Evans (1979) found that *Phytophthora* sp, fusarium wilt, verticillium wilt and didymella were all found to spread when introduced into an NFT system.

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Biotic

A basic hypothesis of disease suppressiveness in soils is that a high microbial biomass and high microbial variability are important components (Chen *et al.*, 1988). Fungi are the largest component of the soil microbial biomass (Lynch & Panting, 1980) and it is therefore essential to study fungal activity. Certain bacterial genera, such as *Pseudomonas* have also been identified as important components of suppressive soils.

Soil organic matter content, the soil management system, and other factors, affect microbial activity and variations in soil microbial communities (Abawi & Widmer, 2000). The microbial population of a soil and its activity strongly influences the behaviour of facultative saprophytic pathogens, such as *Pythium* spp., *Cylindrocarpon destructans*, *Fusarium oxysporum* and *Fusarium solani* (Manici *et al.*, 2005; Mazzola, 2002). Facultative saprophytic root pathogens have been considered as potential bio-indicators of soil fertility for particular crop species (Pankhurst *et al.*, 1995). *Pythium* spp. are considered to be the saprophytic pathogens most sensitive to microbial activity (Chen *et al.*, 1988) and have been used by several research groups as an indicator of soil suppression (Grunwald & Semenov, 2000). A soil is considered to be disease suppressive if there is little growth of *Pythium* when it is added to a soil.

Several microbial preparations are marketed as soil or growing medium amendments and some claim to increase plant growth through increased microbial populations around roots or strengthened plant resistance to root disease.

Microbial amendments

The fungivorous nematode *Aphelenchus avenues* significantly reduced severity of corky root disease (*Pyrenochaeta lycopersici*) when inoculated into the soil (3–23 nematodes/mL) one day after transplanting tomato seedlings; other *Aphelenchoides* species were ineffective (Hasna *et al.*, 2008).

Non-pathogenic strains of *Fusarium oxysporum* have been found to inhibit invasion of tomato roots by *Pyrenochaeta lycopersici* (Dixon, 1981).

The plant growth-promoting fungus *Fusarium equiseti* isolate GF191 reduced vascular staining caused by fusarium crown and root rot (*Fusarium oxysporum* f. sp. *radicis-lycopersici* (Horinouchi *et al.*, 2007). When biodegradable pots were used at transplanting into infested soil to extend the separation time of tomato roots from the pathogen, the protective effect of *F. equiseti* increased to around 85%. Population levels of FORL in soil around roots treated with *F. equiseti* were reduced.

Jarvis (1989) states that the application of non-pathogenic strains of *Fusarium oxysporum* to tomato seedlings reduces disease due to FORL.

Two bacterial isolates, *Bacillus megaterium* (C96) and *Burkholderia cepacia* (C91) were demonstrated to be antagonistic against FORL, and were evaluated alone and in addition to the fungicide carbendazim (Omar *et al.*, 2006).

Mitchell & Hurwitz (1965) inoculated tomato seeds with an *Arthrobacter* species isolated from the rhizosphere of tomato, which resulted in greatly reduced levels of wilt when the seed was planted into soil infested with *F. oxysporum* f.sp. *lycopersici*.

Sharma *et al.* (2007) reported that treatment of tomato seed with certain strains of *Pseudomonas* sp. reduced pre-and post-emergence damping-off of tomato seedlings caused by *Pythium aphanidermatum* and *Phytophthora nicotianae* in both inoculated and naturally-infected field experiments.

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Phillips *et al.*, (1967) reduced levels of fusarium wilt following inoculation of tomato seedlings with a *Cephalosporium* species isolated from the rhizosphere of a healthy tomato plant.

Langton (1968) found that when inoculating tomato seedlings with spore suspensions of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *pisi* (the cause of pea wilt), the wilt symptoms on the tomatoes became less severe as the ratio of the f. sp. *pisi* was increased. Heat-killed spores of f. sp. *pisi* did not produce the same effect.

Homma & Ohata (described by Dixon, 1981) suppressed the severity of fusarium wilt symptoms in glasshouse-grown tomato seedlings by prior inoculation with a range of *formae speciales* of *Fusarium oxysporum* and *Fusarium solani*. Particularly effective were *F. oxysporum* f.sp. *melongenae*, *cucumerinum* and *batatas*, and *Fusarium solani* f.sp. *pisi* and *phaseoli*.

Baker (1981) reports results obtained by other workers on the biological control of verticillium wilt. When a tomato cultivar with resistance to fusarium wilt was inoculated with that pathogen, it developed resistance to verticillium wilt. In another experiment, roots of tomato seedlings were dipped in spore suspensions of avirulent *Verticillium albo-atrum*, *V. tricorpus*, *Fusarium oxysporum* f.sp. *lycopersici* and *F. oxysporum* f. sp. *dianthi* and planted in soil infested with virulent *V. dahliae.* However, only a 'fair' degree of control of verticillium wilt was obtained under commercial conditions.

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Organic matter amendments

A garden waste compost incorporated at 20% by volume into a soil naturally infested with *P. lycopersici* significantly suppressed corky root disease at 10 weeks after planting (Hasna *et al.*, 2008). Green manure and horse manure composts were ineffective.

The microbial community structure in soil-grown tomato was significantly affected by a cover crop of hairy vetch and not by polythene mulch (Carrera *et al.*, 2007). An organic amendment (poultry manure compost) under black plastic mulch also altered the microbial community structure.

Jarvis (1989) comments that the fusarium crown and root rot pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) has good saprophytic ability, and can re-colonise sterile soil rapidly. The build-up of microbial competitors to FORL can be encouraged by growing a green manure such as lettuce or dandelion immediately after taking out the previous crop and sterilising the soil. The green manure is ploughed in prior to planting the new tomato crop.

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Root sampling and detection of fungi by culturing

Introduction

An overall aim of this project is to monitor the diversity and levels of fungi and bacteria associated with the roots of tomato crops. This requires removal of samples of roots at intervals during crop growth. Ideally, root sampling should not result in plant death or significant yield reduction, be representative of the crop area being examined and be relatively quick and easy to do.

Subsequent culturing on nutrient agar is generally required to enable identification of fungi associated with roots. The culturing method should result in recovery of a wide range of the fungal genera associated with roots and provide some quantitative information on their relative levels.

The objectives in the work described here were (1) to examine various root sampling procedures for tomato crops grown in soil, rockwool slabs and by NFT; (2) to investigate the effect of two agar media, surface disinfection and root size on recovery of fungi; (3) to provide isolates of fungi obtained from tomato roots as reference cultures for use in identification of rhizosphere microorganisms by T-RFLP (see section 3).

Materials and methods

Site and crop details

Root samples were collected from three crops (Table 2.1). All of the crops were grown on their own roots.

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Table 2.1: Details of tomato crops where root samples were taken – 2008

Production method	Run-off solution re-circulated	Location	Variety	Fungicides applied to roots	Biologicals applied to roots	Dates sampled
Soil	No	Isle of Wight	Sunstream	None	None	3 June, 23 July
Rockwool	No	Norfolk	Mecano	None	None	10 June
NFT	Yes	Worcs	Flavorina	Derosal (Feb) Aaterra (Mar) Filex (15 Aug)	None	28 August

Root sampling procedures

Roots were collected from the soil-grown crop using a 2 cm diameter soil auger inserted to 20 cm depth (3 June 2008) or by carefully forking soil away from the side of a plant and cutting-off root sections around 5-15 cm long (23 July 2008).

Roots were collected from the rockwool-grown crop using a 10 mm diameter cork borer pushed to the bottom of the slab, and by cutting off a section of roots from one corner of a slab; bags were re-sealed with sticky tape. Roots were collected from the NFT crop by using scissors to cut off a 1 cm wide x 5-10 cm long strip from the mat of roots at the base of channels midway between two plants. For all crops, samples were taken from a single row of plants.

Details of sample positions with reference to the propagation cube and the number of sub-samples within a test sample are summarised below (Table 2.2).

Table 2.2: Summary of root samples collected – 2008

Production method	Sample date	Sample method	Sample positions relative to cube	Number of sub-samples per position	Number of replicates
Soil	3 June	Auger	Adjacent and	10 cores	3

			Midway		
	23 July	Expose and cut	Adjacent [a]	20 root lengths	3
Rockwool	10 June	Cork borer and cut	Adjacent, mid and slab corner	10 cores or sections	3
NFT	28 August	Cut	Midway [b]	One	5

[a] Three root thicknesses compared.

[b] White and brown roots compared.

Culture of fungi from roots

Root samples were picked out of the growing media, washed in tap water to remove soil or other growing media and cut into pieces c. 5 mm in length. For the soil-grown crop sampled on 23 July, roots were graded into three thickness (thin 1-1.5 mm; medium 1.5-3 mm and thick 5-8 mm) prior to cutting into short lengths. The set of root pieces were then divided into two halves, one half was tested for fungi by plating onto agar by ADAS, the second half was tested for fungi and bacteria by T-RFLP by Nottingham University (see section 3 for results).

