MONITORING RHIZOSPHERE MICROBIAL COMMUNITIES OF TOMATO

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ABSTRACT

Tomato is an economically important crop that can be devastated by many root infecting pathogens. The development of alternative and sustainable crop cultivation techniques and disease control methods is a must for the tomato industry, due to more strict government regulations and concerns over the sustainability of conventional chemical-intensive agriculture (Dixon and Margerison, 2009).

In this thesis, the molecular fingerprinting method Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and next generation sequencing method (pyrosequencing) were used, targeting ITS1, ITS2 and 23S ribosomal DNA to characterize and examine microbial community assemblages in the rhizosphere of tomato. These molecular techniques were employed alongside traditional cultivation, microscopy and plant health assessment techniques to determine the effects of growth media, plant age and disease control methods on rhizosphere microbial populations and tomato root health.

Plant age and media were found to significantly affect microbial community assemblages; conversely, microbial populations were not altered by soil amendments or rootstock disease control measures used. These findings suggest that the factors influencing rhizosphere community structure can be ranked by importance. Furthermore, if the most influential factors are kept consistent then rhizosphere microbial structures are robust and difficult to perturb with changes in a factor contributing less control over microbial community composition.

No direct link between crop health assessments and rhizosphere microbial community diversity or presence of root pathogens could be established. Furthermore, high abundance of potential pathogens and poor crop health assessments during the growing season did not always result in poor health or disease symptoms at the end of cropping assessment in our trials. These results imply that many factors control the rhizosphere competence and ecological role of different species, ultimately affecting the outcome of

disease. As no known methods are capable of efficiently assessing the fate of total microorganisms in the rhizosphere over time and space, this study could be considered as part the ‘descriptive phase’ in this field (Kent and Triplett, 2002).

Pyrosequencing increased the resolution and confidence of rDNA analysis compared to T-RFLP, identifying organism within samples to a genus and often species level. Advances in next generation sequencing and analytical tools and pipelines associated with this analysis are likely to develop as these methods become common practice. With this in mind, next generation sequencing represents the future approach for resolving complex microbial communities in environmental samples.

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ABBREVIATIONS AND ACRONYMS

A	adenine
AFLP	amplified fragment length polymorphism
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
ARDRA	amplified ribosomal DNA restriction analysis
ARISA	automated ribosomal intergenic spacer analysis
BLAST	Basic local alignment search tool
bp	base pair
C	cytosine
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
FAO	Food and Agriculture Organization
G	guanine
IPTG	isopropylβ-D-thiogalactopyranoside
ITS	internal transcribed spacer
LSU	large subunit of ribosomal DNA
MID	multiplex identifier
NCBI	National Center for Biotechnology Information
NFT	nutrient film technique
NS	no significance
nt	nucleotide
OTU	operational taxonomic unit
PCA	Principal component analysis
PCR	polymerase chain reaction
PVPP	Polyvinylpyrrolidone
rDNA	ribosomal DNA
RDP	ribosomal database project
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
RW	rockwool
SDS	sodium dodecyl sulphate
SSF	slow sand filter
SSU	small subunit of ribosomal DNA
T	thymine
TBE	Tris-borate-EDTA
TGGE	temperature gradient gel electrophoresis
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
U	uracil
UK	United Kingdom
UV	ultraviolet light
WF	woodfibre
X-GAL	5-bromo-4-chloro-3-indolylbetagalactoside

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1 INTRODUCTION

1.1 THE TOMATO CROP

Tomato (*Solanum lycopersicum)* is the one of the world's most economically important 'vegetable crops', regarding area planted, production and industrial value (Gould, 1983). The plant is grown both commercially and non-commercially in over 140 countries for its diverse edible fruit. China, the United States, India, Turkey and Egypt are the top five tomato fruit-producing countries (Table 1.1) and for more than 20 years annual production has been rapidly increasing. Most recent statistics estimate world production at approximately 145 million tons of fresh and processing tomatoes per year, with such levels having more than doubled since 1990. Levels of production have increased primarily due to improvements in cultivation methods and increased consumer demand (Van de Vooren *et al*., 1986). The tomato is the most widely eaten fruit with global consumption over 118 million tons per year, making the tomato industry worth more than $70 billion (FAOSTAT Database, 2010).

Table 1.1: World production of tomato, 2010.

Location	Area(Ha)	Yield (Hg/Ha)	Production (ton)
World	4336505	335875	145652579
China	870503	480926	41864750
United States	159200	810427	12902000
India	619800	193283	11979700
Turkey	304000	330658	10052000
Egypt	216385	394898	8544990

Source: FAOSTAT, 2010.

The high level of tomato consumption and the concentration and availability of several vitamins, minerals and antioxidants makes the fruit important to human nutrition. The nutritional quality of tomato is mainly determined by its carotenoid, potassium, vitamin C and vitamin A content. Ripe tomatoes have high levels of carotenoids, of which carotenes make up between 90 and 95%

(Guil-Guerrero and Rebolloso-Fuentes, 2009). In particular, the pigment lycopene, which is the most abundant carotene in red tomatoes, has gained much attention due to its antioxidant properties and is known to reduce the risks of many forms of cancer and heart attacks (Dorgan *et al*., 1998; Clinton, 2005). Cultivars of tomato vary greatly in their total carotenoid contents (18.5 to 60.7 mg/kg fw; Abushita *et al*., 1997) and there is scope to look to wild relatives such as *S. pimpinellifolium* (Table 1.2), which have 5 times higher levels of lycopene, to increase the lycopene content of cultivated tomato via breeding programmes (Fernandez-Ruiz *et al*., 2002).

1.1.1 Historical background: taxonomy and domestication

Tomato belongs to the Solanaceae family, which comprises over 1000 species of flowering plant including a number of other economically important crops (e.g. potatoes, peppers and tobacco). The botanical classification of tomato has had an interesting history regarding its nomenclature, with tomato firstly being placed in the largest and most diverse genus of the Solanaceae family; *Solanum* by the Swedish botanist Linnaeus in 1753. A mere 15 years later Miller moved tomato into its own new genus *Lycopersicon*, primarily due to morphological differences in pollen bearing structures between tomato and other members of the *Solanum* genus (Taylor, 1986). Genetic evidence now suggests that Linnaeus was correct to put the tomato into the genus *Solanum*; initially shown by chloroplast DNA restriction site and sequence data (Spooner *et al*., 1993) and more recently by granule-bound starch synthase gene sequence data (Peralta and Spooner, 2001).

Solanum section *Lycopersicon* includes cultivated tomato (*Solanum lycopersicum*) and 12 additional wild relatives listed in Table 1.2 (Peralta *et al.*, 2006). The wild relatives of cultivated tomato originate from the coastal strip of western South America, predominantly Peru and the Galápagos Islands. The most probable ancestor of modern cultivars is the wild cherry tomato, which is the only wild variety found outside of South America where it most likely escaped from cultivation (Rick, 1979). The original site of domestication

is not certain, although the majority of evidence suggests Mexico, where it is considered that the tomato reached a fairly advanced level before being brought to Europe in the 15th century. Further domestication on a much more intense scale occurred throughout Europe from the 18th century onwards. Today, approximately 7500 different cultivars have been created from the single species *S. lycopersicum* producing tomatoes of many shapes, colours and sizes (Sims, 1980).

Table 1.2: Species list for Solanum section Lycopersicon.

Solanum name	Distribution
S. arcanum	N Peru, coastal and inland Andean valleys
S. cheesmaniae	Galápagos Islands, Ecuador
S. chilense	S Peru to N Chile
S. chmielewskii	S Peru (Apurı´mac) to N Bolivia (La Paz)
S. corneliomuelleri	Central to S. Peru, W slopes of the Andes
S. galapagense	Galápagos Islands
S. habrochaites	Central Ecuador to Central Peru
S. huaylasense	N Peru
S. lycopersicum	Known only from cultivation or escapes; worldwide
S. neorickii	S Ecuador to S Peru
S. pennellii	N Peru to N Chile
S. peruvianum	Central Peru to N Chile
S. pimpinellifolium	Central Ecuador to central Chile

Source: Peralta *et al.*, 2006.

1.1.2 Commercial cultivation

Cultivated tomato varieties can be divided into determinate or indeterminate types based on growth habit; the former terminate in a flower cluster topping off at a specific height, and the latter develop into single-stemmed vines that never top off. Determinate cultivars are more appropriate for short-season production; the fruit ripen uniformly making these cultivars suitable for mechanical harvesting. Indeterminate cultivars are for long-season production and fruit continuously for long periods (Jones, 2008).

Large scale tomato growers usually germinate seeds late winter, typically at a propagation house, which are then transplanted at 2-6 weeks into the chosen growth media. Tomato plants develop optimally at day temperatures of

21.5°C-29.5°C and night temperatures of 18.5°C-21°C. Grown in favourable conditions tomato crops can be maintained for periods of up to 6 to 9 months, by training crops up vertical supports and removing older leaves, and for indeterminate plants, older fruit clusters. The cultivated tomato is a self-pollinating species (autogamous) and has been bred extensively to maximize this trait. However, the level of self-fertilization between cultivars varies and in commercial nurseries pollination is usually aided by artificial wind or by cultured bumblebees. The approximate time required to go from planting to market is between 50-60 days for an early variety and 85-90 days for a late variety (Hu *et al*., 2007; Jones, 2008).

Land resources are becoming ever more constrained for agriculture purposes, and to maximise crop yield per unit area there has been a rapid increase in the production of tomato crops grown under glass and plastics (Figure 1.1). Glasshouse production gives growers more control over the environment in which their crop is produced, enabling crops to be grown at optimal rates throughout the growing season, including areas with cooler climates. Environments in commercial glasshouses are often automatically controlled by high-tech heating, cooling, lighting and irrigation systems. Increasingly, commercial tomato growers are producing under contract for supermarkets that require large scale specialist production; this has further enhanced moves towards more intensive forms of horticultural production (Bennett *et al*., 2011).

More intensive cultivation methods have had a negative impact on the standard method of growing tomatoes in soil, as continuous cropping in soil can lead to an excessive build up of soil borne pathogens, which in turn is known to result in large yield reductions (Bennett *et al*., 2011). To help avoid diseases prevalent in soil grown crops, hydroponic soilless growing systems (Figure 1.1) have been commercially developed and are becoming increasingly prevalent amongst commercial tomato growers. Hydroponically cultivated crops are grown with their roots wholly or partially submerged in a nutrient rich solution, with the use of an artificial medium (e.g. rockwool or gravel) or

without (Nutrient Film Technique; NFT). Hydroponic systems can either be closed (recirculating) or open (run-to-waste) systems. Due to environmental concerns and legislation it has become more cost effective to use closed methods, as growers will save on water and the costs associated with the disposal of used solutions (Gould, 1983; Van de Vooren *et al*., 1986).

Figure 1.1: Tomato production under glass and plastics using hydroponics and traditional methods. Image A depicts the earliest form of hydroponic technology; Nutrient Film Technique (NFT), B shows the latest hydroponic method involving drip irrigation into a solid medium and C portrays growing tomato crops in soil. Image D provides an aerial view of tomato nursery glasshouses.

In addition to avoiding the build up of soil-borne pathogens, hydroponic cultivation permits the optimal mineral requirements of specific cultivars to be met by monitoring and adjusting irrigation solutions. As there is access to unlimited nutrients, growing crops hydroponically can result in crops that grow ten times faster than their soil grown counterparts. Consequently this specialist form of crop production allows large scale production with larger economic returns; however, the cost to set up these systems is far greater (Geraldson, 1982). Although theoretically growing tomato crops in hydroponic systems is an effective and economic way of avoiding soil borne diseases, it

has been found that when a pathogen enters such systems they spread rapidly and can result in a disease epidemic, particularly in closed systems and under times of abiotic or biotic stress (Calvo-Bado *et al*., 2006).

1.1.3 Tomato breeding

Selection and breeding to improve desirable agricultural characteristics in tomato has been in progress for more than 200 years, yet emphasis in breeding was initially often upon increasing fruit morphological diversity and yield, whilst grown in optimal conditions. As a result, the cultivated tomato is moderately to highly sensitive to numerous abiotic and biotic stresses including extreme temperatures, drought, overwatering, salinity and diseases (Rick and Cheelat, 1995).

To improve resistance to abiotic and biotic stresses breeders must look to other species for resistance genes, as the cultivated tomato experienced severe genetic bottlenecking when domesticated in South America onwards; where selection was typically done using a single plant with a small number of selected plants. In such a largely inbreed species, the cultivated tomato is genetically poor with very little variation between cultivars. In contrast, wild relatives of cultivated tomato (Table 1.2) have large genetic diversity. Miller and Tanksley (1990) estimated that the genomes of tomato cultivars have <5% of the genetic variation of their wild relatives. Consequently wild relatives provide an invaluable resource for the genetic improvement of the crop and numerous breeding programmes have been set up to develop stress-tolerant crops (Bai and Lindhout, 2007; Robertson and Labate, 2007).

Molecular methods and *in-vitro* culture techniques have facilitated the hybridization between incompatible wild and cultivated relatives, resulting in the introgression of resistance and tolerance genes. For example Patterson (1988) identified cold tolerance genes in the wild relatives *S. peruvianum, S. chilense* and *S. habrochaites*, which are all found at high altitudes and endure night temperatures often below 10°C. In cultivated tomato temperatures below 15°C severely affect the quality and quantity of pollen produced; it has

been suggested that the germplasm of high altitude wild relatives could improve cold tolerance in crops, with crossing programmes to *S. habrochaites* seeming to be the most promising method (Venema *et al*., 2005). Notably, the major breeding efforts have been made towards disease resistance, with over 40 resistance genes to major diseases having been discovered in wild relatives. Many of these resistances have been successfully bred into cultivated crops; for instance *S. peruvianum* is the source of many widely used resistance genes such as: *Tm-2* (Tobacco Mosaic Virus resistance), *Mi* (resistance to root knot nematodes) and *Sw-5* (tomato spotted wilt virus resistance) (Rick and Cheelat, 1995).

1.1.4 Tomato disease and control

The tomato crop is host to over 200 disease causing agents and in certain conditions disease development can result in significant yield loss (Watterson, 1986). The most costly and common diseases of tomato are caused by a number of different organisms namely bacteria, fungi, oomycetes, viruses and nematodes (Table 1.3). A crop cultivar can be defined as resistant, tolerant or susceptible to these biotic stresses, dependent upon ability to prevent infection and the severity of the symptoms caused. In resistant crops, the plant avoids infection by the initiation of plant defence responses and as such, symptoms (if any) are low. On the contrary, if a crop is susceptible then the infection is successful, the disease causing agent colonizes plant tissues and the crop develops disease symptoms. Tolerant crops are unusual in that they are successfully infected and colonized by pathogenic organisms but the plant will exhibit reduced symptoms and similar yields to resistant cultivars. Tomato diseases can be spread by air, soil (media), water, seed or vector and can infect aerial parts of the plant as well as the roots, the latter often being more expensive and difficult to control due to location and microbial complexities in the root environment (Blancard, 1994; Hawks *et al*., 2007; Jones, 2008).

Table 1.3: Commonly occurring and economically important tomato diseases, the causal agent and their control

Disease	Microorganisms	Control
	Fungi	
Late blight	*Phytophthora infestans*	Approved fungicides; resistant varieties; Crop rotation; soil solarization.
Verticillium wilt	*Verticillium albo-atrum*	
Verticillium dahliae		
Anthracnose	*Colletotrichum coccodes*	
	Colletotrichum dematium	
	Colletotrichum gloeosporioides	
Early blight	*Alternaria solani*	
Cercospora leaf mold	*Pseudocercospora fuligena*	
Fusarium wilt	*Fusarium oxysporum f.sp. Lycopersici*	
Fusarium crown and root rot	*Fusarium oxysporum f.sp. radicis-lycopersici*	
Powdery mildew	*Oidiopsis sicula*	
Pythium damping-off and fruit rot	*Pythium aphanidermatum*	
	Pythium arrhenomanes	
	Pythium debaryanum	
	Pythium myriotylum	
	Pythium ultimum	
Rhizoctonia damping-off and fruit rot	*Rhizoctonia solani*	
Gray leaf spot	*Stemphylium lycopersici*	
Septoria leaf spot	*Septoria lycopersici*	
Leaf mold	*Cladosporium fulvum*	Stake and prune to provide air movement
	Bacteria	
Bacterial spot	*Xanthomonas campestris*	Approved bactericides; hotwater-treated seed; avoid planting in affected fields for 3 years.
Bacterial wilt	*Ralstonia solanacearum*	
Bacterial speck	*Pseudomonas syringae pv. Tomato*	
Bacterial canker	*Clavibacter michiganensis*	
Root Mat	*Agrobacterium rhizogenes*	
	Viruses	
Tomato mosaic	*Tomato mosaic virus*	Avoidance of contact by smokers; control of aphid carrier with insecticides; stylet oil; resistant varieties; prevention and eradication; disease-free seed and plant material.
Tomato fern leaf	*Cucumber mosaic virus*	
Curly top	*Curly top virus*	
Tomato bushy stunt	*Tomato bushy stunt virus*	
Tomato etch	*Tobacco etch virus*	
Potato virus Y	*Potato virus Y*	
Tomato necrosis	*Alfalfa mosaic virus*	
Tomato spotted wilt	*Tomato spotted wilt virus*	
Tomato mosaic	*Pepino Mosaic Virus*	
	Nematodes	
Root-knot	*Meloidogyne spp.*	Control methods before planting; resistant varieties; crop rotation; alternate flooding and drying; soil solarization; methylbromidechloropicrin.
Sting	*Belonolaimus longicaudatus*	
Reniform	*Rotylenchus reniformis*	
root lesion	*Pratylenchus spp.*	
false root-knot x	*Pratylenchus spp.*	
potato cyst nematodes	*Globodera spp.*	
Stunt	*Tylenchorhynchus spp.*	

Source: Jones *et al*., 1991

The damage caused by tomato diseases has been significantly reduced over the years due to developments in chemical, biological and genetic control. These control methods are often used in conjunction with one another depending on cultivation methods (e.g. organic production); despite this, tomato diseases are still a major problem and significantly affect profit margins. The application of chemical compounds (e.g. fungicides and bactericides) can raise production costs hugely and their efficacy varies greatly between active compounds and causal agents. For instance, Song *et al*. (2004) tested seven fungicides for their inhibitory activities against *Fusarium oxysporum* resulting in a range of efficacies between 0% to 69.9% control. The overuse of chemical compounds pose potential risk to human health and the environment, and furthermore, they can lead to resistance in causal agents and thus new chemicals with new modes of actions are continuously being produced (Stammler *et al*., 2006). An alternative method to overcome new/evolved disease agents is to develop resistant cultivars (Section 1.1.4); however, resistance genes can also be overcome by further genetic adaptations of the pathogen (Watterson, 1986; Jones, 2008). An alternative to the laborious efforts involved in retaining good desirable fruit qualities of a crop but also the incorporation of disease resistance via tomato breeding is to graft a good fruit yielding crop (called the scion) onto disease resistant roots (named rootstock) (discussed further in Chapter 5).

The fourth control approach is to use biocontrol products, the benefits of which have been recognized for a long time. These products contain live 'beneficial' microorganisms or antigens that either directly affect the pathogen (Section 1.2.3) or are able to induce plant systemic resistance (Stewart *et al*., 2010). Biological control agents that induce plant systemic resistance either elicit a systemic acquired resistance (SAR) or an induced systemic resistance (ISR) response, which can be differentiated by the regulatory pathways involved and the elicitors required. SAR can be induced by virulent, avirulent and non-pathogenic organisms and is mediated by salicylic acid which leads to the production of pathogenesis-related (PR)

proteins such as chitinase and β-1,3-glucanase. ISR is elicited by plant growth-promoting rhizobacteria (PGPR) and endophytes and is regulated by jasmonate and ethylene resulting in the release of defensive compounds in the presence of a pathogen. Both plant systemic resistances induce a state of 'enhanced defensive capacity' resulting in a quicker and potentiated defence response (Vallad and Goodman, 2004).

1.2 THE ROOTS

Research into root systems is difficult not only because of the complexities in structure, and the physical and chemical exchanges that take place, but simply because of the difficulty in viewing them without significant disruption to their structure and habitat (Smit *et al*., 2000). Due to the opacity of most growth media, studies are often conducted on seedlings and small plants (predominantly *Arabidopsis thaliana*) in artificial conditions; thus such results should be interpreted with caution. There is growing evidence that there are developmental and functional differences of roots between species, habitats and young and mature root systems (Waisel and Eshel, 2002; Gregory, 2006).

The anatomies of roots are complex with the production of many types of root and variable structures along the length. However in all there are three types of tissue; dermal, ground and vascular tissue (Figure 1.2). Dermal tissue consists of the epidermis which forms a protective layer around the root, preventing water loss. In young roots the epidermis is composed of specialized absorbent epidermal cells with root hairs projecting to increase the absorbing surface area. Ground tissues contain the cortex and endodermis, the former of which contains three types of cells (Parenchyma, Collenchyma, and Sclerenchyma cells) that perform multiple roles (e.g. wound repair, gas exchange, storage, secretion and structural support); the latter forms the inner most layer of cortex, aiding the regulation of substances to and from the vascular tissues. Vascular tissues comprise of phloem and xylem tissues which are found in the centre of the root and are responsible for the

transport of water, minerals and organic nutrients through the plant (Gregory, 2006).

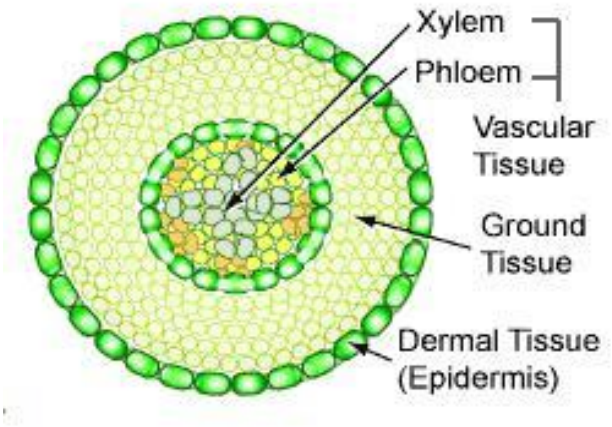

Figure 1.2: Illustration depicting a transverse cross-section of root, highlighting the three tissue types present (Waisel and Eshel, 2002).

1.2.1 Root system development and function

In the tomato (dicotyledonous) plant there are four root classes: Tap root, basal roots, lateral roots and shoot borne roots (Figure 1.3). The first root that emerges from a germinated seed is the tap root, which is also the term given to any root that supersedes a damaged original. Lateral root is the term given to any root branching from another. Lateral roots emerge from the tap root, and then one lateral root from another and so forth. It is the tap root and lateral roots that form the majority of the root system. Basal roots develop, which entail any root that emerges from the hypocotyl (organ between the tap root and base of the shoot). Shoot borne roots can arise from above ground stem tissue and are often formed in response to abiotic and biotic stresses (notably waterlogging) to replace stress damaged roots (Waisel and Eshel, 2002; Vidoz *et al*., 2010).

The root system serves several roles essential for crop success; one major function is to provide sufficient anchorage for the plant to remain upright and intercept sunlight. Formation of shoot borne roots, strengthening in basal areas and production of a fibrous lateral root system increase the stability of a plant. However, if too much branching of lateral roots occurs it can cause

certain media to 'root ball' resulting in overturning (Ennos, 2000). Another imperative function of the root system is to provide an adequate network that can utilize sufficient water and nutrient resources available in growth media. Water is essential for the survival of plants; not only does it provide turgidity and carry nutrients to and from the growth media, but it is also involved in most of the biochemical reactions that take place in the plant. Water is lost from the plant during carbon fixation of CO_2 and via evaporating surfaces, and as a consequence there is a regular demand for water from media (Drew, 1990; Gregory, 2006).

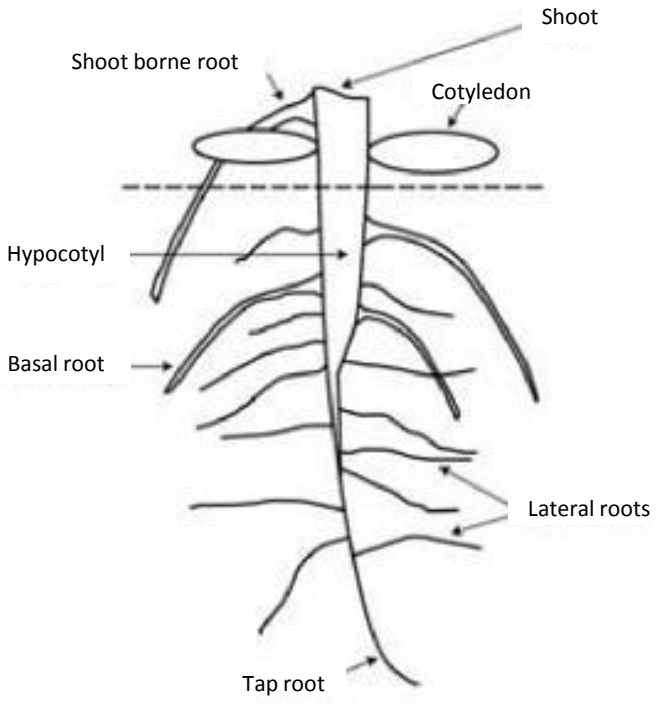

Figure 1.3: Illustration of generic dicotyledonous plant with root nomenclature (Gregory, 2006)

Certain nutrients are also in high demand and essential for healthy crops. The elements acknowledged to be crucial are the macronutrients; carbon (C), oxygen (O), hydrogen (H), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulphur (S) and the micronutrients; boron (B), copper (Cu), iron (Fe), chloride (Cl), manganese (Mn), molybdenum (Mo) and zinc (Zn). Most of these nutrients, with the exception of C and O, are acquired

from the growth media via numerous chemical exchanges to and from the root environment, often involving the microorganisms that coexist in the media (Adams, 1986).

In addition to the well established functions mentioned above, roots also have the ability to produce, store and secrete a vast array of compounds, collectively known as root exudates. These chemical compounds include the secretion of ions, free oxygen and water, enzymes, mucilage and carbon-containing primary and secondary metabolites (Bais *et al*., 2006). The functions of most root exudates have not been established; however there is evidence that certain compounds are involved in lubrication of media and in positive and negative interactions (via attractant and repellent compounds) with indigenous microbes and other plant species. The production and release of root exudates to the root environment is a significant carbon cost to the plant and levels excreted vary with the physiological state of the plant, nutrient availability and growth media (Bais *et al*., 2003; Bais *et al*., 2005). Root exudates are secreted to a narrow zone at the root-media interface termed the rhizosphere; this zone encompasses several component regions of various layers of the root cells that can be colonized by microorganisms (endorhizosphere), the root surface (rhizoplane) and the media directly surrounding the root containing root-associated microorganisms (ectorhizosphere). It is important to note that the boundaries of these component regions within the rhizosphere are not always distinct (Lynch, 1990).

1.2.2 The rhizosphere trinity: factors determining the rhizosphere environment

The word rhizosphere is coined from a form of Greek ‘rhiza’ meaning ‘root’, and ‘sphere’ which denotes ‘one’s field of influence’, this is a very apt term for defining this zone and aids in understanding why this region is so variable. Changes in the rhizosphere can be chemical, physical and biological and this ultimately affects its breadth of influence, particularly in the ectorhizosphere

which can range from <1-2mm from the root surface to >10-20mm, dependant on certain mobile nutrients and water (Gregory, 2006). The rhizosphere is formed by the roots of actively growing plants; as the roots pass through media releasing root exudates they activate the growth of indigenous microbes. It is suggested that there is an initial rapid microbial growth in this zone, reflected by the abundant substrate availability and colonization sites, followed by a slower growth phase and changes in microbial constituents dependent on a number of variables (Bennett and Lynch, 1981; Kent and Triplett, 2002).

The rhizosphere is the main source of microbial activity when compared to the rest of the bulk media and other regions of the plant, due to relatively conducive microbial growth conditions. Nutrient concentrations are much higher in the rhizosphere than other areas of the plant and growth media, with approximately 5% to 21% of all fixed carbon being secreted from plants as root exudates (Marschner, 1995). In addition, moisture levels are less variable in the rhizosphere, with root structure and root products providing physical shelter thus creating a relatively stable habitat for microbes, which in turn form a complex interacting community (Pinton and Varanini, 2001).

In the rhizosphere there are not only chemical and physical plant-microbe interactions but an interacting trinity of the media, the plant and root-associated microorganisms determining the total rhizosphere environment (Figure 1.4; Lynch, 1990). Media influences are often over looked; however, bulk media density and porosity will influence root development as it passes through the media, primarily affecting the roots ability to penetrate the media and to utilise water and nutrient stores (Wiersum, 1957; Bowen, 1981). Furthermore, factors such as aeration, water and nutrient availability are related to the porosity of the media; poor conditions affect root health and the microbial community, resulting in lower microbial diversity and reduction in positive plant-microbe interactions. A good structure for optimal plant health and a positive microbial community consists of a media that permits

free draining of roots, is aerobic and with a high available water capacity (Arshada and Coena, 1992).

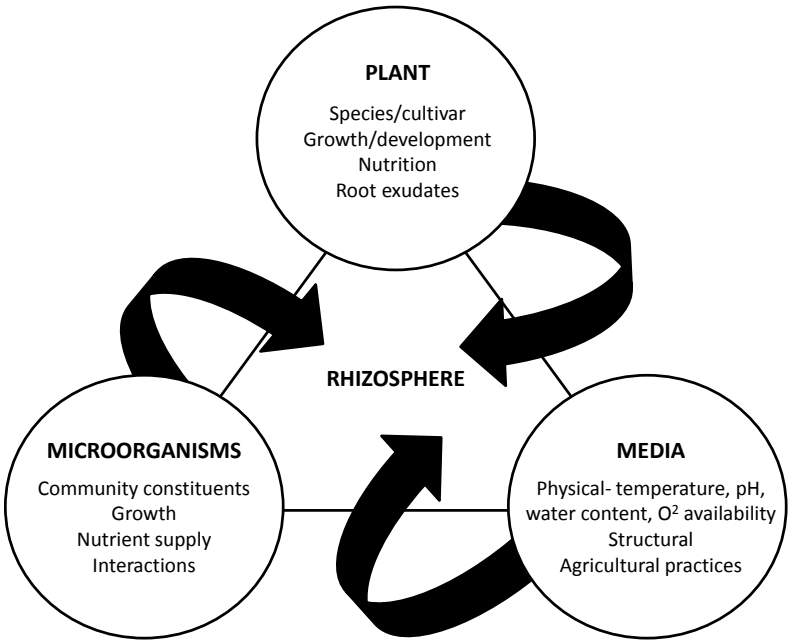

Figure 1.4: The rhizosphere trinity: the interacting factors that determine the environmental conditions of the rhizosphere (based on Lynch, 1990).