Sets of 10-50 root pieces per sample were plated onto potato dextrose agar amended with streptomycin (PDA) and a *Pythium*-selective agar (P_5ARP) directly and after surface sterilisation. Roots plated onto PDA were sterilised with sodium hypochlorite (1% available chlorine for 3 minutes, rinsed in sterile distilled water); those plated onto P_5ARP were sterilised in 70% alcohol (10 seconds). Ten root pieces were plated onto each 9-cm Petri dish of agar. Plates were incubated at 20°C in a black-light incubator (PDA) or in the dark (P_5ARP). After 3 days, the proportion of root sections with any fungal growth was recorded. After 10-14 days, the proportion of root pieces with different fungi was recorded. The proportion of root pieces with no fungal growth ('clean') was also recorded. Fungi were identified by colony colour and morphology and by microscopic examination of selected colony types for spores and other fungal structures. Selected isolates of major colony types were plated onto agar and retained for examination by T-RFLP.

Experiment design and statistical analysis

Three or five replicates samples were collected (Table 2.2) and root sections plated onto agar. Each set of samples collected was treated as a factorial design with four factors (replicate, position of root with reference to the plant, surface sterilisation and agar type). In the soil crop sampled on 23 July, root thickness was examined as a factor rather than sample position. In the NFT crop sampled on 28 August, root colour was examined as a factor rather than sample position. Results were examined by ANOVA. Main factor and 2-way and 3-way interactions are presented.

Results and discussion

Soil grown crop samples - 3 June 2008

The predominant organisms recovered on PDA were *Colletotrichum coccodes* (black dot), *Fusarium* spp. and green-coloured colonies (probably *Trichoderma*). A number of other colony types occurred at a lower incidence and were not identified. Fungi recovered on P_5ARP were generally white and on microscope examination appeared to be *Pythiaceous* or related fungi. It was not possible to examine all colonies microscopically due to the workload.

The proportion of roots from which no fungi were recovered declined with time after plating onto agar (Table 2.3a). After 3 days, there were significant effects from replicate sample (different positions along the same row), sample position (adjacent to cube or midway between cubes), surface sterilisation and surface sterilisation x agar interactions on the % roots clean (Table 2.3b). After 14 days, all the roots not surface sterilised had fungal growth from them (0% clean) (Table 2.3b). Roots sampled from midway between blocks had significantly greater levels of clean roots than those adjacent to blocks; this effect was largely due to differences on the P_5ARP

medium (i.e. there were fewer *Pythiaceous* fungi on roots midway between cubes, presumably the youngest roots).

At 14 days after plating, *Fusarium* was recovered from a significantly greater proportion of roots midway between cubes than adjacent to cubes (Table 2.4). Black dot appeared to occur at greater levels on roots adjacent to cubes than midway between cubes, but the difference was not significant ($p=0.125$).

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Table 2.3a: Effect of sample position, surface sterilisation and agar type on recovery of fungi from tomato roots (soil crop) – sampled 3 June 2008

Sample position	Surface sterilisation	Agar	% roots clean after:	
			3 days	14 days
Cube	–	PDA	0	0
	+	PDA	58.0	20.0
Midway	–	PDA	2.5	0
	+	PDA	71.7	44.8
Cube	–	P_5ARP	0	0
	+	P_5ARP	28.7	0
Midway	–	P_5ARP	15.5	0
	+	P_5ARP	65.0	50.2

Table 2.3b: Analysis of variance of sample position, surface sterilisation and agar type on recovery of fungi from tomato roots – sampled 3 June 2008

Source of variation	Df	F probability on % roots clean after:	
		3 days	14 days
Replicate	2	0.038	0.172
Sample position (P)	1	0.004	0.002
Surface sterilisation (SS)	1	<0.001	–
Agar (A)	1	0.271	0.339
SS x A	1	0.028	–
SS x P	1	0.133	–
A x P	1	0.097	0.122
SS x A x P	1	0.637	–
Residual	14		
Total	23		

Table 2.4: Mean effect of sample position on recovery of fungi from tomato roots plated onto PDA (soil crop) – sampled 3 June 2008, assessed 14 days after plating

Sample position	Mean % roots from which fungi recovered:			
	Fusarium sp.	C. coccodes	Trichoderma sp.	Other
Cube	0.7	22.7	13.3	2.0
Midway	3.9	7.2	11.9	3.0
Significance	0.03	NS	NS	NS
LSD	2.50	–	–	–

NS – not significant at p=0.05

Soil grown crop – 23 July 2008

A similar range of fungal colony types were recovered as at the first sampling. At 14 days after plating there was no significant effect from replicate plant, root thickness or surface sterilisation on the proportion of roots without fungal growth (Table 2.5a). Agar type had a significant effect, with a greater proportion of clean roots on P_5ARP than on PDA (Table 2.5b). Agar type also significantly affected recovery of *Trichoderma*, *Gliocladium*, *C. coccodes* and pythiaceous fungi, as would be expected (Table 2.5b).

Root thickness significantly influenced recovery of *Trichoderma* and pythiaceous fungi (more from thick than thin roots). Surface sterilisation significantly influenced recovery of pythiaceous fungi, but not the other fungal groups. There were significant root thickness x agar (pythiaceous fungi) and Agar type x Surface sterilisation (pythiaceous and other fungi) interactions (Table 2.5b).

The mean effects of root thickness, surface sterilisation and replicate plant on recovery of individual fungal groups are shown in Table 2.6 – 2.8; statistical significance values for fungi commonly isolated and listed in Tables 2.6 and 2.7 are given in Table 2.5b (Agar x surface sterilisation and Root thickness x Agar respectively). *Fusarium oxysporum* and a grey sterile fungus were only recovered from thin roots on PDA and after surface sterilisation (Table 2.6). *Penicillium* sp. was only recovered from thin roots on PDA and without surface sterilisation (Tables 2.6 and 2.7). *Trichoderma* was recovered from all root thicknesses, both before and after surface sterilisation, but only on PDA (Table 2.7).

The level of recovery of the most common fungal groups (*Trichoderma*, *Colletotrichum*, pythiaceous) was reasonably consistent between replicate plants (Table 2.8). The

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greatest range of fungal groups was identified from roots plated directly onto PDA and the least from roots that were surface-sterilised and plated onto P_5ARP (Table 2.9).

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Table 2.5a: Effect of root thickness, surface sterilisation and agar type on recovery of fungi from tomato roots (soil crop) – sampled 23 July 2008

Root thickness	Surface sterilisation	Agar	% roots:					
			Clean	Tri	Coll	Gli	Pyth	Other
Thick	–	PDA	0	18	42	8	0	38
	+	PDA	0	25	60	7	0	20
	–	P_5ARP	0	0	0	0	100	0
	+	P_5ARP	7	0	0	0	62	32
Medium	–	PDA	0	13	47	25	0	27
	+	PDA	0	28	73	2	0	7
	–	P_5ARP	3	0	0	0	97	0
	+	P_5ARP	20	0	0	0	17	33
Thin	–	PDA	0	10	40	0	0	40
	+	PDA	8	0	42	0	0	23
	–	P_5ARP	50	0	0	0	50	0
	+	P_5ARP	30	0	0	0	0	20

Coll – *Colletotrichum coccodes*; Tri– *Trichoderma*; Gli– *Gliocladium*; Pyth – Pythiaceous fungi. Unidentified fungi were grouped and classed as 'other'.

Table 2.5b: Analysis of variance of root thickness, surface sterilisation and agar type on recovery of fungi from tomato roots – sampled 23 July 2008

Source of variation	Df	F probability					
		Clean	Tri	Coll	Gli	Pyth	Other
Replicate	1	0.875	0.045	0.330	0.233	0.356	0.832
Root thickness (RT)	1	0.106	0.042	0.280	0.602	0.004	0.783
Sterilisation (SS)	1	0.808	0.499	0.117	0.305	<0.001	0.481
Agar (A)	1	0.043	<0.001	<0.001	0.005	<0.001	0.109
RT x A	1	0.240	0.042	0.280	0.602	0.004	0.454
RT x SS	1	0.761	0.208	0.560	0.118	0.368	0.945
A x SS	1	0.917	0.499	0.117	0.305	<0.001	0.003
RT x A x SS	1	0.486	0.208	0.560	0.118	0.368	0.876
Residual	16						
Total	23						

Coll – *Colletotrichum coccodes*; Tri– *Trichoderma*; Gli– *Gliocladium*; Pyth – Pythiaceous fungi

Table 2.6: Mean effect of agar type and surface sterilisation on recovery of fungi from roots of tomato grown in soil - sampled 23 July 2008

Agar and surface sterilisation	Mean percentage pieces with:									
	Clean	Tri	Coll	Glio	Pen	Fus	Foxy	GS	Pyth	Other
PDA+SS	2.8	17.8	58.3	6.1	0.0	0.0	1.1	3.3	0.0	16.7
PDA	0.0	13.9	42.8	12.2	1.7	1.1	0.0	0.0	1.7	35.0
P_5+SS	18.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	26.1	28.3
P_5	17.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	82.2	0.0

PDA – potato dextrose agar; P_5 – P_5ARP agar: SS – surface sterilised Tri - *Trichoderma*; Coll – *Colletotrichum coccodes*; Glio - *Gliocladium*; Fus - *Fusarium* sp., Foxy - *F. oxysporum*; GS - grey sterile fungus; Pyth- Pythiaceous fungi.