Yet it is the plant-microbe relationship that gains the most research interest where such interactions can be beneficial, harmful or neutral. However, these effects are often dependant on the media conditions and therefore media must be regarded as an important variable. In most instances the plant-microbe relationship is predominantly beneficial, where microorganisms can take advantage of the nutrients provided by the plant. In return, microorganisms are usually mutually beneficial by making complex, inaccessible nutrients available to the plant or by being antagonistic towards potential pathogens of the plant. Such knowledge of the ability of certain microorganisms to inhibit potential pathogens and to promote plant health has stimulated a plethora of research into desirable microbiota with the aim of improving crop yields with particular focus on the prevention of root disease (Pinton and Varanini, 2001; Kent and Triplett, 2002).

1.2.3 Root diseases of tomato and beneficial root-associated microbes

The future of a crop can often depend on the speed and accuracy of disease identification. In the case of root disease, it is frequently very hard to monitor and quantify the development of a pathogen due to the difficulty in viewing the roots. In many instances an infection problem may not become apparent until there are above ground symptoms such as wilting or reduced yield. As a consequence, the early stages of disease are commonly missed, allowing the pathogen to become well established and potentially more difficult to control. The frequently delayed response to root disease often results in greater economic loss than aerial disease and can result in greater yield loss (Blancard, 1994; Hawkes *et al*., 2007).

Soil grown crops and hydroponic soilless crops differ in their root disease problems, primarily due to different crop management practices involved in each method and also due to the effects of media on the rhizosphere environment. Fungal root diseases predominantly confront tomato producers with huge losses in production, with *Fusarium oxysporum* and *Verticillium dahlia* being particular problems in soil grown crops and *Collectotrichum coccodes* and numerous *Pythium* species (oomycetes) are prominent pathogens in soilless hydroponic systems. (Watterson, 1986; Blancard, 1994). Such root pathogens enter the root environment via a number of sources including seed-borne or contaminated transplants, soil-borne, plant debris, staff with contaminated hands or shoes, contaminated tools, water-borne and air-borne. Notably the likelihood of a pathogen entering the root environment via these sources can be greatly reduced by good crop management practices (Watterson, 1986).

Microbes present in the rhizosphere of a healthy plant primarily consist of saprophytic organisms or biotrophs which are considered to be generally beneficial to root health (Whipps, 2001). It is thought that certain levels of these organisms can help prevent or suppress root disease by a number of methods including:

- Direct antagonism: parasitism, extracellular enzyme production, surfactant production and release of antibiotics.
- Niche exclusion: by utilizing nutrients, colonization sites and other vital resources e.g. production of siderophores.
- Stimulation of systemic plant defence responses (section 1.1.4).

As a result, many of these microbes have been extensively researched for their potential biological control properties. Recently, interest has increased in biocontrol fuelled by public concerns over the use of chemicals in the environment, resulting is a higher demand for alternative methods (Whipps, 2001; Hawkes *et al*., 2007). Most interest resides in the development and application of specific biocontrol agents in the form of seed dressings or media amendments for the control of diseases on roots. Notably the rhizobacteria *Bacillus* species and *Pseudomonas* species have been used in a vast amount of papers; for example, Sharma *et al*. (2007) found that seed bacterization with several *Pseudomonas* sp. reduced pre-emergence of damping off in tomato by 60-70% and Nihorimbere *et al*. (2010) found that *Bacillus subtilis* was an effective biocontrol agent against fusarium disease with a disease reduction of 65 -70% after seed bacterization. Not only are these two biological agents antagonistic towards certain pathogens they also have positive effects on plant health and are collectively known as plant growth promoting rhizobacteria (PGPR) (Van Loon *et al*., 1998). PGPRs improve plant health by promoting plant growth and induction of plant systemic resistance (see section 1.1.4) (Shoresh *et al*., 2010).

Fungi are also used as biocontrol agents and can also induce plant systemic resistance, markedly species of *Trichoderma* have been examined and used successfully for these purposes (Sivan and Chet, 1989; Yedidia *et al*., 1999), primarily due to their ease of growth and wide host range (Whipps and Lumsden, 2001). Research into fungal biocontrol agents are on a par with bacterial biocontrol products. However, fungi have a greater potential than bacteria to grow and spread through media via hyphal growth often resulting

in more reliable and systemic control (Whipps, 2001). Despite this, most successful biocontrol products contain specific combinations of both fungal and bacterial biocontrol agents. For example, Akkopru and Demir (2005) found that suitable combinations of the arbuscular mycorrhizal fungus *Glomus intraradices* and other rhizobacteria reduced fusarium wilt in tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* by 8.6-58.6%.

It is important to note that most biocontrol products use culturable organisms for obvious reasons, namely they are easier to study and can be produced and stored on a mass scale via known culturing methods. However, it is estimated that only 0.1-10% of total microbial populations can be cultivated from many environmental communities (Forney *et al*., 2004; Ghazanfar *et al*., 2010) and the effect of unculturable organisms are just as important in determining the rhizosphere environment and consequently the probability of root disease. Due to continuous advances in culture-independent technology the vast diversity of unculturable organisms in microbial communities is becoming gradually clearer (Kent and Triplett, 2002).

1.3 MICROBIAL COMMUNITY ANALYSIS

The composition and diversity of microbial communities in natural environments have been of interest to scientists for many years; however they were hindered by an inability to characterize unculturable organisms. The advent of nucleic acid-based methods has been key in identifying numerically significant non-culturable organisms. From late 1980s onwards different culture-independent methods based on the analysis of total community DNA have been developed, giving microbial ecologists new tools to describe microbial communities in evermore depth (Pace *et al*., 1986; Giovannoni *et al*. 1988, Torsvik *et al*., 1990).

Traditional microbial community analysis relies on separating microbes from their habitat, followed by cultivation on artificial, often selective media; with metabolic, morphological, and physiological traits being used to determine their taxonomic classification. The use of culturing techniques has been

reported to vastly underestimate microbial diversity of many environments and cannot provide any information on the population sizes of the majority of species within a community (Hawksworth, 1991; Amann *et al*., 1995). Furthermore, the alteration of the original environment during cultivation, by necessity, modifies the original structure of the community through the imposition of new selective conditions thus providing an unrepresentative view of the natural population (Dunbar *et al*. 1997). In addition, classical methods of taxonomic classification are less revealing of evolutionary relationships among microorganisms than molecular structures and sequence analysis (Woese *et al*., 1990). From this it is clear that culture dependant methodologies are inadequate when looking to describe microbial community diversity of environmental samples and their phylogenetic relationships (Kent and Triplett, 2002).

None of the advances in molecular microbial ecology would have been possible without the revolutionary work of the Carl Woese and Norman Pace groups who initiated the use of molecular markers for inferring phylogenetic relationships and for characterizing microbial communities (Pace *et al*., 1986; Giovannoni *et al*. 1988; Woese *et al*., 1990). Their work revolved around comparing DNA sequences of ribosomal RNA (rRNA) molecules of microorganisms. However, such methods can be applied to any gene that is present in target organisms. Despite this, rDNA molecules are the most commonly used molecular markers for numerous procedures in molecular microbial ecology (Head *et al*., 1998).

1.3.1 The nature of rDNA molecules and their use to infer phylogenetic relationships

Ribosomal DNA molecules make excellent molecular markers due to their ubiquity and are in all cellular life forms, with 18S, 5.8S, 25S/28S subunits in Eukarya and 16S, 23S subunits in Prokarya (Figure 1.5). The rRNA genes are comprised of highly conserved sequence domains interspersed with more variable regions, which have independent rates of sequence change

dependant on their structural and functional conservation (Head *et al*., 1998). This sequence variation in rDNA can be used for inferring phylogenetic relationships among microorganisms, where the degree of sequence similarity between rDNA fragments from other organisms reflects phylogenetic distances. Furthermore, certain signature structures are unique for different *taxa* due to structural and functional conservation, again highlighting the useful nature of rDNA molecules as targets for phylogenetic analysis of microbial communities (Kent and Triplett, 2004).

The most common method used to compare rDNA sequences is to construct phylogenetic trees, which involve the alignment of sequences via their conserved regions, using different mathematical measures to ascertain phylogenetic groupings and relationships (Swofford and Olsen, 1990). However, a number of methods have been developed to exploit the sequence variation of rDNA molecules among *taxa*, most of which involve polymerase chain reactions (PCR) to amplify molecular markers (rDNA sequences) from total community DNA using 'universal' primers designed from conserved regions (Forney *et al*., 2004; Kent and Triplett, 2004).

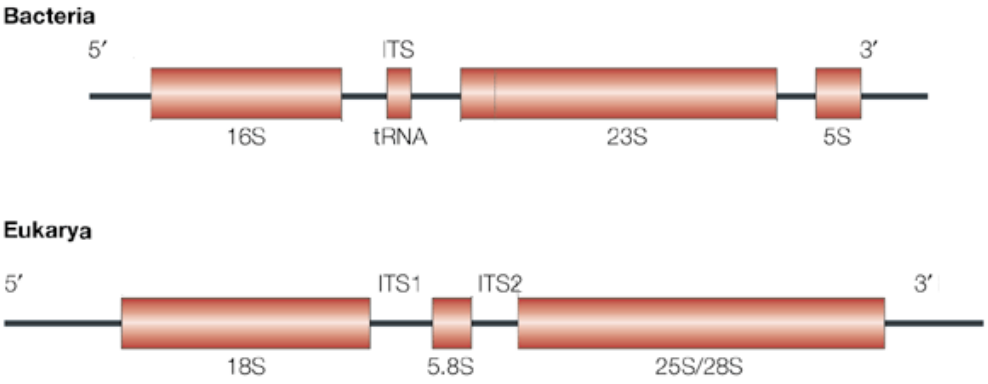

Figure 1.5: The ribosomal RNA (rRNA) molecules are ubiquitous in all cellular life forms. There are small rRNA subunits (16S in bacteria and archaea; 18S in eukaryotes) and large rRNA subunit (23S in bacteria and archaea; 25S/28S rRNA plus 5.8S rRNA in eukaryotes). The rRNA sequences are separated by one or more internal transcribed spacers (ITS). The 5S rRNA is present in most bacteria and archaea (Lafontaine and Tollervey, 2011).

1.3.2 PCR based methods of microbial community analysis

Initial attempts to determine microbial community compositions in environmental samples based on PCR amplification of marker genes, involved the construction and analysis of clone libraries (Forney *et al*., 2004). The construction involves DNA sequencing following the cloning of PCR products, producing a clone library of target gene amplicons. Identification of amplicons is achieved by sequence comparisons, such as Blast searches, or by phylogenetic analysis, such as the construction of phylogenetic trees. The latter is more precise particularly when performed for many sequences from the same environment and can lead to the discovery of novel phylogenetic groups (Liu and Jansson, 2010). However, this method is not well suited for the analysis of numerous samples because of the laborious, time consuming protocols and also the large expense associated with producing sufficient clone libraries. But, if a clone library of sufficient scale is created for a specific environment then new sequences can be compared to a sequence database generated from the clone library and phylogenetic analysis can be carried out (Forney *et al*., 2004).

To gain an understanding of changes in microbial community structure on temporal and spatial scales, high-throughput methods of analysis are required to allow the analysis of a sufficient numbers of samples, so that specific hypotheses can be statistically tested. A number of microbial community 'fingerprinting' techniques have been developed to allow the simultaneous analysis of many samples and provide a good compromise between information gained and number of samples processed, at relatively low costs. Fingerprinting techniques such as Denaturing Gradient Gel Electrophoresis (DGGE; Muyzer *et al*. 1993; Ercolini, 2004) and Temperature Gradient Gel Electrophoresis (TGGE; Heuer and Smalla, 1997) give complex community profiles which allow comparisons of community composition but do not directly give the taxonomic composition. Both methods involve the analysis of electrophoretic profiles and the differences between samples reflect differences in microbial community constituents and their relative abundance.

However, these methods are hindered by insufficient methods of quantifying the results and as a consequence it is difficult to compare data from differing studies (Forney *et al*., 2004)

Alternative methods have been developed to improve the detection and resolution of early fingerprinting analysis such as Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Fisher and Triplett, 1999; Ranjard *et al*., 2001) and Terminal Restriction Fragment Length Polymorphisms (T-RFLP; Liu *et al*., 1997; Osborne *et al*., 2000; Kitts, 2001). These techniques involve the separation of PCR products by high resolution gel electrophoresis on automated sequencers, following amplification with fluorescently labelled oligonucleotide primers. The combination of the use of automated sequencers and fluorescence detection increases the number of fragments detected compared to standard gel electrophoresis. Furthermore, band intensity can be measured more precisely by fluorescence detection, allowing more accurate comparisons of community profiles. Notably T-RFLP is the only fingerprinting method that infers taxonomic identity without further sequencing of the fragments by comparing and matching observed T-RFs with an *in silico* database of known taxa and their corresponding putative T-RFs (Kent and Triplett, 2004).

T-RFLP analysis is consequently one of the most used molecular fingerprinting methods. This method has been used to study communities of different organisms such as bacteria (Dunbar *et al*., 2001; Mceniry *et al*., 2008), fungi (Dickie and Fitzjohn, 2007; Bennett *et al*., 2008), archea (Wu *et al*., 2006; Stres *et al*., 2008) and to understand interaction between different organisms (Carletto *et al*., 2008; Edel-Hermann *et al*., 2008). The T-RFLP analysis of rRNA genes 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 (T-RFs) of differing lengths are generated relating to their rDNA sequences and consequently their phylogenetic identity. T-RFs are then separated by high resolution gel electrophoresis on automated sequencers, which record the

fragment length and relative abundance. The resulting data are easy to analyse as they 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 culture independent methods for rapidly comparing microbial communities from environmental samples (Marsh, 1999).

1.3.3 Biases associated with PCR based methods and limitations to molecular microbial ecology

PCR based community analyses have a number of steps that may introduce biases that must be recognized and minimized where possible. The introduction of biases starts with the extraction of community DNA, where extraction methods must optimize lysis of structurally different cells and take into account the possible coextraction of humic substances from soil which can interfere with PCR. It has been shown that methods that include mechanical lysis using a bead beater coinciding with freezing and thawing yield the most consistent results (Moré *et al*., 1994). Furthermore, it has been found that commercial extraction and clean up kits used on environmental samples provide the broadest spectrum of organisms with good recovery of PCR yields and less PCR inhibition compared to chemical and enzymatic lysis without clean up steps (Niemi *et al*., 2001).

In addition there are biases that may occur during the PCR step. These biases may originate from preferential annealing of universal primers to certain *taxa* as even the sequences of conserved regions of rRNA genes are divergent (Suzuki and Giovannoni, 1996). In addition, the copy number of rRNA genes present within the genomes of different organisms varies greatly (Cole and Girons, 1994; Bellemain *et al*., 2010) which can result in skewed relative abundance data. However, all of the biases mentioned can be minimized if microbial ecologists acknowledge them and take steps to reduce their effects. For example, Fernández *et al*. (1999) found that the study of relative changes

in the same ecosystem and the production of replicate community profile minimized the effects of molecular microbial methods.

PCR based methods struggle to describe the vast microbial diversity of certain environments; *taxa* that comprise less than 1% of the total community are usually not represented, but could be present in high numbers and affect ecosystem function (Head *et al*., 1998). Furthermore, using these methods allows dominant taxa within a community to be identified but not their ecological significance as there are no known methods capable of assessing the functional roles of thousands of organisms in environmental samples over time and space. In addition, when changes are observed it is not known whether these changes affect ecosystem function. While these methods have limitations and provide an incomplete and occasionally distorted view, it is better than no view and is indeed a step closer to understanding the complexities of microbial ecosystems (Forney *et al*., 2004). Kent and Triplett (2002) aptly describe this period of study as 'the descriptive phase' and define it as a necessary step before a theoretical 'testing phase' where the role and function of millions of organisms in many ecological niches will be discovered. Notably the dawn of the testing phase has already begun with huge advances in sequencing technologies referred to as 'next generation sequencing' or metagenomic technologies, which enable analysis of the links between phylogenies created from rRNA genes and functional genes, consequently facilitating the identification of microorganisms performing particular ecosystem functions (Metzker, 2010).

1.4 AIMS AND OBJECTIVES OF THIS STUDY AND THESIS OVERVIEW

The overall aim of this research is to study the microbial ecology of the tomato rhizosphere and to examine the relationship between microbial community structure and root health. To facilitate the core aim, high throughput molecular methods and classical methods were employed to monitor eukaryotic and prokaryotic communities present in the rhizosphere of both commercial and experimental tomato crops.

Preliminary objectives to obtain consistent and robust data were to:

1. Standardize root sampling methods to ensure reliable coverage of rhizosphere microorganisms.
2. Optimize the molecular fingerprinting method Terminal Restriction Fragment Length Polymorphism (T-RFLP) to identify the presence and relative abundance of microbial constituents of the tomato rhizosphere.
3. Validate molecular experimental procedures by comparisons with classical methods (plating and microscopy) and by identifying causal agents of diseased crops.

Experiments were then carried out to:

4. Determine the effects of growth media, plant age, organic disease control methods and active and passive water purification treatments on rhizosphere microbial communities, plant health and disease occurrence.

An outline of the chapters within this thesis is stated below:

Chapter 2: GENERAL MATERIALS AND METHODS. In this chapter the general materials and basic experimental procedures used in this study are described.

Chapter 3: OPTIMIZATION AND VALIDATION OF ROOT SAMPLING AND T-RFLP ANALYSIS METHODS FOR THE EXAMINATION OF TOMATO RHIZOSPHERE MICROORGANISMS. The root sampling methods used throughout this study were optimized to ensure reliable coverage of rhizosphere microorganisms. Furthermore, the molecular fingerprinting method Terminal Restriction Fragment Length Polymorphism (T-RFLP) was optimized to identify the presence and relative abundance of microbial constituents of the tomato rhizosphere. T-RFLP protocols were validated by comparisons with classical methods and ability to detect causal agents of root diseased crops.

Chapter 4: EFFECT OF GROWTH MEDIA ON RHIZOSPHERE MICROBIAL COMMUNITIES, ROOT HEALTH AND PLANT SURVIVAL. T-RFLP was used to study the effect of media on microbial communities of the tomato rhizosphere throughout the growing season. Routine root sample analysis took place over 2 growing seasons on commercial crops with 2 crops each growing in soil, in NFT solution, on rockwool slabs, coir slabs and woodfibre slabs, taken on three occasions per growing season. In addition, these samples were analysed by pyrosequencing to further characterize the microbial ecology of the tomato rhizosphere. Root health and plant survival assessments took place at the end of each season.

Chapter 5: EFFECT OF SOIL AMENDENTS AND ROOTSTOCK VARIETY ON ORGANIC TOMATO RHIZOSPHERE MICROBIAL COMMUNITIES, ROOT HEALTH AND PLANT HEALTH. Two one-year trials took place at an organic nursery in glasshouses with a history of root disease problems. The trials aimed to determine the effect of some biological amendments and some commonly used rootstocks on tomato root health, plant health and microbial populations associated with roots.

Chapter 6: EFFECT OF RECYCLED NUTRIENT SOLUTION WATER PURIFICATION TREATMENTS ON TOMATO RHIZOSPHERE MICROBIAL COMMUNITIES AND ROOT PATHOGENS. T-RFLP was used to study the effect of two different water purification treatments, namely ultraviolet (UV) irradiation and a slow sand filter (SSF), used to treat recirculated water in NFT systems, on rhizosphere microbial populations and root pathogens.

Chapter 7: SUMMARY AND FINAL CONCLUSIONS. In this chapter all results are discussed together. This chapter is followed by the reference list related to all chapters and appendices of chapter 3 and 4.

2 GENERAL MATERIALS AND METHODS

The molecular fingerprinting method Terminal Restriction Fragment Length Polymorphism (T-RFLP) was optimized to identify the presence and relative abundance of microbial constituents of the tomato rhizosphere (see Chapter 3). To characterize and monitor changes in the microbial community of the tomato root environment a number of experiments were conducted on root samples from commercial nurseries and experimental Nutrient Film Technique (NFT) systems. In this chapter the general materials and basic experimental procedures used in this study are described.

2.1 COMMERCIAL NURSERIES

Numerous experimental trials took place at six commercial nurseries in the UK to characterize microbial communities of the tomato rhizosphere as well as examine the effect of growth medium, biological amendments, rootstocks and disease occurrence on the microbial population.

2.1.1 Growth media

T-RFLP was used to study the effect of crop growth media on microbial communities of the tomato rhizosphere throughout the growing season. Routine root sample analysis occurred over two growing seasons on 10 commercial crops with two crops each growing in soil, in NFT solution, on rockwool slabs, coir slabs and woodfibre slabs. Although it was not possible to use a common tomato variety at all sites, the range of varieties used was kept to a minimum. Root samples from each crop were taken on three occasions per growing season: at 2-4 weeks after rooting into the growing medium (early), around first pick (mid) and in peak production (late). At each visit, root samples were collected from three plants in one row. Each sample was split into three sub-samples in the laboratory to provide nine microbial population profiles. Sampled plants were labelled and adjacent plants in the same row were used at sequential visits. All crops were grown to the commercial

standards according to normal practice of the host nursery. The experiment was a factorial design with two factors (growing medium and sample timing) at five levels (rockwool, soil, NFT, coir and woodfibre) and three levels (early, mid, late season) respectively. Further crop details are discussed in Chapter 4 section 4.2.

2.1.2 Biological amendments and rootstocks

Two one-year trials took place at an organic nursery in glasshouses with a history of root disease problems, continuous cultivation of tomato crops in soil and no soil disinfestation treatment between crops. The trials aimed to determine the effect of some biological amendments and some commonly used rootstocks on tomato root health, plant survival and microbial populations associated with roots.

The soil amendment trial took place in a glasshouse where organic tomatoes had been grown for five consecutive years. Poor growth and plant death caused by a range of fungal root pathogens had become an increasing problem over successive years, even with plants grown on resistant rootstocks.

Soil was amended with five different amendments (Table 2.1) in winter 2009 prior to planting cv. Piccolo on Beaufort rootstock in February 2010. There were also plots with no amendments added to the soil acting as untreated controls. The crop was grown to commercial standards according to normal practice of the host nursery.

The rootstock trial was done using an organic tomato crop cv. Roterno grafted to six different rootstocks with different resistances (Table 2.2). Organic tomatoes had been grown in the house for at least 10 years. The experiment was located in an area where leaf yellowing and poor growth occurred in 2009. The crop was grown according to normal nursery practice which included incorporation of green waste compost prior to planting and monthly drench treatment with PHC Compete Plus and Colonise AG in alternation (Table 2.1). The crop was planted in February 2010.

Table 2.1: Soil amendments and their application methods used in the biological amendment trials on an organically grown tomato crop.

Treatments	Application
Untreated	N/A
PHC Compete Plus	Applied at 0.23 g/pot in alternation with PHC Colonize AG at 0.23 g/pot in 340 ml water/pot at monthly intervals. PHC Compete Plus was also applied in propagation.
Trianum-P	Applied in propagation at 1.5 g/m^2 in 2.5-5 litres water immediately after sowing, and at 15 ml/1000 plants (0.088 ml/L) in 340 ml/pot 1 week after planting and again 1 month later.
Composted green waste	Applied at 25 kg/linear m of bed and incorporated to around 23 cm depth at 1 month before planting.
Composted Fine Bark	Applied at 0.345 m^3/m^2 and incorporated as above (i.e. 1 part bark to 3 parts soil by volume).
Biofence pellets	Applied at 250 g/m^2, incorporated to 23 cm depth as above, watered in and covered with polythene.

Table 2.2: Rootstocks and their resistances used in the rootstock trial on organically grown tomato crops.

Rootstocks	Resistances
Beaufort	HR: ToMV/Fol/For/Pl/Va/Vd/Ma/Mi/Mj
Efialto	HR: ToMV/Ff/Va/Vd/ Fol /For; IR: Ma/Mi/Mj
Emperador	HR: ToMV/Fol/For/Pl/Va/Vd/Mi/Mj/Ma
Optifort	HR: ToMV/Fol/For/Pl/Va/Vd/Ma/Mi/Mj
Stallone	HR: ToMV/Fol/For/Pl/Va/Vd
Unifort	HR: T0MV/ Ff/Fol/For/Va/Vd/Ma/Mi/Mj

HR- high resistance/ IR-intermediate resistance/ ToMV-Tomato Mosaic Virus/ Fol- *Fusarium oxysporum* f. sp. *lycopersici*/ For- *Fusarium oxysporum* f. sp. *radicis-lycopersici*/ Pl - *Pyrenochaeta lycopersici*/ Ff - *Cladosporium fulvum*/ Va- *Verticillium albo-atrum*/ Vd- *Verticillium dahliae*/ Ma- *Meloidogyne arenaria*/ Mi- *Meloidogyne incognita*/ Mj- *Meloidogyne javanlca*.

Both trials were randomised block designs with six fold replication. There were six blocks containing six plots. Individual plots consisted of an island bed of 18 planting pots (36 plants) spaced at 50 cm (plot dimension was 9.5 m x 0.8 m). The six plots in a block were arranged along one row. The six blocks

were arranged in adjacent bays of crop. Two heads were taken per plant to give a density of 4/m^2. Root samples from each plot (36 samples) were collected on three occasions per growing season; 5 weeks after rooting into soil, around first pick and in peak production. Further experimental procedures are discussed in Chapter 5 section 5.2.

2.1.3 Disease occurrence

Root samples were taken from various commercial crops as opportunities for specific disease symptoms versus healthy root comparisons arose during 2009. Comparisons had to be from a single uniform crop grown in identical conditions from the same glasshouse. Details of the crops sampled and disease symptoms examined are given in Table 2.3. The aim of this investigation was to establish whether the optimized molecular method T-RFLP could identify the causal agent for an unhealthy crop.

Each comparison consisted of a single factor occurring at two levels (disease roots/healthy roots). For each paired comparison, three samples of each level were collected on one occasion, usually from plants in the same crop row. Three sub-samples from each were examined by T-RFLP giving nine T-RFLP profiles per factor. Further sample details are discussed in Chapter 3.2.

Table 2.3: Details of crops sampled for comparison of the effect of disease symptoms on populations of microorganisms associated with tomato roots.

Disease symptoms vs. healthy comparison	Growth media
Root mat roots vs. healthy (at first symptom)	Rockwool
Brown roots vs. healthy (at first symptom)	Rockwool
Yellowing leaves vs. healthy (when obvious)	Soil

2.2 EXPERIMENTAL NFT SYSTEMS

Two duplicate recirculating NFT systems (Figure 2.1) were established in a glasshouse (Sutton Bonington Campus, University of Nottingham). Each NFT system consisted of three rows of polyvinyl chloride (PVC) channels kept at an

inclination of 1.5° to allow the nutrient solution to return to a 130 L tank by gravity before being recirculated by a pump. The flow of irrigation water through the channels was regulated at 2 L/min. Irrigation water was obtained from nutrient stock solution (1, VITAX, Leicester, UK, Table 2.4) added to freshwater.

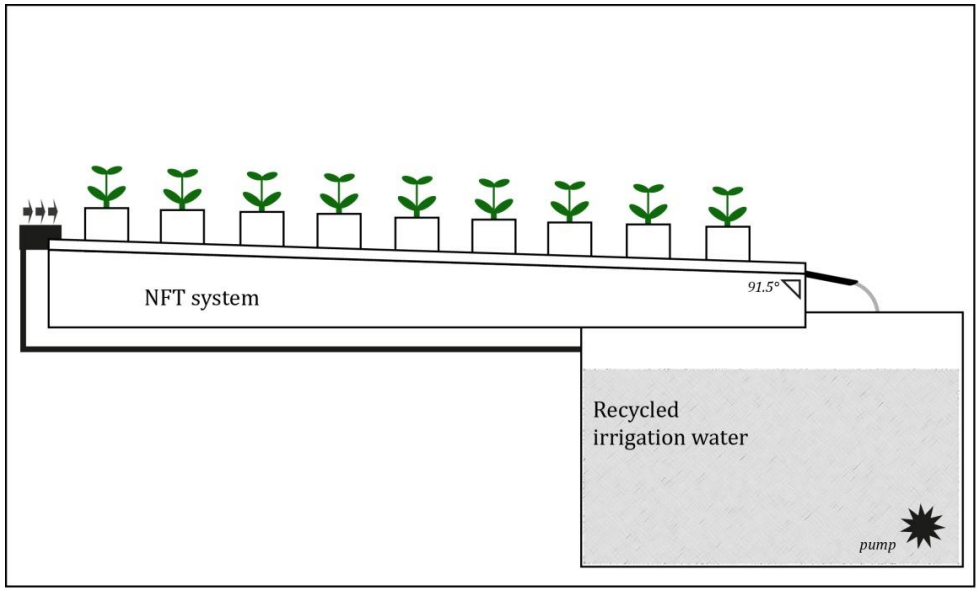

Figure 2.1: A schematic of the experimental NFT system. Recycled irrigation water was directed to the top of the channels by a pump. Arrows illustrate the direction of recycled irrigation water (Cafà, 2012).

A slow sand filter (SSF) was connected to one of the two experimental NFT systems (Figure 2.2). In this SSF NFT system the water was directed by the pump in two directions: half of the irrigation water was pumped to the top of the PVC channels whilst the other half was sent to the top of the sand filter and directed through the sand of the filter.

The filter was prepared with 2 m of 20 cm diameter Terrain PVC pipe (Geberit, Aylesford, Kent, UK) mounted vertically. The bottom of the filter was filled with a 30 cm depth of gravel. The column was constructed with a sand depth of 1 m and a 60 cm deep head of water above it. The water flow through the column was gravity assisted with an outflow of water regulated at a speed of 4 L/h by a valve (Calvo-Bado *et al.*, 2003). An overflow pipe was used to maintain a constant water level above the sand column.

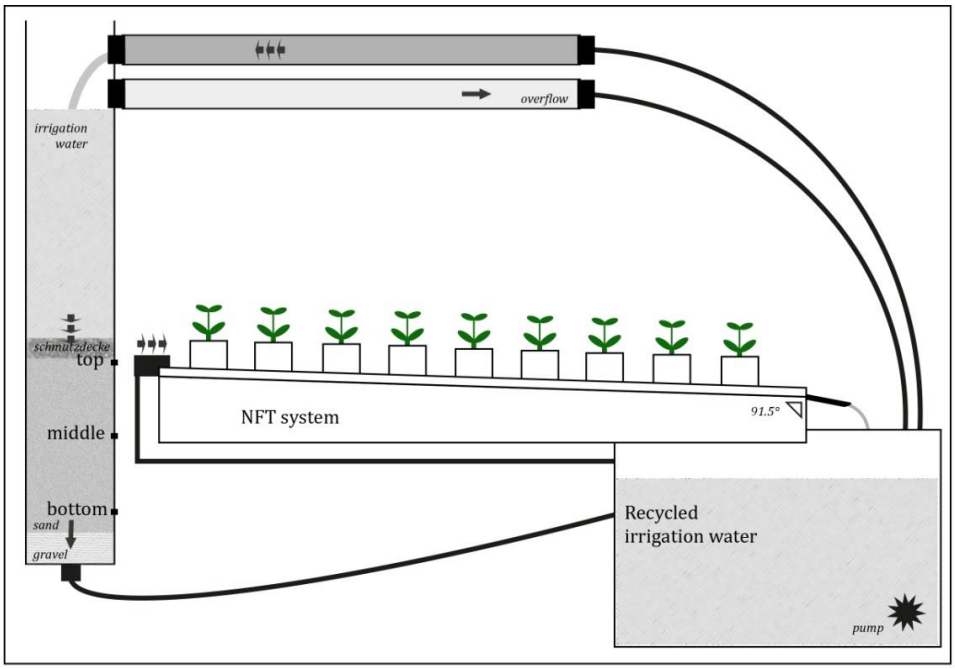

Figure 2.2: A schematic of the experimental NFT system connected to the slow sand filter. Recycled irrigation water was directed to the top of the column of the sand filter and to the top of channels by a pump. Arrows show the direction of recycled irrigation water (Cafà, 2012).