Table 2.7: Mean effect of root size on recovery of fungi on two agars from roots of tomato grown in soil - sampled 23 July 2008

Agar and root thickness	Mean percentage pieces with:									
	Clean	Tri	Coll	Glio	Pen	Fus	Foxy	GS	Pyth	Other
PDA										
Thick	0	21.7	50.8	7.5	0	0	0	0	0	29.2
Medium	0	20.8	60.0	13.3	0	1.7	0	0	0.8	16.7
Thin	4.2	5.0	40.8	6.7	2.5	0	1.7	5.0	1.7	31.7
P_5ARP										
Thick	3.3	0	0	0	0	0	0	0	80.8	15.8
Medium	11.7	0	0	0	0	0	0	0	56.7	16.7
Thin	40.0	0	0	0	0	0	0	0	25.0	10.0

Tri – *Trichoderma*, Coll – *Colletotrichum coccodes*, Glio – *Gliocladium*, Pen – *Penicillium*, Fus – *Fusarium*, Foxy – *Fusarium oxysporum*, GS – grey sterile fungus, Pyth – *Pythiaceous* fungus
Thin, 1-1.5 mm; Medium, 1-2.5 mm; Thick, 3-7 mm diameter

Table 2.8: Comparison of recovery of fungi on two agars from roots of three tomato plants around 2 m apart in the same row grown in soil - sampled 23 July 2008

Agar and replicate	Mean percentage pieces with:									
	Clean	Tri	Coll	Glio	Pen	Fus	Foxy	GS	Pyth	Other
PDA										
Plant 1	4.2	25.8	40.8	10.0	0.8	0.0	0.0	5.0	0.0	26.7
Plant 2	0.0	7.5	52.5	15.0	1.7	1.7	1.7	0.0	0.0	24.2
Plant 3	0.0	14.2	58.3	2.5	0.0	0.0	0.0	0.0	2.5	26.7

P_5ARP										
Plant 1	10.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	64.2	9.2
Plant 2	19.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	42.5	14.2
Plant 3	25.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	55.8	19.2

Tri – *Trichoderma*, Coll – *Colletotrichum coccodes*, Glio – *Gliocladium*, Pen – *Penicillium*, Fus – *Fusarium*, Foxy – *Fusarium oxysporum*, GS – grey sterile fungus, Pyth – *Pythiaceous* fungus

Table 2.9: Effect of agar type, surface sterilisation and root size on number of identified fungal groups recovered from soil-grown tomato plants - sampled 23 July 2008

Agar type and root size	Number fungal groups recovered (of 9)	
	Direct plated	Surface sterilised
PDA		
Thick	4	3
Medium	5	3
Thin	6	5
P_5ARP		
Thick	1	1
Medium	1	1
Thin	1	0

Rockwool grown crop - 10 June 2008

The major colony types obtained from sampled roots were white (pythiaceous and *Fusarium*), pink-red (mostly *Fusarium*), black dot (*C. coccodes*). Black dot occurred less frequently than on roots of soil-grown crops. On surface-sterilised roots, there was a trend to cleaner roots in moving from the cube, to midway between cubes to the slab corner (Table 2.10a).

At 3 days after plating on agar, there were significant effects of replicate (distance along a row), sample position (cube, midway between cubes or slab corner), agar type and surface sterilisation on the proportion of roots with no fungal growth (% clean) (Table 2.10b).

At 14 days after plating on agar, there was a significant agar x surface sterilisation interaction, and also an effect of replicate (distance along the row) on the proportion

of roots clean. On PDA, there was a significant sampling position x surface sterilisation interaction influencing recovery of white, pink/red colonies and black dot (Table 2.11a). White colonies were most common from unsterilised roots from midway or corner positions and from sterilised roots near the cube; pink/red colonies were most common from unsterilised roots adjacent to the cube and none were recovered from roots at the slab corner. *C. coccodes* was most common on unsterilised roots from adjacent to the cube with little recovery from roots at the slab corner (Table 2.11a).

Table 2.10a: Effect of sample position, surface sterilisation and agar type on recovery of fungi from tomato roots (rockwool crop) – sampled 10 June 2008

Sample position	Surface sterilisation	Agar	Growth after 3 days	
			Mean No. colonies/root	Mean % clean roots
Cube	-	PDA	0.96	5.9
	+	PDA	0.55	45.8
Midway	-	PDA	0.99	1.4
	+	PDA	0.35	65.1
Corner	-	PDA	0.90	9.8
	+	PDA	1.00	88.3
Cube	-	P_5ARP	0.99	1.4
	+	P_5ARP	0.67	32.6
Midway	-	P_5ARP	1.00	0.0
	+	P_5ARP	0.63	38.1
Corner	-	P_5ARP	0.87	12.7
	+	P_5ARP	0.41	59.3

Table 2.10b: Analysis of variance of sampling position, surface sterilisation and agar type on recovery of fungi from tomato roots

Source of variation	Df	F probability	
		No. colonies/root	% clean
Replicate	2	0.030	0.032

Sample position (P)	2	0.729	0.037
Surface sterilisation (SS)	1	<0.001	<0.001
Agar (A)	1	0.612	0.080
P x A	2	0.014	0.935
P x SS	2	0.122	0.272
A x SS	1	0.609	0.109
P x A x SS	2	0.027	0.795
Residual	22		
Total	35		

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Table 2.11a: Effect of sample position, surface sterilisation and agar type on recovery of fungi from tomato roots (rockwool crop) sampled 10 June 2008 – growth after 14 days

Sample position	Surface sterilisation	Agar	Mean % roots				
			Clean	White fungus	Pink/red fungus	Black dot	Total No. fungi/root
Cube	–	PDA	4.4	20.0	61.5	10.3	0.96
	+	PDA	44.4	38.5	16.4	0.7	0.55
Midway	–	PDA	0	90.5	5.8	2.9	1.00
	+	PDA	54.1	11.9	22.2	5.1	0.40
Corner	–	PDA	0	100.0	0	1.3	1.01
	+	PDA	50.0	50.0	0	0.7	0.51
Cube	–	P_5ARP	0	–	–	–	1.00
	+	P_5ARP	29.8	–	–	–	0.76
Midway	–	P_5ARP	0	–	–	–	1.00
	+	P_5ARP	30.4	–	–	–	0.70
Corner	–	P_5ARP	2.1	–	–	–	0.98
	+	P_5ARP	14.0	–	–	–	0.86

White fungus – mostly pythiaceous and *Fusarium*; pink fungus – mostly *Fusarium*.

Table 2.11b: Analysis of variance of sampling position, surface sterilisation and agar type on recovery of fungi from tomato roots – sampled 10 June 2008

Source of variation	Df	F probability				
		% clean	% white fungus	% pink fungus	% black dot	Total no. fungi/root
Replicate	2	0.029	0.354	0.465	0.870	0.039
Sample position (P)	2	0.778	0.002	<0.001	0.116	0.622
Surface sterilisation (SS)	1	<0.001	<0.001	0.046	0.129	<0.001
Agar (A)	1	0.026	–	–	–	0.015
P x A	2	0.848	–	–	–	0.952
P x SS	2	0.688	0.001	<0.001	0.035	0.534
A x SS	1	0.036	–	–	–	0.015
P x A x SS	2	0.576	–	–	–	0.704
Residual	22	–	–	–	–	–
Total	35	–	–	–	–	–

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NFT crop – Sampled 28 August 2008

At 3 days after plating, there was a significant effect of surface sterilisation on recovery of fungi from roots (Table 2.12a and b). From both white and brown roots, and on both agar types, there were more clean roots (no fungal growth) after surface sterilisation. At this assessment time, agar type had a significant effect on recovery of white colonies (present on PDA only) and surface sterilisation significantly affected recovery of fast-growing colonies (few were recovered after surface sterilisation).

The predominant fungi recovered were *C. coccodes*, *Fusarium* sp., *Mucor/Rhizopus* and pythiaceous fungi. *Thielaviopsis basicola* was recovered once at 14 days after plating onto agar. There was no significant effect of replicate (five locations along a row) or root colour (white or brown) on any of the variables assessed. There was a significant root colour x surface sterilisation interaction influencing recovery of *C. coccodes*; the fungus was recovered at a high level from unsterilised white roots and from sterilised brown roots. *Mucor/Rhizopus* was only recovered from unsterilised roots and only on PDA. Recovery of pythiaceous fungi was significantly influenced by agar type (greater recovery on P_5ARP) and surface sterilisation (greater recovery from unsterilised roots). *Fusarium* sp. appeared to be recovered more frequently from brown than white roots, but the difference was not quite statistically significant ($p=0.072$).