In each experiment 60 tomato plants of cv. Alicante were grown from seed in an incubator on rockwool plugs for 14 days and then transferred to rockwool cubes before being placed into NFT systems (10 plants per PVC channel), with 100 L of irrigation water placed in each tank. All experiments were carried out for four weeks during which root samples were collected every 14 days. These short experiments allowed for the investigation of the SSF on tomato rhizosphere microbial communities and its effect on the occurrence of root disease. Further details of these experiments are discussed in Chapter 6.

Table 2.4: Chemical composition of 100 g of nutrient stock solution VITAFEED 214.

VITAFEED 214	in 100 g (g)
Nitrogen	16
Phosphorous	8
Potassium	32
Boron	0.013
Copper	0.025
Iron	0.05
Manganese	0.025

2.3 ROOT SAMPLE COLLECTION

Thin or young roots were targeted in all sample collections. It has been established that young roots are more likely to be the site of pathogen entry due to *higher levels of root exudates* and root abrasions caused by active growth (Olivain *et al*., 2006). Young roots are subsequently the most probable location to allow the early detection of root disease. To optimize the recovery of young roots and to minimise plant death, samples were taken from mid-way between plants (soil and NFT grown crops) or from slab corners (woodfibre, rockwool and coir grown crops). Root samples from commercial nurseries were collected from three locations from a uniform crop and then each sample divided into three technical replicates. A minimum of 2 g root fresh weight were collected from each plant. Samples were posted directly to the University of Nottingham and processed immediately on arrival. Root samples from experimental NFT systems were collected from each of the three rows per system and each sample divided into three technical replicates.

2.4 ROOT SAMPLE RECOVERY

Soil crops: Larger roots were picked out from soil using sterile tweezers. To obtain smaller roots samples were placed in Petri dishes and bathed in sterile distilled water (SDW) to release them from the soil.

Rockwool, woodfibre and coir crops: Media fibres were teased apart from each bulk sample using sterile tweezers and roots were picked out.

NFT crops: samples only consisted of roots.

2.5 DNA EXTRACTION

Root samples (≥100 mg) were roughly chopped using a 10A sterile scalpel blade (Swann Morton, Sheffield, UK). Samples were placed in a 2.0 mL graduated skirted NAT tube (Starlab, Ahrensburg, Germany) containing 10 acid washed 1 mm glass beads (Sigma-Aldrich, Haverhill, UK). Root tissue was

disrupted by vigorous shaking in a Fastprep (QBiogene, Cambridge, UK) for 3 cycles of 45 seconds at 6.5 m s^{-1}. Soil grown crop DNA was extracted using the PowerSoil DNA kit (Mo-Bio Laboratories Inc., Carlsbad, California, USA) following the manufacturers protocols. All other media grown crop DNA was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Crawley, UK), following the manufacturers protocols. In instances where there was PCR inhibition, extracts were cleaned using polyvinylpolypyrrolidone (PVPP; Cullen and Hirsch, 1998). DNA extracts were stored at -20^{o}C prior to PCR amplification.

2.6 PCR AMPLIFICATION OF RIBOSOMAL DNA

One µL of DNA extract from root samples was used in PCR amplification of the 23S ribosomal subunit for bacteria and the ITS-2 region for Eukarya with primer pairs shown in table 2.5.

Table 2.5: Primer pairs used in this study.

Target Gene	Primer	Sequence 5'→3'	Tm	Reference
Prokaryotic	23sfor	GCGATTTCYGAAYGGGGRAACCC	59	Anthony *et al*., 2000
23S rDNA	23srev	TTCGCCTTTCCCTCACGGTACT		
Eukaryotic	ITS3for	GCATCGATGAAGAACGCAGC	53	White *et al*., 1990
ITS-2 region	3126Trev	ATATGCTTAAGTTCAGCGGGT		Ranjard *et al*., 2001

Tm- annealing temperature (^{o}C); Y- is C or T; R- is A or G

Amplifications were performed in 25 µL reactions containing 12.5 µL of 2xPCR master mix (Promega, Southampton, UK), 10.5 µL of SDW and 0.5 pmol of each primer in a Techne Progene thermal cycler (Techne, Cambridge, UK). PCR conditions were as follows with specific annealing temperatures for primer pairs (Table 2.1): 94^{o}C for 2 min, followed by 30 cycles of 94^{o}C for 0.5 min, annealing for 1 min and 72^{o}C for 1 min, completed with a final extension step of 72^{o}C for 10 min. Amplification of ribosomal regions was confirmed by running PCR products (2 µL) on a 1% agarose gel in 1X TBE buffer and

ethidium bromide (0.5 µg/mL). Gels were run for 1 hr at 90V and viewed under UV light.

When PCR reactions were performed for T-RFLP the reverse primers were fluorescently labeled; 23Srev was labeled with D4 Beckman WellRED dye (Sigma Proligo) and 3126Trev was labeled with D3 Beckman WellRED dye (Sigma Proligo). Both fluorescent labels are suitable for analysis on the CEQ8000 fragment analysis system (Beckman-Coulter, High Wycombe, UK).

2.7 RESTRICTION DIGEST

PCR products for T-RFLP analysis were digested with two restriction enzymes to increase the resolution of closely related organism; *Mse*I and *Hae*III or *Hae*III and *Alu*I (New England Biolabs, Hitchin, UK) for 23S rDNA and ITS-2 rDNA respectively (Table 2.6). Two different restriction enzymes are used to produce two distinct, although sometimes matching, terminal restriction fragments (T-RFs) for each amplicon generated by PCR amplification. The combination of pairs of T-RFs was used for the identification of microorganisms. Five µL of PCR product was used in a 10 µL reaction volume containing 1U of restriction enzyme. Digests were incubated at 37^{o}C for 2 hours followed by denaturation of enzymes by heating to 80^{o}C for 20 min. Digestion products were verified by gel electrophoresis of aliquots of digestion mixture (3 µL) in 1% of agarose in 1X TBE buffer and ethidium bromide (0.5 µg/mL). Gels were run for 1 hr at 90V and then viewed under UV light.

Table 2.6: Restriction enzymes used in this study with corresponding recognition site.

Enzyme	Recognition site (5'→3')
Alu I	AG▼CT
*Hae*III	GG▼CC
*Mse*I	T▼TAA

2.8 T-RFLP ANALYSIS

Restriction digests were mixed at a 2:1 ratio of ITS-2 rDNA and 23S rDNA digests respectively due to differences in signal strength of the two wellRED dyes. Three µL of the digest mix 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 (Beckman-Coulter). The Samples were overlaid with mineral oil and separated by electrophoresis on a CEQ 8000 DNA analysis system (Beckman-Coulter).

After electrophoresis, the length and signal intensity (peak height) of fluorescently labelled fragments were determined by comparison with internal standards using the Fragment Analysis Module of the Genetic Analysis System v. 8.0 (CEQ™ 8000; Beckman Coulter Inc.). Fragments with fluorescence >1% of the total fluorescence and length between 50 bp and 700 bp were considered for analysis. T-RFs that differed by <0.5 bp in size between replicated profiles were considered identical and only T-RFs that occurred in at least two of the three technical replicates were included in the analyses (Dunbar *et al*., 2001). Analysis parameters were set to a quartic calibration curve, PA ver.1 dye mobility calibration. T-RFLP profiles were checked for stable current and baselines, and were repeated if the size calibration correlation coefficient was <0.999 or size calibration standard deviation was >0.75 nt (Bennett *et al*., 2008).

T-RFLP datasets were normalized by dividing each peak height value by the sum of the total peak height value within the same profile. Normalization of data removes differences in sample loading that would result in differences in the overall profile intensity among samples (Hartmann *et al*., 2005). From this analysis *n* x *m* matrices were produced with rows (*n*) containing samples and columns (*m*) containing T-RF lengths (bp). Information from these matrices were used to identify putative taxonomic units and for statistical analysis (Hartmann and Widmer, 2008).

Putative taxonomic identities of T-RFs were assigned by importing T-RFLP profile information into FRAGSORT version 5.0, a computer sorting tool, which compares experimental results with assigned primers and enzymes to a defined database (Michel and Sciarini, 2003).

2.9 STATISTICAL ANALYSIS OF T-RFLP DATA

A number of statistical tests were performed on T-RFLP data, which primarily comprised of a three step approach; testing the null hypothesis by Analysis of Similarities (ANOSIM), representing the data in an ordinational space with Principal Component Analysis (PCA) and describing the α-diversity (diversity of species found within a site) and β-diversity (difference in species composition between sites) of the samples using species richness and diversity indices (Shannon diversity index, Simpson diversity index, Species richness; Formula in Table 2.8)

2.9.1 ANalysis Of SIMilarities - ANOSIM

ANOSIM (Clarke, 1993) was performed using the software PAST (PAlaeontological STatistics, ver.1.12; Hammer *et al*., 2004) to statistically test the null hypothesis (H_0) of difference between groups of T-RFLP datasets (Klaus *et al*., 2005; Ramette, 2007). Normalized datasets (Section 2.8) were transformed into Bray-Curtis dissimilarity matrices (Bray and Curtis, 1957), from which either one-way or two-way ANOSIM tests were conducted with the significance test obtained by 9999 permutations.

The Bray-Curtis dissimilarity equation was used to calculate a distance matrix (*n x n* matrix) of correspondence between samples, transforming normalized data into ranks. The values of ranks in the distance matrix range between 0-1, where 0 means the two samples have the same composition and 1 means the two samples do not share any species.

ANOSIM (Equation 2.1) is based on the rank similarities between samples in distance matrices and reports an R statistic which can range from -1 to 1 and

indicates the level of separation. An R value of 0 indicates the null hypothesis is true. An R statistic greater than 0 indicates objects are more dissimilar between groups than within groups. R values >0.75 are commonly interpreted as well separated, >0.5 as separated, but overlapping, and <0.25 as barely separated (Clarke and Gorley, 2001). A *p* value indicating level of significance is also produced for the analysis based on 9999 permutations (randomization) of all ranks. The null hypothesis that there were no significant differences between groups was rejected when the significance level, *p*, was <0.05.

$$R = \frac{\bar{r}_b - \bar{r}_w}{\frac{1}{4}\left[n\,(n-1)\right]}$$

r_b = mean rank of between group dissimilarities
r_w = mean rank of within group dissimilarities
n = total number of objects

Equation 2.1: ANOSIM Formula

2.9.2 Principal component analysis - PCA

The variation of bacterial and eukaryotic microbial communities in both commercial nurseries and the experimental Nutrient Film Technique (NFT) system experiments were analysed by Principal Component Analysis (PCA) based on variance-covariance matrices (Ramette, 2007; Culman *et al*., 2008). Glimm *et al*. (1997) suggest the variance-covariance matrix does not sacrifice data within large multivariate datasets, such as those produced from T-RFLP analysis. PCA calculations were carried out on normalized T-RFLP datasets (section 2.8), with each T-RF considered as a different variable, using GenStat 14th edition (Payne *et al*., 2011).

This statistical method reduces the dimensionality of the data, from which the mean principal component (PCs) scores are then plotted in two dimensions. PCA procedure calculates a new set of synthetic variables (PCs) that

correspond to linearly independent combinations of the original variables (T-RFs). The aim is to represent the objects (the samples) and the variables (the T-RFs) of the dataset into a new system of coordinates (normally the first and the second PCs). The new variables describe as much of the variance in the data as possible with as few variables as possible (Ramette, 2007; Culman *et al*., 2008). The first principal component, the first new variable, normally describes the largest amount of variation in the data. The second principal component, which is orthogonal to the first principal component, takes into consideration the second largest amount of variation. PCs were further analysed by ANOVA to determine if factors under examination are significant factors for groupings in PC1 and PC2.

When the new system of coordinates is created, the original descriptors (the T-RFs) assume values (loadings) that relate them to the principal components (Legendre and Legendre, 1998). Loadings (also called latent roots or eigenvalues) represent the influence of each original variable on the new system of coordinates, that is the sum of the squared loadings is equal to 1 (Abdi and Williams, 2010). Loadings were analyzed by defining a threshold of significance of their values. Pio *et al*. (1996) estimated that loading values <-0.25 or >0.25 have a significant effect on the total variance of the system under statistical analysis. Once significant loadings were established, statistically significant T-RFs could be identified and differences in the relative abundance between samples could be analyzed.

2.9.3 Species richness and diversity indices

Species richness and diversity are commonly used in microbial ecology to compare microbial communities between sites, over time, and under different treatments. It has been stated that in typical environmental samples there are usually many *taxa* or species present in low numbers and few *taxa* or species present in high abundance. As a result, there are two descriptors necessary to define microbial community assemblages: the number of species present (species richness) and relative abundance of each species present (species

evenness); mathematical approaches that account for both these descriptors are termed diversity indices (Legendre and Legendre, 1998). In this study species richness was interpreted by the number of T-RFs produced. Furthermore, Simpson's Diversity Index (Simpson, 1949) and the Shannon Diversity Index (Shannon, 1948) were used to determine and compare microbial community diversity of T-FRLP datasets (Table 2.7). The Simpson diversity index values range between 0 and 1, the greater the value, the greater the sample diversity. The Shannon Diversity index values are between 0 and 5, where values above 3 indicate stable community assemblages and values under 1 indicate poor diversity. ANOVA was used to test whether diversity scores between treatments were significantly different. p-values <0.05 were considered to be significant.

Several diversity indices were chosen to increase the accuracy of the analysis when comparing estimations between treatments. As diversity indices represent a theoretical estimation of complex assemblages into a single value and as a result the information they provide can be limiting and incomplete for a single observation, but, they are useful tools for comparing microbial communities between sites, over time, and under different treatments.

2.10 PCR PURIFICATION

In an attempt to identify unknown T-RFs PCR products containing a relatively high abundance of the unknown T-RF were purified and cloned. PCR products were purified with the QIAquick PCR purification kit (Qiagen) following the manufacturers protocols and quantified on a Nanodrop spectrophotometer.

2.11 LIGATION AND CLONING

Approximately 50 ng of purified PCR product was used for ligation reactions using the pGEM cloning kit pGEM-T Easy Vector Systems (Promega), following the recommendations of the manufacturer. Promega *Escherichia coli* JM109 cells were transformed according to the manufacturer's protocol, using the plasmids obtained from the ligation reactions.

Transformed cells were incubated at 37°C in Petri dishes with Luria Bertani (LB) medium, containing agar 15 g/L, IPTG (Isopropyl β-D-1-thiogalactopyranoside) 0.05 mM, X-GAL (5-bromo-4-chloro-3-indolyl-betagalactoside) 80 µg/mL and Ampicillin 100 µg/mL (Sambrook *et al*., 1989). White colonies (potential positive clones) were selected and screened for inserts by colony PCR using vector specific primers M13for (5'-GTAAAACGACGGCCAGT-3') and M13rev (5'-CAGGAAACAGCTATGAC-3'). Colony PCR reactions contained 12.5 µL of 2xPCR master mix (Promega, Southampton, UK), 10.5 µL of SDW and 0.5 pmol of each primer performed in a Techne Progene thermal cycler (Techne, Cambridge, UK). PCR conditions were 94^{o}C for 2 min, followed by 30 cycles of 94^{o}C for 0.5 min, 56°C for 1 min and 72^{o}C for 1 min, completed with a final extension step of 72^{o}C for 10 min. Insert containing vectors were confirmed by running PCR products (2 µL) on a 1% agarose gel in 1X TBE buffer and ethidium bromide (0.5 µg/mL). Gels were run for 1hr at 90V and viewed under UV light. PCR products containing inserts were then purified and sequenced.

2.12 SEQUENCING

Purified PCR products were sequenced on a CEQ 8000 GeXP Genetic Analysis System (Beckman Coulter). Sequence similarity searches were performed in the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) data library using the BLAST algorithm (Altschul *et al*., 1990). Best BLAST matches were recorded and compared to putative T-RFLP fragment lengths. Organisms that were not present in the existing database were further analysed identifying restriction recognition sites and added to the database.

2.13 CULTURES

Microbial cultures were acquired from various sources (see Chapter 3 Table 3.5 for details). Fungal and oomycota cultures were kept on Potato Dextrose Agar (PDA; Oxoid, Basingstoke, UK), and bacteria on Nutrient Agar (Oxoid).

DNA was extracted, ITS2 or 23S rDNA sequences were amplified, cloned, sequenced and examined by T-RFLP (section 2.5-2.8 and 2.10-2.12) to validate T-RFLP methods (See chapter 3). Furthermore, tomato root samples were collected throughout the study, were plated onto PDA amended with streptomycin and P5ARP (Oxoid; Jeffers and Martin, 1986), with and without surface sterilisation by ADAS for comparison with molecular results (Chapters 3-5). All cultures were stored at 4^{o}C.

2.14 PYROSEQUENCING

In an attempt to identify unknown T-RFs and to further characterize the microbial ecology of the tomato rhizosphere DNA extracts from the growth media trials taken place at commercial nurseries (section 2.1.1) were further analysed using 454 pyrosequencing technology.

Pyrosequencing reads were obtained from PCR amplicons of the ITS1 region using a Roche 454™ pyrosequencer (Roche, Basel, Switzerland). PCR amplifications of the ITS1 region were performed in a Techne Progene thermal cycler (Techne, Cambridge, UK) at FERA. The primers used for this reaction (Table 2.7) consisted of 3 parts an adapter, a multiplex identifier and the previously published ITS primers ITS1 as the forward primer and ITS2 as the reverse primer (White *et al*., 1990). The adapter sequences are 30 nucleotide (nt) and were used during the library preparation step of the pyrosequencing reaction to create a bond between single stranded amplicons and glass beads. To reduce costs and increase efficiency up to 12 samples can be multiplexed onto one plate by including short unique barcode sequences or multiplex identifiers (MIDs) that are added to the 3'-end of the adapter and to the 5'-end of the forward primer. Ten different MIDs were used to combine the 10 crop samples from commercial nurseries in the same reaction mixture, which were then separated at the end of the pyrosequencing procedure.

The PCR reaction was set up using KAPAHiFi™ HotStart PCR kits (KAPABIOSYSTEMS, Boston MA, USA) in 25 µL reactions containing 1 µL of

DNA extract, 5 µL of 5X KAPAHiFi fidelity buffer, 0.75 µL of KAPA dNTPmix (10 mM each dNTP), 0.74 µL of the forward primer (10 µM), 0.74 µL of the reverse primer (10 µM), 0.5 µL KAPAHiFi™ HotStart DNA polymerase and 16.25 µL of SDW. The following PCR conditions were used: 95°C for 3 min followed by 35 cycles of 98°C for 20 sec, annealing at 60°C for 15 sec and 72°C for 30 sec, with a final extension step of 72°C for 5 min. Amplified DNA was confirmed by running PCR products (2 µL) on a 1% agarose gel in 1X TBE buffer and ethidium bromide (0.5 µg/mL). Gels were run for 1hr at 90V and viewed under UV light. PCR products were quantified using a Nanodrop spectrophotometer. Equal amounts of the 10 samples mixed (containing 10 different MIDs) and run overnight by FERA for the pyrosequencing reaction. Further sample details can be found in Chapter 4 section 4.2.

Table 2.7: Adapters MIDs and primers used for the amplification of the ITS1 region for pyrosequencing.

Primer component	Sequence (5'→3')
Adapters*	
F Adapter	CCATCTCATCCCTGCGTGTCTCCGACTCAG
R Adapter	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG
MIDs	
M1	ACGAGTGCGT
M2	ACGCTCGACA
M3	AGACGCACTC
M4	AGCACTGTAG
M5	ATCAGACACG
M6	ATATCGCGAG
M7	CGTGTCTCTA
M8	CTCGCGTGTC
M9	TAGTATCAGC
M10	TCTCTATGCG
ITS primer*	
F ITS1	TCCGTAGGTGAACCTGCGG
R ITS2	GCTGCGTTCTTCATCGATGC

*F- forward, R- reverse.

2.15 PYROSEQUENCING DATA ANALYSIS

Following quality filtering provided by the 454 pyrosequencer, raw pyrosequencing data was further processed (trimmed) using the software Mothur (Schloss *et al*., 2009) to remove low quality reads. Trimming

parameters were set to remove reads with lengths <180 nt or >500 nt, with Q-average scores <25, reads containing Ns and reads with >8 homopolymers. After trimming Mothur was used along with the statistical and graphics software package R (RDC Team, 2011) for both the phylogenetic analysis and *de novo* operation taxonomic unit (OTU) analysis of the pyrosequencing data. Some output data from both analysis methods could be compared with T-RFLP output data.

2.15.1 Phylogenetic analysis

For the phylogenetic assignment of the reads of pyrosequencing; unique sequences were identified and aligned against ITS1 reference sequences acquired from NCBI based upon the fungal phylogenetic tree developed by James *et al*. (2006). To identify reads that did not match reference sequences; reads were clustered using the furthest neighbor clustering algorithm at 10% dissimilarity cut-off, using *de novo* OTU clustering methods (Sogin *et al*., 2006). Unclustered reads were discarded from the analysis. Clusters underwent a sequence similarity search performed in NCBI data library using the BLAST algorithm (Altschul *et al*., 1990), and best BLAST matches were added to reference sequences. Best matches of the BLAST searches were likely to be closely related species, to confirm these relationships and to group unidentified Eukarya from blast results, a phylogenetic tree was constructed. Selecting only the regions between the ribosomal universal primers; reference sequences along with sequences with unique identities from cluster analysis were aligned in MEGA5 (Tamura *et al*., 2011) using ClustalW (Thompson *et al*., 1994). From the alignment, phylogenetic analysis was conducted in MEGA5 using the maximum likelihood method (Felsenstein, 1981) based on the Tamura-Nei model (Tamura and Nei, 1993), and a consensus tree was obtained from a 1000 replicates (bootstrap, Felsenstein, 1985), representing the evolutionary relationship of the organisms analysed. Each maximum likelihood placement provides not only a most likely branching position for the query sequence, but also branch length information, indicating the

approximate number of sequence changes. Furthermore, from all qualifying clusters, a bar chart was created to highlight the major taxonomic identity (determined by best BLAST matches) of microbial community constituents contributing to >1% of population in the tomato rhizosphere in different media.

2.15.2 OTU analysis

De novo OTU analysis begins with labelling clustered reads (explanation of clustering found in section 2.15.1) as unique, 0.03 (3% dissimilarity), 0.05 (5% dissimilarity), and 0.10 (10% dissimilarity) OTU definitions (Sogin *et al*., 2006). These labelled clusters provide OTU groupings used for generating rarefaction curves and for calculating species richness estimators.

The statistical and graphics software package R (www.r-project.org) was used to generate rarefaction curves, and the software Mothur was used to produce species richness estimators (Table 2.8) for the characterisation of the data, in order to gain information regarding the community assemblages of the samples and also to ascertain if the sampling was sufficient to show 'true' community species richness.

A consistent problem that all microbial ecologists face is whether environmental samples taken reflect the 'true' microbial community under examination. Molecular methods involving the use of rRNA genes as molecular markers often underestimate species richness, as *taxa* that represent ≤1% of the community are usually not represented. However, various statistical approaches have been developed to estimate 'true' species richness, named species richness estimators (Hughes *et al*., 2001).

Non-parametric species richness estimators compare the proportion of species that are abundant with the proportion of species that are rare. In communities with low diversity, it is more probable that most species will be observed multiple times; whilst in communities with high diversity it is more probable that most of the species present will be observed rarely. In this research the non-parametric species richness estimators Chao1 (based on

whether an individual is observed once or more; Chao, 1984) and ACE (based on whether an individual is observed ≤10 times or > 10 times; Chao and Lee 1992) are used to estimate 'true' species richness of Pyrosequencing data and compared to number of OTUs and reads, indicating if sampling represents the microbial community under examination (Table 2.7).

Several species richness estimators were chosen to increase the accuracy of the analysis when comparing estimations between treatments. 'True' species richness is often limiting for a single observation and cannot be accurately determined unless there is sufficient sampling to assess the community, but because most studies of community assemblages involve relative comparisons, problems with sampling biases can be overcome.

2.15.3 Comparison of Pyrosequencing analysis and T-RFLP analysis

The fingerprinting molecular method T-RFLP and the sequence-based molecular method pyrosequencing used in this study to examine eukaryotic populations associated with roots grown in five media were compared to determine if one method provides better community coverage than the other. OTUs generated from pyrosequencing cluster analysis and T-RFLP analysis was compared, as well as, Principal Component Analysis (PCA) plots and relative abundance data of identified major *taxa*.

Relative abundance of pyrosequencing clustered sequence data and normalized T-RFLP data were analyzed by PCA to examine overall patterns of variation in microbial community assemblages and ordination plots were visually compared. For the comparison of identified *taxa* and their relative abundances; pyrosequencing data from the phylogenetic analysis (section 2.15.1) and T-FRLP data from FRAGSORT output (section 2.8) were compared looking at major taxa contributing to >1% of total microbial communities.

Table 2.8: Diversity indices and species richness estimators used in this study and their corresponding mathematical equations

Diversity indices/species richness estimators	Equations
Simpson's Diversity Index 1-D (D)	$D = 1 - \left[\frac{\Sigma n(n-1)}{N(N-1)}\right]$ N = the total number of organisms of all species n = the total number of organisms of a particular species
Shannon Diversity Index (H')	$H' = -\sum_{i=1}^{S} Pi \ln Pi$ P_i = fraction of the entire population made up of species *i* S = numbers of species encountered Σ = sum from species 1 to species *S*
Chao1 (S_{Chao1})	$S_{Chao1} = S_{obs} + \frac{n_1^2}{2n_2}$ S_{obs} = the number of observed species n_1 = the number of singletons (species captured once) n_2 = the number of doubletons (species captured twice)
ACE (S_{ACE})	$S_{ACE} = S_{abund} + \frac{S_{rare}}{C_{ACE}} + \frac{F_1}{C_{ACE}}\gamma^2_{ACE}$ S_{rare} = the number of rare samples (sampled abundances ≤10) S_{abund} = the number of abundant species (sampled abundances >10 $S_{rare} + S_{abund}$ = the total number of species observed C_{ACE} = 1 - F_1/N_{rare} estimates the sample coverage F_1 = the number of species with *i* individuals N_{rare} = $\sum_{i=1}^{10} iF_i$ γ2 = the distribution that estimates the coefficient of Fi variation
Species Richness (S)	***S*** **S =** is the number of *taxa*

3 OPTIMIZATION AND VALIDATION OF ROOT SAMPLING AND T-RFLP ANALYSIS METHODS FOR THE EXAMINATION OF TOMATO RHIZOSPHERE MICROORGANISMS.

3.1 INTRODUCTION

The major root diseases of tomato predominantly belong to the Kingdoms Fungi and Chromista (Jones *et al*., 1991). Such diseases are traditionally identified by disease symptoms and from metabolic, morphological, and physiological traits after cultivation on artificial media. Cultivation techniques can be difficult, time consuming and require highly skilled plant pathologists to classify causal agents accurately. Furthermore, cultivation methodologies are inadequate when looking to examine the effects of community assemblages on root disease, due to recognized underestimations of the total microbial diversity of only 0.1-10% of microbial populations being cultivated from most environments (Kent and Triplett, 2002; Forney *et al*., 2004; Ghazanfar *et al*., 2010).

In this study the culture-independent molecular fingerprinting method Terminal Restriction Fragment Length Polymorphism (T-RFLP) is used to describe and compare the composition and structure of microbial communities associated with roots of tomato crops, using ribosomal RNA (rRNA) gene sequence targets. The technique relies on the variation of restriction enzyme sites within the target gene sequences in different organisms. Amplification of target genes from total community DNA involves the use of fluorescently labelled primers, following which rDNA amplicons are digested with one or more restriction enzymes, resulting in a multitude of terminal restriction fragments (T-RFs) of differing lengths relating to their rDNA sequences and consequently their phylogenetic identity. T-RFs are then separated by high resolution gel electrophoresis on automated sequencers,

which record the fragment length and relative abundance (Kent and Triplett, 2002; Schütte *et al*., 2008).

T-RFLP profiles can be affected by biases that are common in all PCR based methods of microbial community analysis (Discussed in Chapter 1 section 1.3.2) but also biases related to the restriction analysis, such as a partial digestion of the PCR products (Clement *et al*., 1998) and variation in the observed length and sequence length of T-RFs (Osborn *et al*., 2000; Kaplan and Kitts, 2003). Furthermore, accurate phylogenetic identification of community members is dependent upon comparisons of T-RFs with a robust database of known species. These potential limitations must be taken into consideration to obtain reliable conclusions from T-RFLP profiles (Avis *et al*., 2006).

In this chapter the methods used to reduce biases and optimize crop sampling and T-RFLP protocols for the identification of microbial community assemblages in the tomato rhizosphere are described. In addition, the optimized protocols are then verified by comparisons with traditional cultivation methods and by identification of the causal agents of diseased tomato crops.

3.2 METHODS

3.2.1 Creation of a database of known species

To obtain accurate phylogenetic identification of microbial community members from T-RFLP profiles, a database containing putative T-RFs of known species associated with the tomato rhizosphere was created via numerous steps, discussed below.

Firstly, a literature review of previously reported fungi, bacteria (pathogenic species only) and oomycota associated with tomato roots was conducted via web searches, numerous public databases and referencing books (all sources used in the literature review are available in Appendix I). A list was created and information regarding whether an organism had been reported in the United Kingdom (UK) or if an organism is commonly found in the rhizosphere were noted. However, a list of bacterial saprophytes was not produced as the number of organisms associated with roots was considered too high and in many studies individual species of bacteria are not reported.

From the list, putative T-RFs were calculated from published ITS2 and 23S rDNA sequences from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) database. To reduce inaccuracies associated with DNA sequences in public sequence databases (Nilsson *et al*., 2006) the online programme Cap3 (Huang and Madan, 1999) was used to align and compare numerous sequences of the same species, from which a consensus sequence of most commonly occurring bases was created. Sequences were aligned and edited removing bases outside of rDNA target regions using BIOEDIT 7.0 (Hall, 1999). An *in silico* digestion of the edited sequences with restriction enzymes *Alu*I, *Hae*III and *Mse*I was performed using pDRAW32 (Kield, 2006) producing restriction profiles, from which putative T-RFs were determined.