Table 2.12a: Effect of surface sterilisation and agar type on recovery of fungi from tomato roots (NFT crop) – sampled 28 August 2008 (assessed 3 days after plating)

Root colour	Surface sterilisation	Agar	Mean % roots:		
			Clean	White fungus	Fast colonies
White	–	PDA	0	20	40
	+	PDA	38	10	0
Brown	–	PDA	0	12	44
	+	PDA	10	32	0
White	–	P_5ARP	54	0	46
	+	P_5ARP	98	0	2

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Root colour	Surface sterilisation	Agar	Mean % roots:		
			Clean	White fungus	Fast colonies
Brown	–	P_5ARP	26	0	74
	+	P_5ARP	92	0	8

Table 2.12b: Analysis of variance of surface sterilisation and agar type on recovery of fungi from tomato roots (NFT crop) – sampled 28 August 2008

Source or variation	Df	F probability		
		Clean	White colonies	Fast colonies
Replicate	4	0.581	0.380	0.908
Root colour (C)	1	0.082	0.463	0.175
Surface sterilisation (SS)	1	<0.001	0.599	<0.001
Agar (A)	1	0.276	<0.001	0.103
C x A	1	0.583	0.463	0.281
C x SS	1	0.891	0.122	0.349
A x SS	1	0.276	0.599	0.349
C x A x SS	1	0.107	0.122	0.515
Residual	28			
Total	39			

Table 2.13a: Effect of surface sterilisation and agar type on recovery of fungi from tomato roots (NFT crop) – sampled 28 August 2008 (assessed 14 days after plating)

Root colour	Surface sterilisation	Agar	Mean % roots:			
			Colletotrichum	*Fusarium*	*Muc/ Rhi*	Pythiaceous
White	-	PDA	38	0	8	80
	+	PDA	12	8	0	0
Brown	-	PDA	4	2	22	100
	+	PDA	54	18	0	0
White	-	P_5ARP	0	0	0	100
	+	P_5ARP	0	2	0	40
Brown	-	P_5ARP	0	0	0	100
	+	P_5ARP	2	20	0	40

Table 2.13b: Analysis of variance of surface sterilisation and agar type on recovery of fungi from tomato roots (NFT crop) – sampled 28 August 2008

Source or variation	Df	F probability			
		Colletotrichum	*Fusarium*	*Muc/ Rhi*	Pythiaceous
Replicate	4	0.357	0.137	0.865	0.160
Root colour (C)	1	0.640	0.072	0.090	0.604
Surface sterilisation (SS)	1	0.229	0.008	<0.001	<0.001
Agar (A)	1	<0.001	0.711	<0.001	0.014
C x A	1	0.778	0.711	0.090	0.604

C x SS	1	<0.001	0.116	0.090	0.604
A x SS	1	0.306	0.902	<0.001	0.127
C x A x SS	1	0.002	0.538	0.090	0.604
Residual	28				
Total	39				

Method selected for routine crop monitoring

The chosen root sampling procedure should not result in plant death, be relatively quick, provide sufficient roots, and be representative of the area being examined. It was decided that these criteria were best met by taking samples from mid-way between plants (soil and NFT crops) or from slab corners (rockwool). These samples are also more likely to contain young, actively growing root than samples taken adjacent to the propagation cube. Testing by T-RFLP found a greater range of microorganisms on young roots than older roots (see section 3). Samples will be tested for fungi and bacteria by T-RFLP only on nine replicates each of around 0.5 g fresh weight of root. Root samples will be collected from three replicate locations in a crop row and each sample divided into three sub-samples.

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Monitoring rhizosphere microbial communities using T-RFLP

Introduction

The diversity of microbial communities in natural environments has been of interest to scientists and growers for many years; however, the methods classically used to identify constituents of samples were based upon isolation and culturing as described in section 2. Such methods are not representative of the whole microbial community as it is estimated that only 0.1-10% of total population can be cultured from many communities. In recent years, advances in culture-independent methods have provided opportunity to identify numerically significant non-culturable organisms (Atkins & Clark, 2003; Calvo-Bado *et al.*, 2006; Postma *et al.*, 2001).

The majority of culture-independent approaches for the study of microbial diversity focus on the direct extraction of total community DNA from samples and the selective amplification by polymerase chain reaction (PCR) of ribosomal RNA (rRNA) genes. rRNA genes are ubiquitous in all cellular life forms. The rRNA genes comprise highly conserved sequence domains interspersed with more variable regions. Identification of conserved sequence domains allows for the design of 'universal' primers which can be used to amplify all homologous markers from a community. One such approach, called clone libraries, provides detailed information about phylogeny of community members. PCR products of rRNA genes are cloned into a vector and subsequently sequenced. This method is not well suited for the analysis of numerous samples because of the laborious time consuming protocols and also the expense associated with producing numerous clone libraries.

DNA fingerprinting techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis of rRNA

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genes allows simultaneous processing of a high number of samples at a relatively low cost. Analysis of microbial communities by DGGE is hindered by insufficient methods of quantifying the results and as a consequence it is difficult to compare data from differing studies.

Alternatively, T-RFLP analysis allows high sample throughput but importantly the data obtained can be analysed using numerous statistical methods such as analysis of variance, principal component analysis (PCA) and correspondence analysis (CA) (Culman *et al.*, 2008).

The T-RFLP analysis method uses a fluorescently labelled oligonucleotide primer for PCR amplification of rRNA genes and relies on the variation of restriction sites within the sequences of different organisms. Once PCR products have been digested with one or more restriction enzymes, a multitude of terminal restriction fragments (TRFs) of differing lengths are generated. TRFs are then separated by high resolution gel electrophoresis on automated sequencers, which record the fragment length and relative abundance. The resulting data is easy to analyse as it can be represented as figures for statistical analysis and also graphically for quick visual interpretation. As a result of its simplicity, T-RFLP analysis of rRNA genes is currently one of the most powerful methods for rapidly comparing microbial communities from environmental samples.

The main aim of this study was to optimise T-RFLP test protocols in preparation for a 2 year study using T-RFLP to investigate the occurrence and relative abundance of micro-organisms around the rhizosphere of tomato. The specific objectives are:

1. To create a database of theoretical/confirmed fragment lengths for common tomato root microflora.
2. To examine and compare differences in methods of DNA extraction and rRNA gene amplification.
3. To confirm T-RFLP results by comparing with findings from conventional diagnostic techniques.

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4. To compare various sampling methods, and identify those that result in significant and representative levels of DNA recovery of a wide range of microorganisms.

Materials and methods

Root material

Tomato root samples were collected by ADAS from commercial nurseries (see Table 2.1). Samples were sent by first class post to Nottingham. On arrival, samples were stored at 4ºC.

DNA extraction

Direct method. Root samples (≥100 mg) were roughly chopped using a sterile scalpel blade. Samples were placed in a 2.0 ml tube containing 10 acid washed glass beads (710-1,180 µm diameter, Sigma) and then disrupted by Fastprep (QBiogene, Cambridge, UK) bead-beating for 2 cycles of 45 s at 6.5 m s^{-1}. DNA was extracted by using Qiagen DNeasy Plant Mini Kit following the manufacturer's protocols. In instances where there was PCR inhibition due to phenolics in samples contaminated with soil, a PVPP clean up stage was used prior to PCR amplification (Cullen & Hirsch, 1998).

Wash Method. The second DNA extraction method was to use larger volumes of root material and wash off the microbes present. One gramme of root material was weighed out, roughly chopped using a sterile scalpel blade and placed in a 50 ml centrifuge tube with 5 ml of sterile 50 mM potassium phosphate buffer pH7.0 solution and shaken for 30 min. The resulting buffer was poured into a sterile filter housing containing a sterile 0.2 µm cellulose acetate filter to concentrate the cells washed from the leaves. The cells were re-suspended in 1 ml of 50 mM potassium phosphate buffer and centrifuged at 13,000 x *g* for 10 min at room temperature. The resulting

pellet was transferred to a 2.0 ml tube containing 10 acid washed glass beads and the wash method followed the direct method from this stage onwards.

Amplification of microbial communities

One µl of DNA extract was used in the PCR amplification of the 23S ribosomal subunit for bacteria and the ITS-2 region for fungi. Primers used to amplify the 23S rRNA gene have been previously published (Hodgetts *et al.*, 2007). The 23S reverse primer (23Srev*) was fluorescently labelled with D4 Beckman dye. The fungal primers amplify the ITS-2 region by priming from 5.8S ribosomal subunit (5' GCATCGATGAAGAACGCAGC 3') and from the 5' end of 28S ribosomal subunit (primer 3126T, 24). The fungal 28S reverse primer (FITSrev1*), was fluorescently labelled with D3 Beckman dye. Both fluorescent labels are suitable for analysis on CEQ8000 fragment analysis system. Amplifications were then performed as described in Hodgetts *et al.*, (2007).

Restriction digest

Following PCR, 5 µl of PCR product was used in a 10 µl reaction volume containing 1U of restriction enzyme *Mse*I (T/TAA) or *Hae*III (GG/CC) for bacteria and fungi respectively, according to the manufacturer's protocols. Digests were incubated at 37°C for 2 hours followed by denaturation of enzymes by heating to 80°C for 20 min.

Terminal Restriction Fragment Length Polymorphism

One µl of the digests were loaded into a 96 well plate with each well containing 38.5 µl of GenomeLab sample loading solution and 0.5 µl of GenomeLab size standard-600. The Samples were overlaid with mineral oil and separated on a CEQ 8000 DNA analysis system. Fragments between 60 and 640bp were considered for analysis.

Cultures

Cultures of common tomato rhizosphere microorganisms were acquired from ADAS and CSL. Fungal cultures were kept on potato dextrose agar and bacteria on nutrient agar. All cultures were stored at 4°C. Isolates were analysed by sequencing the 23S ribosomal gene or ITS2 region and by T-RFLP sequence analysis. In addition, theoretical fragment sizes of common root microflora were calculated from published sequences using the NCBI website.

Purification of DNA

PCR products from pure culture plates were purified using QIAquick PCR purification kit (Qiagen) and sequenced on a CEQ 8000 GeXP Genetic Analysis System. Sequences were edited and BLASTED using Chromas version 2.33. Results were recorded and compared with confirmed and theoretical T-RFLP fragment lengths.