In addition to a database of specific fungi, oomycota and pathogenic bacteria known to be associated with the tomato rhizosphere, a larger but less specific database was created from the UNITE database for fungal sequences

(Abarenkov *et al*., 2010) and the SILVA ribosomal database for oomycota and bacterial sequences (Pruesse *et al*., 2007). Target sequence regions were selected and T-RFs were determined using T-RF Generator (Bradshaw, 2011).

3.2.2 Confirmation of putative T-RFs

The database of putative T-RFs of known species was tested for accuracy using a number of methods. One method was to collect cultures of fungi, oomycota and bacteria. ITS2 or 23S rDNA sequences were then amplified, cloned, sequenced and examined by T-RFLP (see Chapter 2 section 2.5-2.8 and 2.10-2.12 for details of the methods). Cultures were acquired from University of Nottingham stocks, ADAS, FERA and CBS (Table 3.5). In addition, tomato roots (Table 3.1: sample details) were plated onto Potato Dextrose Agar (PDA; Oxoid, Bakingstoke, UK) amended with streptomycin, and a pythium-selective agar (P5ARP). Selected isolates of major colony types were sub-plated onto PDA agar and examined by T-RFLP. All fungal and oomycota cultures were kept on Potato Dextrose Agar (Oxoid) and bacteria on Nutrient Agar (Oxoid). All cultures were stored at 4^{o}C. If the T-RFs produced from cultures were different to putative T-RFs then the database was updated with the new findings.

Furthermore, a clone library was created; crop DNA samples tested by T-RFLP in 2008 (Table 3.1) with unidentified T-RFs were cloned and sequenced (section 2.10-2.12). Similarity searches were performed at NCBI using the BLAST algorithm (Altschul *et al.,* 1990). Best BLAST matches were recorded and compared to putative T-RFLP fragment lengths; following sequence alignment and editing in BIOEDIT 7.0 (Hall, 1999) and identification of restriction recognition sites in pDRAW32 (Kield, 2006). Organisms that were not present in the existing database were added with their corresponding T-RFs. Lastly, all commercial crops sampled in 2009 and 2010 were analyzed by Pyrosequencing; the methods and results are discussed in Chapter 4.

3.2.3 Optimization of root sampling

To devise a root sampling procedure for tomato crops in different growth media, several samples were taken from different locations relative to a propagation cube. Furthermore, roots of different thickness were sampled (thin 1.0-1.5 mm; medium 1.5-3.0 mm and thick 3.0-8.0 mm). Samples were tested by traditional methods by ADAS and T-RFLP to identify which sampling method provided the best coverage of microbial communities. Notably, the plants examined were commercial crops, so any samples taken had to be representative of the microbial community of the rhizosphere but also not result in plant death or significant yield reduction.

Three root sampling methods were compared for tomatoes grown in hydroponic systems with media (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. Two sampling locations were compared for soil grown crops: auger-borings adjacent to the propagation cube; and mid-way between cubes. For nutrient film technique (NFT) grown tomato, the only valid method, with regards to plant health, was to a cut a wedge of roots from a channel midway between two plants. However samples taken from NFT crops were examined by both T-RFLP and traditional methods for a comparison of the methods (section 3.2.4).

Roots were collected from the soil-grown crop using a 2 cm diameter soil auger inserted to 20 cm depth or by carefully forking soil away from the side of a plant and cutting-off root sections around 5-15 cm long. 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. Root pieces were then divided into two halves; one half was tested for fungi or oomycota by plating onto agar by ADAS (section 2.2.4) and the second half was tested by T-RFLP (section 2.5-2.8). Plating results based on percentage of roots with fungal or oomycota growth were examined by ANOVA with sampling position as a factor.

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 in Table 3.1.

Table 3.1: Summary of root samples collected in 2008

Production method	Sample date	Sample method	Sample positions relative to cube	Number of sub-samples per position	Number of replicates
Soil	03-Jun	Auger	Adjacent & Midway	10 cores	3
Soil	23-Jul [ab]	Expose & cut	Midway	20 root lengths	3
Rockwool	10-Jun	Cork borer & cut	Adjacent, midway & slab corner	10 cores or sections	3
NFT	28-Aug[b]	Cut	Midway	1	5

a= Three root thicknesses compared
b= Samples used for section 3.2.4 only

3.2.4 Comparisons of T-RFLP results with traditional methodology

To verify the accuracy of T-RFLP methods, root samples collected in 2008 (Table 3.1) were analyzed to identify the fungi and oomycota present by T-RFLP and compared to results from traditional cultivation methods. Root samples were cut into 5 mm length pieces. The set of root pieces were then divided into two halves; one half was tested for fungi and oomycota by plating onto agar by ADAS and the second half was tested by T-RFLP (section 2.5-2.8).

Plating methods involved plating sets of 10-50 root pieces per sample onto potato dextrose agar amended with streptomycin (PDA) and a pythium-selective agar (P5ARP) with and without surface sterilisation. Roots plated onto PDA were sterilised with sodium hypochlorite (1% for 3 minutes, rinsed in sterile distilled water); those plated onto P5ARP were sterilised in 70% alcohol (10 seconds). Ten root pieces were plated onto each Petri dish of agar. Plates were incubated at 20°C in a black-light incubator (PDA) or in the dark (P5ARP). After 14 days, the proportion of root pieces with different fungi or oomycota was recorded. Organism growth was identified by colony colour

and morphology and by microscopic examination of selected colony types for spores and other fungal structures.

3.2.5 T-RFLP as a diagnostic tool for root disease

To further check the validity of the optimized T-RFLP protocol and sampling protocols, the methods that are used throughout the project were implemented to identify the causal agents of diseased crops by comparing with healthy crops.

Root samples were taken from various commercial crops as opportunities of specific disease symptoms arose during 2009. Healthy root samples were taken as comparisons to identify differences in community assemblages. Comparisons had to be from a single uniform crop grown in identical conditions, from the same crop row. Details of the crops sampled and disease symptoms examined are given in Table 3.2. Each comparison consisted of a single factor occurring at two levels (diseased roots/healthy roots). For each paired comparison, three samples of each level were collected on one occasion. Three sub-samples from each were examined by T-RFLP (section 2.5-2.8) giving nine T-RFLP profiles per factor. Roots of diseased crops were also plated out on PDA to identify pathogens present using surface sterilization methods previously described (section 3.2.4).

From the resulting T-RFLP profiles, the null hypothesis (H_0) of there being no difference between healthy and diseased root T-RFLP datasets was tested by Analysis of Similarities (ANOSIM; section 2.9.1). T-RFLP data was also represented in an ordinational space with Principal Component Analysis (PCA; section 2.9.2) and α-diversity was calculated using species richness and diversity indices (section 2.9.3).

Table 3.2: Details of crops sampled in 2009 for comparison of the effect of disease symptoms on populations of microorganisms associated with tomato roots

Dataset	Disease symptom versus healthy crop	Growth media	Variety	Date sampled
Brown root	Brown roots vs. white roots	Rockwool	Roterno	06-Mar
Root mat	Root mat present vs. absent	Rockwool	Lucino	20-Apr
Yellowing	Roots from yellowing crop vs. healthy crop	Soil	Roterno	07-May

3.3 RESULTS

In this section the results regarding the creation of a database of known species are discussed, as well as some methods used to verify putative T-RFs.

Furthermore, results from the optimization of sampling methods (sample location and root thickness) are shown, including comparisons of T-RFLP findings with traditional methodology results. Finally, experiments using the optimized T-RFLP protocol for the identification of causal agents of diseased crops are shown.

3.3.1 Creation of a database of known species

From the literature review it was found that at least 66 fungal/oomycota pathogens and five bacterial pathogens have been previously reported to cause root disease on tomato plants. Of the fungal and oomycota pathogens, 33 have been reported to occur in UK, whereas all five bacterial pathogens have been reported in the UK, but are relatively uncommon with the exception of *Agrobacterium radiobacter* (Table 3.3; full detailed list in Appendix I). Seventy-five fungal saprophytes were found to have been previously recorded in the tomato rhizosphere, with 68 having been found in the UK (Table 3.4; full detailed list in Appendix I). From these lists a database of putative T-RFs (described in 3.2.1) was produced and compared with T-RFLP datasets for the phylogenetic identification of microbial community constituents.

As suggested by Jones *et al*. (1999), the majority of pathogens associated with tomato roots from previous reports were found to be from the phyla Oomycota, Ascomycota, and Basidiomycota. Saprophytes identified from the review predominantly belong to the phyla Ascomycota and Basidiomycota but also a few in the Chytridiomycota and Zygomycota.

Table 3.3: Fungal/oomycota and bacterial pathogens previously reported on tomato roots

Pathogen identity	
Alternaria solani	*Pyrenochaeta lycopersici*
Aphanomyces cladogamus	*Pyrenochaeta terrestris*
Botrytis cinerea	*Pythium* (19 species)
Calyptella campanula	*Rhizoctonia solani*
Collectotrichum coccodes	*Spongospora subterranean*
Didymella lycopersici	*Thielaviopsis basicola*
Fusarium (7 species)	*Verticillium* (5 species)
Humicola fuscoatra	*Agrobacterium rhizogenes*
Macrophomina phaseolina	*Agrobacterium tumefaciens*
Plectosphaerella cucumerina	*Pseudomonas syringae* pv. *tomato*
Phymatotrichopsis omnivora	*Clavibacter michiganensis*
Phytophthora (18 species)	*Ralstonia solanacearum*

Table 3.4: Fungal saprophytes previously reported on tomato roots

Saprophyte identity		
Acremonium atricum	*Lycoperdon* sp.	*Cylindrocarpon didymium*
Acremonium (2 species)	*Mortierella polycephala*	*Doratomyces microsporus*
Agaricus arvensis	*Mortierella zychae*	*Epicoccum purpurascens*
Alternaria (2 species)	*Mortierella* sp.	*Fusarium* (2 species)
Aspergillus (5 species)	*Mucor* sp.	*Gelasinospora reticulata*
Aureobasidium pullulans	*Mycotypha microspora*	*Gilmaniella humicola*
Blastomyces sp.	*Myrothecium roridum*	*Gliocladium roseum*
Calyptella capula	*Nectria gliocladioides*	*Idriella lunata*
Cephalosporium (2 species)	*Neurospora crassa*	*Lepiota efibulis*
Chaetomium (4 species)	*Oedocephalum sp.*	*Pyronema amphalodes*
Chromalosporium ochraceum	*Olpidium* (2 species)	*Rhizopus* (2 species)
Conidiobolus coronatus	*Paecilomyces lilacinus*	*Rhodotorula glutinis*
Coprinopsis gonophylla	*Penicillium* (15 species)	*Sporobolomyces roseus*
Cryptococcus albidus	*Petriella asymmetrica*	*Torulopsis famata*
Cunninghamella echinulata	*Peziza ostracoderma*	*Tricocladium adspersum*
Volutella ciliata	*Trichurus spiralis*	*Trichoderma* (3 species)

A larger less specific database was created from ITS2 sequences and 23S rDNA sequences from the UNITE database (Abarenkov *et al*., 2010) and the SILVA ribosomal database (Pruesse et al., 2007).

Table 3.5: Number of sequences available in the UNITE and SILVA databases and the portion of species where T-RF length could be determined. Results are based on searches in 2011.

Database	Number of sequences available	Number of full sequences available	Portion of Species where T-RFs could be determined
UNITE	204,660	65,542	19,361
SILVA	269,240	23,600	819

From >200,000 sequences available in both the UNITE database and SILVA database, only 19,361 and 819 species could be used to determine T-RFs respectively (Table 3.5). This is because the databases have many sequences for the same species and many organisms were not identified to a species level. Furthermore, the contents of the database are sequences from public sequence databases, and as a consequence many sequences were of low quality or were partial sequences preventing T-RFs from being determined (Nilsson *et al*., 2009). The number of sequences in the two databases and the number of T-RFs determined pale in comparison to the total estimated numbers of 1.5 million fungal species (Hawksworth, 2001) and an estimated range from 1 million to 100 million of prokaryotic species (Hammond, 1995). With this in mind and the approximation of 86% to 91% of existing species yet to be described, it is not unexpected that some T-RFs produced cannot be given a phylogenetic identity (Mora et al., 2011).

To confirm the putative T-RFs generated from literature results and published sequences from NCBI, a culture collection was assembled from various sources (Table 3.6 key). Cultures were analyzed by T-RFLP (Figure 3.1 for four examples) and the resulting T-RFs produced were recorded (Table 3.6) and added to the database if they differed from the putative results. Notably, the 12 cultures of *A. radiobacter* examined were found to have varying restriction

profiles, suggesting that this species has high variability in 2SS rDNA regions, emphasizing the importance of verifying putative T-RFs.

Furthermore, roots sampled in 2008 (Table 3.1) with unknown T-RFs produced in T-RFLP profiles were cloned and sequenced. Best blast matches were recorded along with percentage identity. Restriction analysis was conducted and T-RFs were identified (Table 3.7). Seven unique clones were identified from soil crops, six from rockwool crops and eight from NFT crops. Major pathogens belonging to Oomycota and Ascomycota were found, as well as saprophytes (Ascomycota, Chytridiomycota and Zygomycota) and unculturables. Notably, sequences from three species of protozoa and a nematode species were cloned, suggesting that the primers used to target the ITS2 region of fungi and oomycota also target the ITS2 regions of other eukaryotic organisms. Thirteen organisms had not been previously reported on tomato in the UK (based upon the literature review) (denoted by '*' Table 3.7); however four of these were unculturable and another four were from kingdoms not under selection for culturing methods or searched for in the literature review. Organisms not already in the database or with differing results to putative T-RFs were added.