Results and discussion

Database of T-RFLP peaks for tomato root associated organisms

From the list of fungi and bacteria associated with tomato roots (section 1.1), theoretical TRFs of many of the root microflora were calculated from published rRNA gene sequences. The theoretical TRF length has been calculated for 45 fungal pathogens identified to species or *forma specialis* level. These include *Colletotrichum coccodes*, *Fusarium oxysporum* f. sp. *lycopersici* (FOL), *Fusarium oxysporum* f. sp. *radicis-lycopersici* (same fragment length as FOL), *Phytophthora cryptogea*, *Pyrenochaeta lycopersici*, *Pythium irregulare*, *Rhizoctonia solani*, *Spongospora subterranea*, *Thielaviopsis basicola*, *Verticillium albo-atrum* (Vaa) and *Verticillium dahliae* (same fragment length as Vaa). The theoretical TRF length has also been calculated for 39 fungal saprotrophs recorded on tomato roots including *Aspergillus flavus*, *Mucor* sp., *Olpidium brassicae*, *Penicillium* (10 species) and *Trichoderma viride*. For bacterial pathogens of tomato roots, the theoretical TRF length has been calculated for *Agrobacterium tumefaciens*, *Pseudomonas syringae* pv. Tomato and

Ralstonia solanacearum. The theoretical TRF length of other fungi and bacteria recorded associated with tomato roots is being sought, and theoretical sizes for more than 460 bacteria and 150 fungi associated with various crops have now been determined using the primers and restriction enzymes that we use.

In addition, where possible, cultures of fungi and bacteria have been collected, sequenced and examined by T-RFLP to confirm theoretical TRFs, as shown for *F. oxysporum* (Fig 3.1).

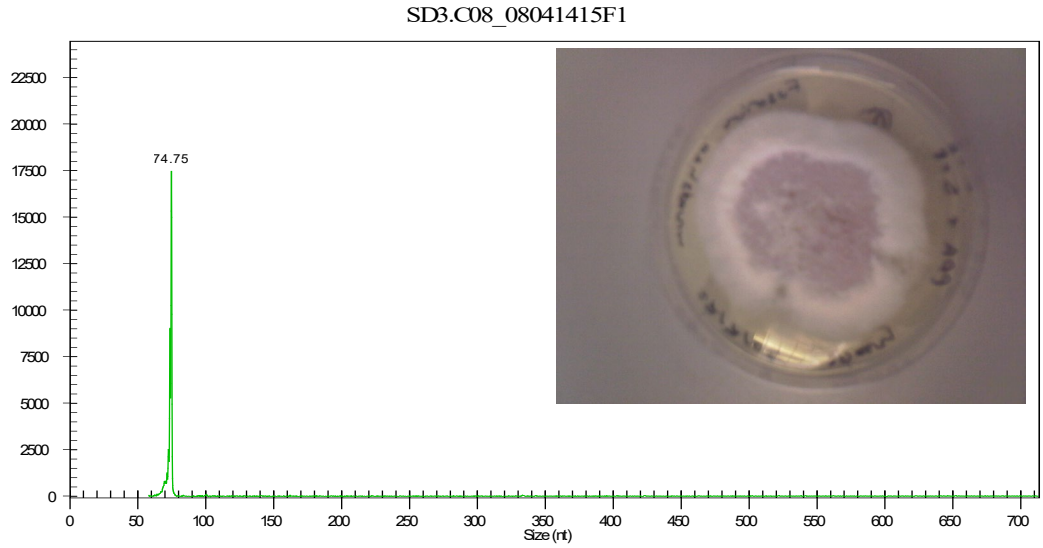

Figure 3.1: T-RFLP analysis of *a Fusarium oxysporum* culture, confirming the ITS-2 region fragment length when cut with a specific restriction enzyme to be 75 base pairs.

It is important to confirm theoretical TRFs either by T-RFLP analysis of identified/sequenced cultures or by cloning techniques, as true fragment length and observed fragment length have been reported to differ by 1 to as much as 7bp. Discrepancies between true and observed fragment length (TRF drift) have previously affected identification of microbes from environmental samples. TRF drift was positively correlated with true TRF length and negatively correlated with TRF purine content,

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implying that subtle difference in molecular weight, caused by purine content or dye label, can affect the observed TRF.

So far, pure cultures of eighteen fungi identified to genus and/or species level by morphological features have been treated with HaeIII and their TRF length determined. Details of the fungi and the TRFs obtained are shown in Table 3.1. Further cultures of other identified fungi and bacteria will be tested in Years 2 and 3 and their TRFs added to the database.

Table 3.1: Details of fungal cultures and TRF length obtained on treatment with HaeIII restriction enzyme; calculated theoretical TRF values are also shown where known

Sample reference	Fungus	TRF length with HaeIII	TRF length (theoretical)	Source[a]	Previously recorded on tomato
VerLec - 04/08	*Verticillium lecanii*	76		Nottingham	No
FusOxy - 04/08	*Fusarium oxysporum*	74	73	Nottingham	Yes
RhiSol - 04/08	*Rhizoctonia solani*	105	105	Nottingham	Yes
PenExp - 04/08	*Penicillium expansum*	79		Nottingham	No
FusAve – 04/08	*Fusarium avenaceum*	75		Nottingham	No
PytIrr - 04/08	*Pythium irregulare*	656	651	Nottingham	Yes
ColCoc – 07/08	*Colletotrichum coccodes*	153	153	Rockwool tomato CW	Yes
PhyCry – 07/08	*Phytophthora cryptogea*	604	604	Rockwood tomato CW	Yes
CW1 – 07/08	*Cladosporium* sp.	322		Rockwool tomato CW	No
CW6 – 07/08	*Plectosphaerella cucumerina*	138	138	Rockwool tomato CW	Yes
PytDic – 07/08	*Pythium diclinum*	242	241	Rockwool tomato CW	Yes
1IOW – 07/08	*Pythium* sp.	639		Soil tomato, IoW	-
8JS – 10/08	*Gliocladium* sp.	120		Unknown	-
9JS – 10/08	*Phytophthora* sp.	291		Unknown	-
PhyCin – 10/08	*Phytophthora cinnamomi*	310	294-311	Unknown	No
11JS – 10/08	*Pythium intermedium*	68		Unknown	No
12JS – 10/08	*Botrytis* sp.	319		Unknown	-
13JS – 10/08	*Pythium* sp.	654		Unknown	-

[a]Nottingham - Nottingham University Biosciences Dept culture collection; Rockwool CW - rockwool crop at Cornerways Nursery, Norfolk; IoW- WSG Nursery, Isle of Wight.

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Development of T-RFLP test protocol

To optimise results of T-RFLP analysis, DNA extraction methods and rRNA gene primers were examined and compared. Two DNA extraction methods were evaluated, a direct method; analysing ≥100 mg of root was used and also a wash method; using much larger samples of root material (at least 1 g).

From testing numerous samples using both techniques it was found that fungal fingerprints were similar when using both extraction methods on the same root sample. However, the direct method gave more representative results of microbial community, with the wash method failing to pick up bacterial organisms (Fig. 3.2). In addition, the 5.8S and FITSrev1* primers amplify part of the tomato plant chloroplast, which can act as a semi-quantitative control. This potentially allows semi-quantitative analysis of both fungal and bacterial genera in a single test. When using the wash method the tomato plant peak is lost as no plant tissue will be present when using the DNA extraction kit. Further repeats are being done to confirm that using ≥100 mg is sufficient to obtain a representative view of the microbial community present in tomato plant rhizosphere.

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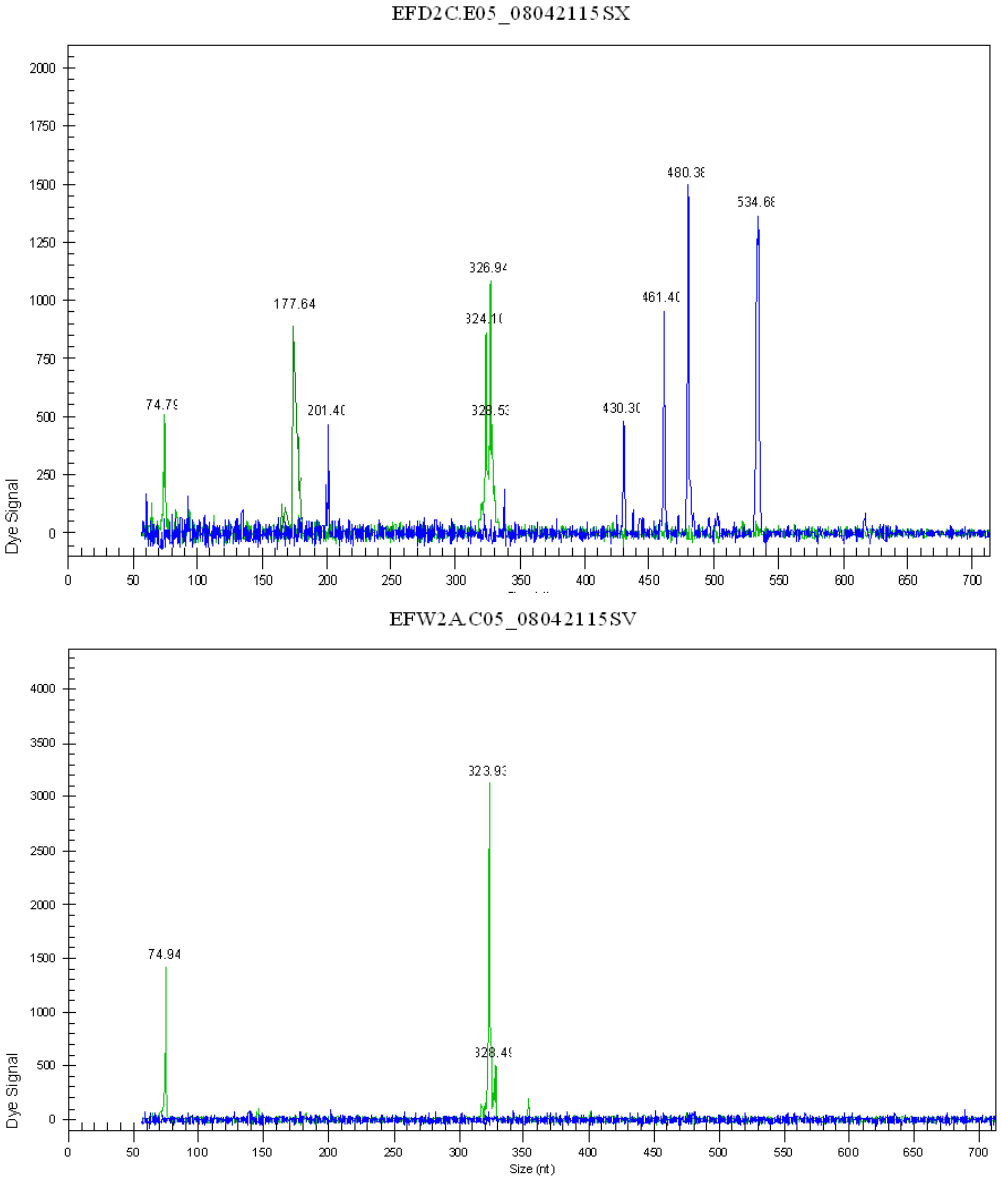

Figure 3.2: T-FRLP analysis of tomato root samples from plants grown on Ecofibre (woodfibre) using the direct method of DNA extraction (above) and the wash method of DNA extraction (below). Note the plant peak at 178 bp as a semi-quantitative internal control when using the direct method.

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In addition to experimenting with DNA extraction methods, several bacterial primers amplifying 23S rRNA genes and some that simultaneously amplify tomato plant genes were examined for their consistency and ability to bind to conserved regions of this domain, resulting in a more representative insight into the bacterial organisms present in root samples.

From comparing numerous results, it was found that the most reliable and representative results were gained from pairing 23Sfor and 23Srev* primers. For example, when 23Sfor and 23Srev* was compared with MJD4 and 23Srev, it was found that although MJD4 could amplify *Pseudomonas* and other bacteria producing much larger fragments, they were missing a large family known as the Enterobacteriaceae. 23Sfor and 23Srev* on the same samples were found to produce TRFs for *Pseudomonas* and larger TRFs as well as Enterobacteriaceae (Fig. 3.3) This may be due to the 23Sfor and 23Srev* primers set binding better or perhaps the homologous markers amplified by 23Sfor are more conserved among bacteria. Using this set of primers, however, did result in losing an internal semi-quantitative control, but this was not considered detrimental as there is a tomato plant peak semi-quantitative control produced by the fungal primers.

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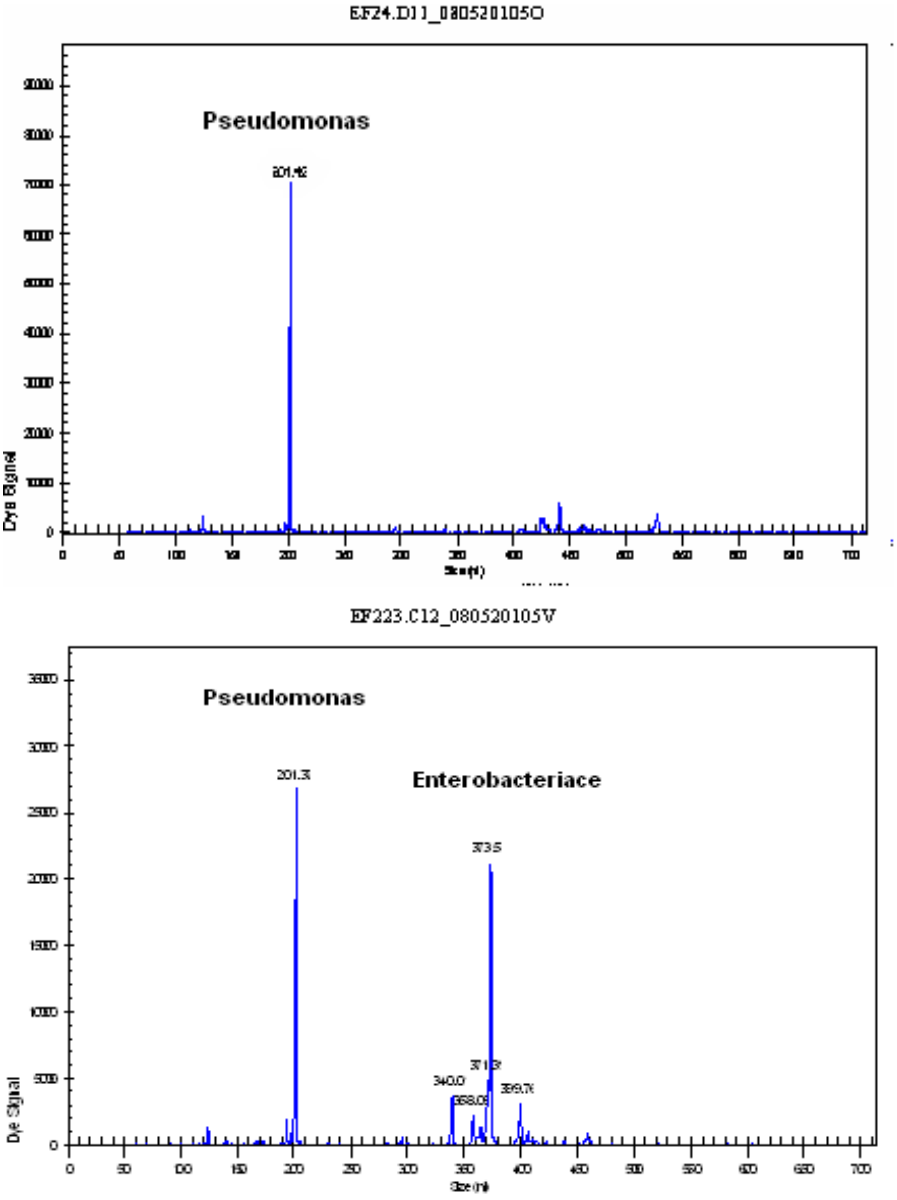

Figure 3.3: DNA extract from a plant root grown in Ecofibre amplified with MJD4 with 23Srev*(above) and 23Sfor with 23Srev*(below). Note that Enterobacteriaceae are not amplified from the sample when using MJD4.

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Comparison of sampling methods used on soil, rockwool and NFT-grown tomato crops

Samples obtained using different methods of root sampling (section 2) were examined by T-RFLP analysis to identify methods that result in significant and representative levels of DNA recovery. DNA was extracted from roots of different thickness from a soil grown tomato crop and amplified using both fungal and bacterial primers. Samples were then digested and sent for T-RFLP analysis to confirm whether there was a difference in microbial community present on thick, medium and thin roots. It was found that there was a greater variety of microbes on the younger, actively growing thin roots and, as the roots thicken, the number of organisms decrease giving way to, perhaps, a more established group of organisms (Fig. 3.4). There also appeared to be a decrease in the relative levels of organisms present when compared to the internal semi-quantitative control (tomato plant peak- 178bp).

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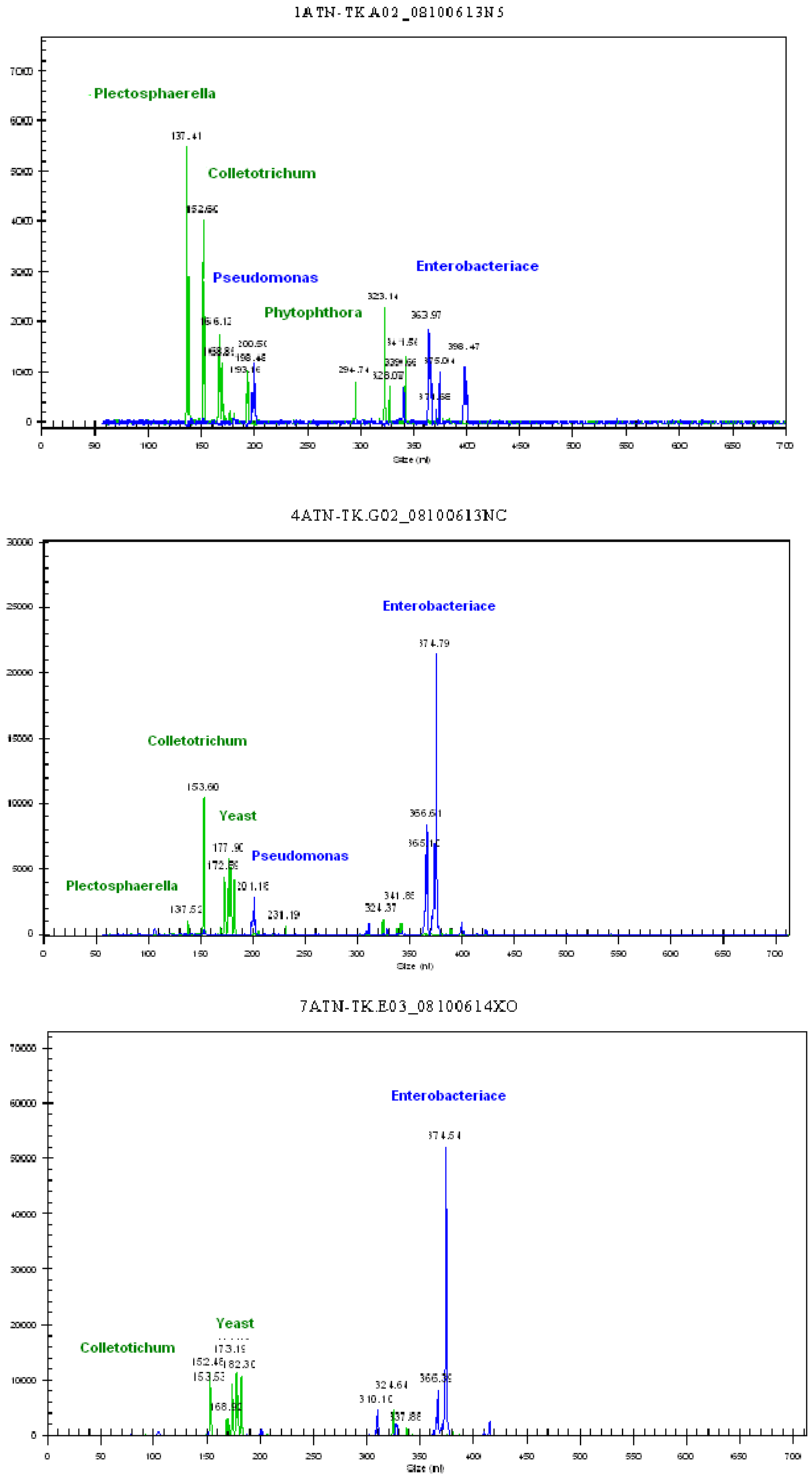

Figure 3.4: T-RFLP analysis of roots of different thickness from the same soil grown tomato plant. Note that there are more peaks on the thin root (top) in comparison with the medium (middle) and thick (bottom). This suggests that there were more micro-organisms present on the thin root.

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Three sampling methods were tested for analysing roots grown in rockwool slabs; 1) cork borings adjacent to a propagation cube, 2) cork borings between two propagation cubes, 3) a corner cut from a slab using a knife. Ten rockwool cores were collected in each of 3 replicate blocks along the row, as well as 3 bulk samples taken by the corner slice method. DNA was extracted using the direct method, amplified using fungal and bacterial primers and sent for T-RFLP analysis

From analysing these three different sampling methods by T-RFLP, it was revealed that there was little difference between DNA recovery and the microbes present in all three methods (Fig. 3.5). However, it was noted that it was far easier and less invasive to take roots from the corners of slabs. In addition, more roots were recovered when using this method. T-RFLP results were very similar between replicates.

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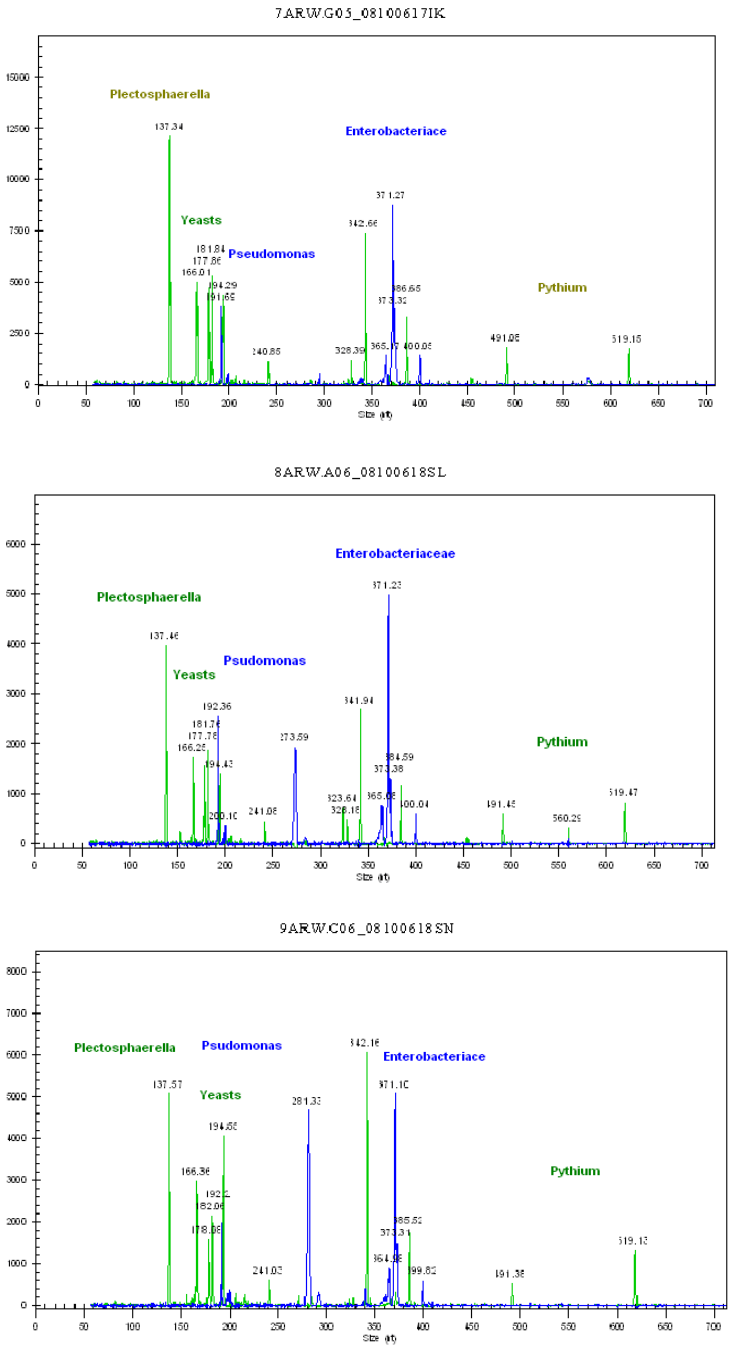

Figure 3.5: T-RFLP analysis of cork borings from adjacent (top) midway between propagation cubes (middle) and a corner slab (bottom) cutting from the same rockwool slab. Note the same peaks are present when using all three sampling methods.

When sampling from the NFT crop, 5 samples were taken equally spaced along one row (sample 1 closest to inlet and sample 5 closest to outlet). In addition, an inlet and outlet nutrient solution sample (30 ml) was taken. Root samples from NFT crops were analysed using T-RFLP and it was found that there was little variation among the five samples taken (Fig. 3.6). This suggests that the microbial community throughout this row is ubiquitous on all roots. Furthermore, results were also very similar between replicates, once again emphasising the robustness of the T-RFLP protocol being used.

With regards to the solution samples, it was found that there was little difference between the inlet and outlet samples. However there were a far greater number of organisms present in the solution samples than the root samples. This could be due to some of the organisms in the solution being unable to colonise the roots (Fig. 3.6).

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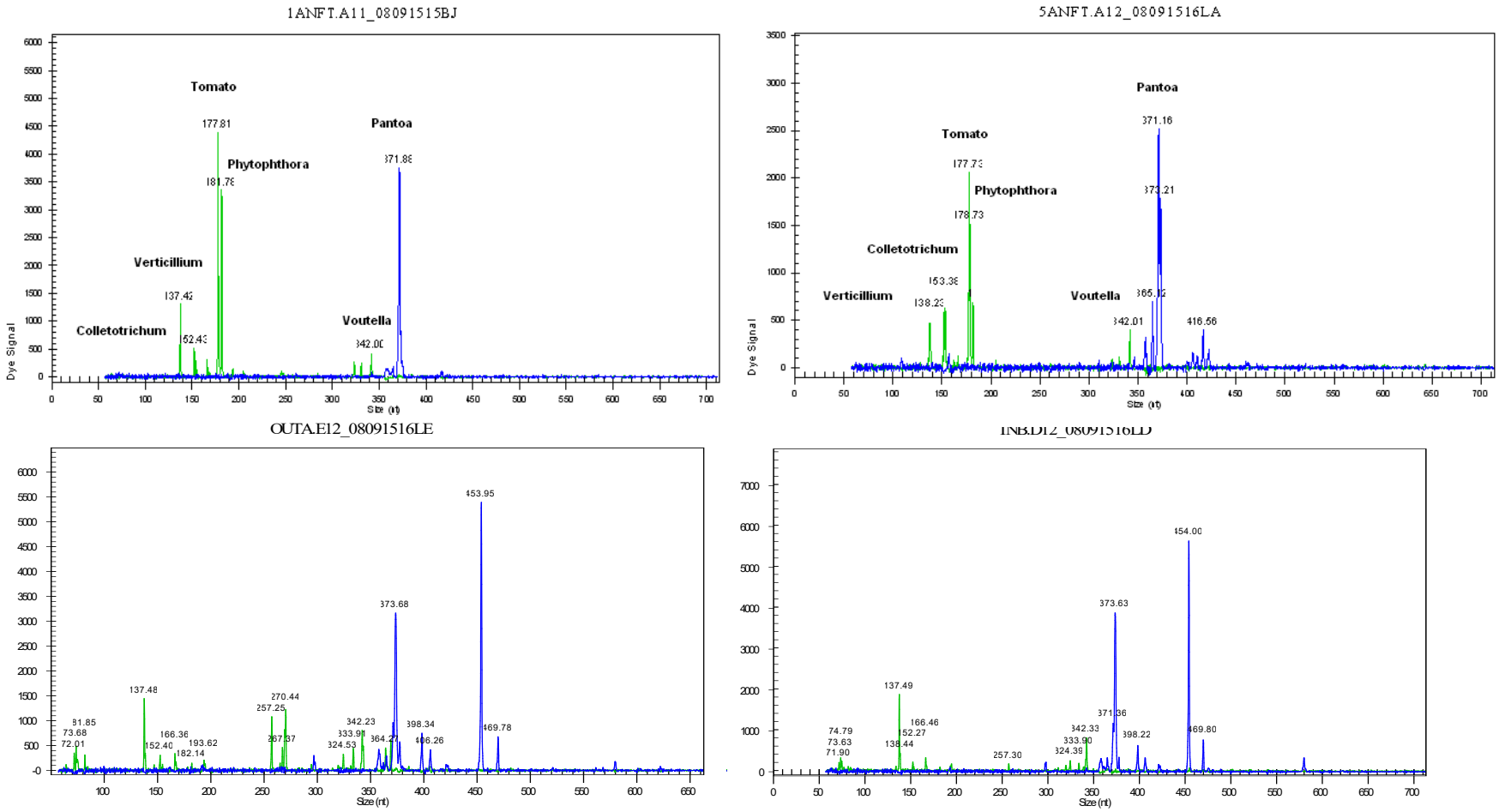

Inlet

Outlet

Figure 3.6: T-RFLP analysis of roots from NFT crop from closest to inlet (sample 1, top left) to closest to outlet (sample 5, top right) and of nutrient solution at inlet (bottom left) and outlet (bottom right). Note similar fingerprints between sample 1 and 5 and between inlet and outlet samples. In addition, there are more organisms present in the nutrient solutions.

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Confirmation of T-RFLP results by comparing those with findings from conventional diagnostic techniques

All samples that were tested for T-RFLP analysis were also examined using conventional methods for isolating fungi from roots (section 2). Isolates were identified from their colony morphology and morphological features and compared with results obtained from T-RFLP analysis (Table 3.1). It was found that most major organisms recovered by conventional techniques were also recovered from T-RFLP analysis, with the exception of some isolates from soil. This could be due to an insufficient DNA extraction, as roots from soil crops tended to be woodier than those from non-soil crops. On the other hand, it could be that there was such a high level of other organisms present in the sample and that organisms selected for by conventional methods were too low in numbers to be picked up by T-RFLP.

In addition certain major organisms were picked up by T-RFLP and not by conventional methods, particularly in the case of NFT samples. This could perhaps be due to culture plates being swamped by other faster growing organisms or the T-RFLP technique detecting unculturable microbes.

Table 3.2: Detection of major fungal groups and species, from roots of tomato grown in soil, rockwool and NFT, by conventional and T-RFLP methods

Fungal group or species	Detected in:		
	Soil crop	Rockwool crop	NFT crop
Colletotrichum coccodes	Both	Both	Both
Fusarium sp.	Both	Both	Both
Fusarium oxysporum	Conventional		T-RFLP
Penicillium	Both		T-RFLP
Pythiaceous	Both	Both	T-RFLP
Trichoderma	Both		T-RFLP
Verticillium			T-RFLP
Other	Both	Both	Both

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Agreed root sampling and testing protocol for crop monitoring in 2009 and 2010

The main aim of the project is to use T-RFLP to investigate the occurrence and relative abundance of micro-organisms around the rhizosphere of tomato, monitoring the changes in microbial profile over two seasons (2009 and 2010), and then to determine if this information can be used to help predict disease occurrence.

Root sampling: Based on results obtained in Year 1, it was agreed by the project consortium that sampling methods for routine monitoring over the next two seasons from the different substrates will consist of:

- Rockwool - roots cut from a slab corner to the full depth of the slab
- NFT - 1 cm wide strip of roots cut from midway between two plants, from the edge of the channel to the centre
- Soil - Soil augured from midway between two plants and roots sifted out (minimum of 2 g of root required, approx 5-10 soil cores per sample), with aim of collect young root where there is likely to be more microbes present.

There will be three replicate samples taken from a single row of crop on each sampling occasion. The same crop area will be used for repeat samples over time. Crops will be sampled on three occasions throughout the season at different growth stages: (1) early - around 2-4 weeks after rooting into slab/soil/NFT; (2) at peak fruit load - just before first fruit pick; (3) at main season – during the first two weeks in August.

Additional crops will also be monitored, including Ecofibre (woodfibre) and coir. This is because both these methods are becoming significantly more popular due to the recent move towards more sustainable methods of tomato production.

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T-RFLP test protocol: The direct extraction method will be used rather than root washes, as this was found to give reliable and robust detection of micro-organisms within roots. Furthermore, this method incorporates some plant DNA, providing the potential benefit of an internal control on success of the test.

It was apparent that the most consistent results with the widest range of bacterial peaks came from using 23Sfor and 23Srev∗. However, more experiments need to be conducted to ensure that the best primer pairs are chosen for 'universal' amplification of both fungi and bacterial communities. In addition, the advantages of labelling both primers with different fluorescent dyes will be assessed. The main reason for tagging both ends would be to increase the resolution of T-RFLP analysis by potentially differentiating between sequences which produce similar sized TRFs with a single tag.

Database of TRFs: From conducting T-RFLP analysis on numerous root samples it became evident that there were many peaks present that did not correspond with TRFs on the database created. To improve the identification of TRFs, PCR products of regularly occurring unknown peaks will be gel purified, cloned into a suitable vector and sequenced. Once this has been completed, the sequence data can be compared to sequences of previously published sequences to identify the organism. In addition, to help improve the database, culture plates will continue to be collected and observed TRFs will be confirmed by T-RFLP analysis.

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Conclusions

1. A review of scientific literature indicates a world total of at least 66 fungal pathogens and 75 fungal saprotrophs have been found associated with tomato roots.

2. Determination of the diversity and relative levels of fungi associated with tomato roots by culturing on agar is time-consuming and requires skilled mycological expertise to identify reliably the organisms obtained.

3. Fungi commonly isolated from the roots of UK commercial tomato crops in 2008 were *Colletotrichum coccodes*, *Fusarium* spp., pythiaceous fungi, *Trichoderma* spp. and mucoraceous fungi. *Penicillium* spp., *and Thielaviopsis basicola* were isolated occasionally. Many fungi isolated were not identified.

4. Fungi are commonly present in and on roots, including visibly healthy white roots, of tomato crops grown in soil, NFT and rockwool.

5. *Fusarium* spp., *pythiaceous* fungi and *Penicillium* sp. were isolated more commonly from young thin roots than from older thicker roots, and vice-versa for *C. coccodes.*

6. Direct extraction of DNA from tomato roots for a T-RFLP test gives more reliable and representative results than a wash extraction method, whilst keeping the benefits of having an internal semi-quantitative control due to the presence of plant material.

7. Bacterial primers 23Sfor and 23Srev* were found to be the best combination for obtaining a wide range of bacteria from tomato roots, and probably therefore a more representative picture of the bacterial community. This could be because there primers are better at binding to highly conserved regions of the 23S rRNA

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domain or that the other primers were homologous to regions that are less well conserved among bacterial organisms.

8. With regards to sampling techniques, the T-RFLP test found that there was more diversity among microbial organisms on thin roots than medium and thick roots. These results could be explained by the thin roots being younger and actively growing, thus the site for most chemical activity and ultimately more attractive to more organisms.

9. T-RFLP test results from different root sampling methods for tomato grown in rockwool and NFT suggest that it makes little difference where you sample from within one slab or one row of a crop. This gives confidence that a relatively small number of samples can be taken for future studies whilst still obtaining representative results.

10. Many organisms isolated from conventional techniques were also found from T-RFLP analysis. This gives assurance in the reliability of the T-RFLP protocol being used and because of this, and the relative ease of the test compared with isolation and culturing, all future samples will be analysed by T-RFLP alone.

11. A database is being created that identifies fungi and bacteria according to the DNA fragment length (TRF) produced using the restriction enzymes described in this work. So far, the database contains the theoretical fragment lengths for 84 fungi and 3 bacteria. The theoretical fragment lengths have been confirmed for 18 fungi by use of reference cultures.

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Technology transfer

Meetings

Project start-up meeting, ADAS Arthur Rickwood, 10 April 2008.

T-RFLP techniques meeting with Warwick HRI, Sutton Bonington, 1 October 2008.

Project progress meeting, Sutton Bonington, 8 October 2008.

Statistics meeting, Sutton Bonington, 24 October 2008.

Presentations

Tomato root health-project objectives and plans, Wight Salads Group, Isle of Wight, 23 July 2008.

Project progress report, Wight Salad Group, Isle of Wight, 30 October 2008.

Articles

Friendly microbes: healthy roots. *HDC News* **143**, p.6. New projects section.

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