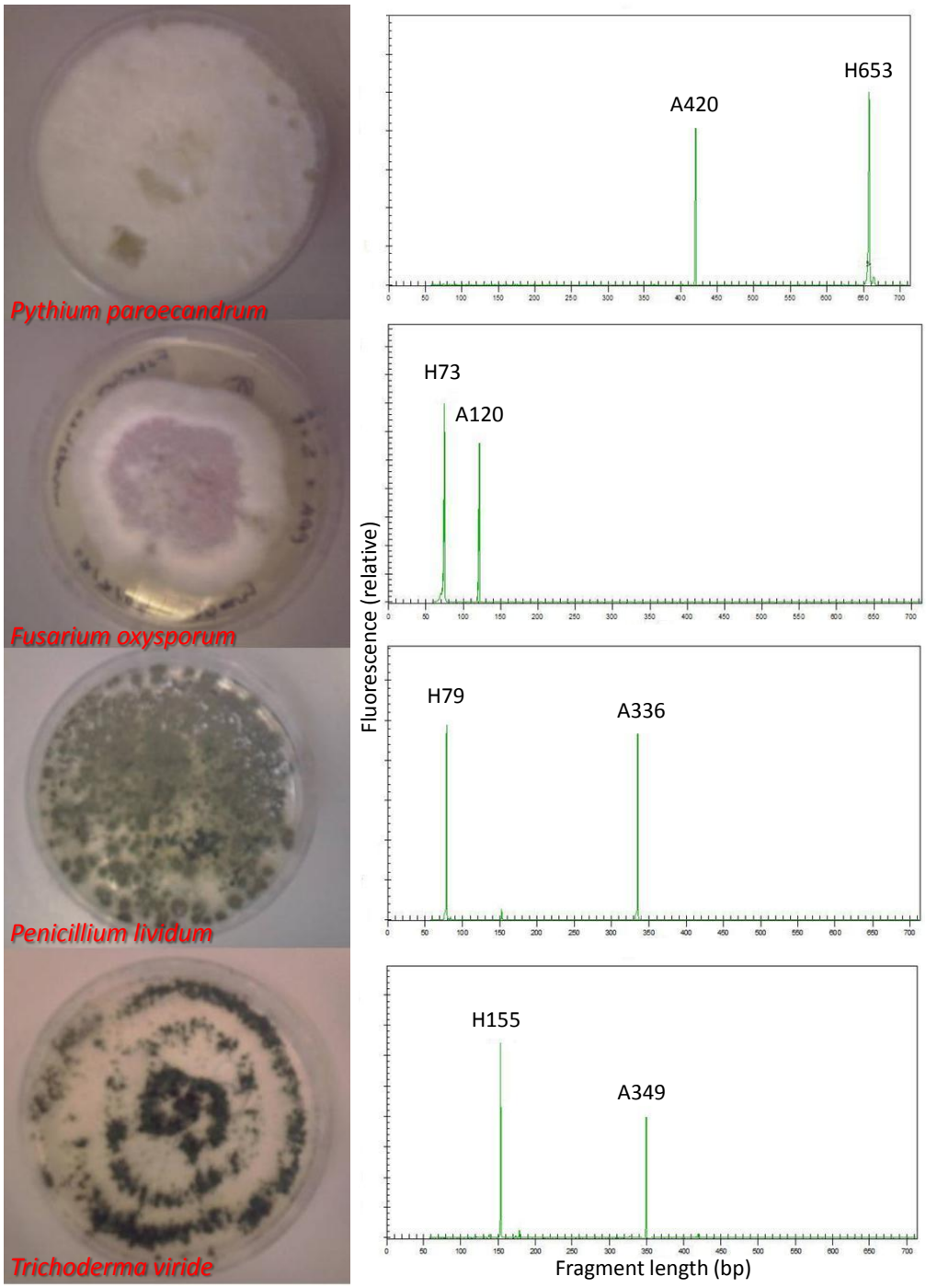

Figure 3.1: Examples of T-RFLP overlay electropherograms of fungal and oomycota cultures, confirming the T-RF length when cut with the restriction enzymes *Hae*III (H) and *Alu*I (A) with length in basepairs along the x axis. Cultures on agar of the organisms tested are illustrated

Table 3.6: Culture stocks, sources of cultures and whether organisms have been previously recorded in the UK. T-RF lengths are shown from restriction analysis.

Sample	Identity	T-RFs		Source[a]	Previously recorded
		***Alu*I**	***Hae*III**		
PCCBS	*Plectosphaerella cucumerina*	342	138	CBS	Yes
TBCBS	*Thielaviopsis basicola*	101	157	CBS	Yes
FusAve	*Fusarium avenaceum*	104	74	Nottingham	No
FusOxy	*Fusarium oxysporum*	120	73	Nottingham	Yes
FusOxyRL	*Fusarium oxysporum* f.sp. *RL*	120	73	Nottingham	Yes
8JS	*Gliocladium roseum*	339	156	Nottingham	Yes
PenChr	*Penicillium chrysogenum*	323	80	Nottingham	Yes
PhyCin	*Phytophthora cinnamomi*	161	311	Nottingham	No
9JS	*Phytophthora* sp.	161	291	Nottingham	Yes
11JS	*Pythium intermedium*	653	68	Nottingham	No
PytIrr	*Pythium irregulare*	419	651	Nottingham	Yes
RhiSol	*Rhizoctonia solani*	175	103	Nottingham	Yes
CW2	*Cladosporium* sp.	323	323	RW tomato	No
CW1	*Colletotrichum coccodes*	189	153	RW tomato	Yes
EXI	*Exophiala pisciphila*	365	184	RW tomato	No
CW1	*Fusarium solani*	105	75	RW tomato	Yes
PHYCRY	*Phytophthora cryptogea*	106	604	RW tomato	Yes
CW6	*Plectosphaerella cucumerina*	342	138	RW tomato	Yes
PytDic	*Pythium diclinum*	384	205	RW tomato	Yes
CW4	*Verticiliium dahliae*	110	134	RW tomato	Yes
CW7	*Trichoderma viride*	349	155	RW tomato	Yes
ASP1	*Aspergillus niger*	340	82	Soil tomato	Yes
2IOW	*Cylindrocarpon destructans*	119	75	Soil tomato	No
2IOW	*Mortierella alpina*	119	58	Soil tomato	No
2IOW	*Olpidium brassicae*	39	424	Soil tomato	Yes
2IOW	*Pyrenochaeta lycopersici*	189	328	Soil tomato	Yes
2IOW	*Pythium dissocotum*	115	241	Soil tomato	No
4IOW	*Penicillium lividum*	326	79	Soil tomato	Yes
"	"	***Hae*III**	***Mse*I**	"	"
AR3478	*Agrobacterium radiobacter 3478*	157	360	FERA	Yes
AR3555	*Agrobacterium radiobacter 3555*	157	360	FERA	Yes
AR3475	*Agrobacterium radiobacter 3475*	202	517	FERA	Yes
AR3576	*Agrobacterium radiobacter 3576*	157	360	FERA	Yes
AR3813	*Agrobacterium radiobacter 3813*	168	371	FERA	Yes
AR4143	*Agrobacterium radiobacter 4143*	157	633	FERA	Yes
AR5013	*Agrobacterium radiobacter 5013*	157	472	FERA	Yes
AR6322	*Agrobacterium radiobacter 6322*	157	388	FERA	Yes
AR6371	*Agrobacterium radiobacter 6371*	157	633	FERA	Yes
AR6392	*Agrobacterium radiobacter 6392*	157	633	FERA	Yes
AR6399	*Agrobacterium radiobacter 6399*	157	633	FERA	Yes
AR6994	*Agrobacterium radiobacter 6994*	157	360	FERA	Yes

CBS-Centraalbureau voor Schimmelcultures; Nottingham-University of Nottingham; RW tomato-isolated from rockwool crops; Soil tomato-isolated from soil crops; FERA-Food and Environment Research Agency

Table 3.7: Fungal and oomycota blast analysis of clones, with closest match identity in the NCBI database, together percentage identity of query sequence with database best blast match and restriction analysis.

Clone	Closest NCBI Database Match	Identity (%)	Accession No.	T-RFs *Alu*I	T-RFs *Hae*III
ITS2N1B1	*Pythium dissotocum**	98	AB259313.1	115	241
ITS2N1B2	*Penicillium olsonii**	99	DQ117963.1	325	79
ITS2N2A1	*Plectosphaerella cucumerina*	99	DQ779781.1	342	138
ITS2N2B1	*Colletotrichum coccodes*	98	GQ485588.1	189	153
ITS2N2B2	Uncultured Eukaryote*	100	GU928478.1	240	106
ITS2N3A1	*Pythium dissotocum*	97	AB531499.1	115	241
ITS2N3A3	*Colletotrichum coccodes*	100	GQ485588.1	189	153
ITS2N3A4	*Exophiala pisciphila**	99	AF050272.1	365	147
ITS2N3B1	*Pythium dissotocum*	98	AB259313.1	115	241
ITS2N3B2	*Vorticella* sp.*	99	GU187057.1	88	320
ITS2N3B3	*Paramecium tetraurelia**	99	JF304166.1	322	322
ITS2N3B4	Uncultured fungus*	93	GU559079.1	349	324
ITS2R2A1	*Carchesium polypinum**	85	FJ810386.1	235	323
ITS2R2B1	*Fusarium solani*	100	EF017210.1	105	75
ITS2R3A2	Uncultured eukaryote*	82	AB222616.1	184	99
ITS2R3A3	*Fusarium solani*	100	EF017210.1	105	75
ITS2R3A4	*Colletotricum coccodes*	100	GQ485588.1	189	153
ITS2R3A6	*Colletotricum coccodes*	100	GQ485588.1	189	153
ITS2R3B1	*Plectosphaerella cucumerina*	96	AB685486.1	342	138
ITS2R3B2	*Rhizoctonia* sp.	100	AY927341.1	407	105
ITS2R3B3	*Fusarium solani*	100	EF017210.1	105	75
ITS2R3B4	*Fusarium solani*	99	EF017210.1	105	75
ITS2R3C1	*Colletotricum coccodes*	100	FJ545227.1	189	153
ITS2S1	Uncultured Soil Fungus Clone*	90	GU083316.1	115	413
ITS2S2	*Actinomucor elegans**	99	AB470907.1	105	69
ITS2S3	*Mortierella alpina **	98	EF192184.1	119	58
ITS2S4	*Plectosphaerella cucumerina*	96	AB685486.1	342	138
ITS2S5	*Mortierella alpina**	98	EF192184.1	119	58
ITS2S6	*Pratylenchus goodeyi**	85	FJZ12925.1	164	184
ITS2S7	*Exophiala pisciphila**	99	JN650536.1	365	147
ITS2S8	*Olpidium brassicae*	97	AY997067.1	39	424

*: organisms not previously identified by literature search

3.3.2 Optimization of sampling methods and comparisons of T-RFLP results with traditional methods

Comparisons of sampling methods were undertaken to obtain reliable and representative data of microbial communities in the rhizosphere. Root thickness and sample position were examined by culturing methods and T-RFLP. Furthermore, T-RFLP datasets were compared with traditional methods of identifying fungi and oomycetes present in the rhizosphere.

From the examination of thin (1.0-1.5 mm), medium (1.5-3.0 mm) and thick, (3.0-8.0 mm) roots, both culturing methods and T-RFLP analysis found that there was a greater range of eukaryotic organisms associated with thin roots than medium or thick roots. Furthermore, thick roots had the least number of species associated with them (Table 3.8; Table 3.9 and Figure 3.1). It is perhaps not unexpected that there are differences in the microbial communities with different root thickness or root age, as it is well known that root exudates differ during the development of roots and in turn affect the microbial community present (Lynch and Whipps, 1990; Gregory, 2006). Young or thinner roots are known to excrete more root exudates than older thicker roots, which could explain higher species richness (Table 3.9) due to higher levels of organic nutrients and space associated with young roots (Bowen and Rovira, 1999).

However, from culturing methods it was found that where major groups were identified in all root thickness datasets, the mean percentage of pieces with colony growth was higher in thick and medium roots than thin roots (Table 3.8). Notably, although thin roots have lower mean percentage of root pieces with colony growth in these instances, all major organisms detected on medium and thick roots are also present on thin roots, suggesting thin roots could provide good coverage of the microbial community in the rhizosphere. Furthermore, from looking at the electropherograms from T-RFLP analyses (Figure 3.2) it can be seen that all root thickness datasets give similar T-RFLP profiles, with a greater variety of microbes on the younger thin roots and as the root thickness increases certain peaks are gradually eliminated from the

profile. This suggests subtle changes in rhizosphere microbial communities with root thickness, as opposed to major taxonomic shifts, which is in agreement with findings from other studies (Heuer and Smalla, 1997; Felske *et al.,* 1999).

From these findings, it would seem that the targeting of young roots for the examination of the tomato rhizosphere microbial community would provide good coverage of communities present and would not result in major *taxa* being missed from medium of thick roots.

Table 3.8: Effect of root thickness on the recovery of major identifiable fungi and oomycetes from tomato roots – sampled 23 July 2008

Agar and root thickness	Mean percentage pieces with:								
	Clean	Tri	CC	Glio	Pen	Fus	GS	Pyth	Other
PDA									
Thick	0	21.7	50.8	7.5	0	0	0	0	29.2
Medium	0	20.8	60.0	13.3	0	1.7	0	0.8	16.7
Thin	4.2	5.0	40.8	6.7	2.5	1.7	5.0	1.7	31.7
P5ARP									
Thick	3.3	0	0	0	0	0	0	80.8	15.8
Medium	11.7	0	0	0	0	0	0	56.7	16.7
Thin	20.0	0	0	0	0	0	0	35.0	18.0

Tri – *Trichoderma*, CC –*Colletotrichum coccodes*, Glio – *Gliocladium*, Pen – *Penicillium*, Fus – *Fusarium*, GS- grey sterile fungus., Pyth – Pythiaceous. Thin, 1.0-1.5 mm; Medium, 1.5-3.0 mm; Thick, 3.0-8.0mm diameter.

Table 3.9: Species richness calculated from T-RFLP datasets for ITS regions under examination, for thin (1-1.5 mm), medium (1.5-3.0 mm) and thick (3.0-8.0 mm) root datasets.

Dataset	S
Thick	3.2±1.23
Medium	5.66±0.63
Thin	8±1.12

S: Species richness: number of taxa or species present
±- Standard deviation of the average

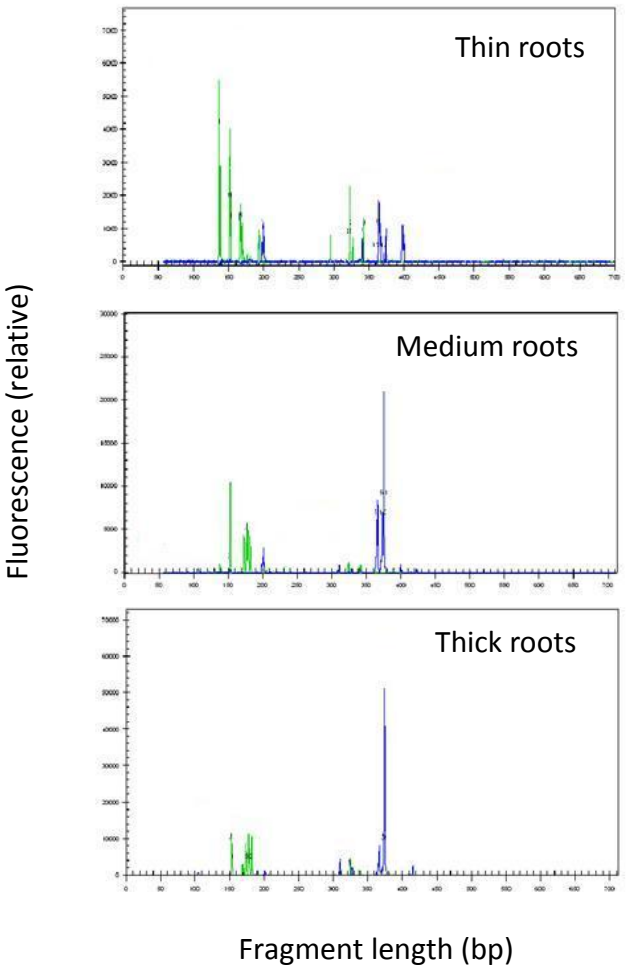

Figure 3.2: Examples of electropherograms from T-RFLP analysis of thin (1-1.5 mm), medium (1.5-3.0 mm) and thick (3.0-8.0 mm) roots. Eukaryotic population T-RFS are represented by green peaks and bacterial populations by blue peaks.

The predominant organisms recovered from the soil crop (sampled on 3rd June; Table 3.1) on PDA were; *Colletotrichum coccodes*, *Fusarium* sp. and green-coloured colonies (probably *Trichoderma* spp.). A number of other colony types occurred at a lower incidence and were not identified. Organisms recovered on P5ARP were generally white and on microscopic examination appeared to be pythiaceous or related organism. These organisms were cultured from both roots sampled from midway between plants and adjacent to the propagation cube. ANOVA results with sampling position as a factor found there was mostly no significant difference between the numbers of roots with organism growth between the two sample

positions, with the exception of *Fusarium* species, which were found on significantly more root pieces from midway root samples (Table 3.10).

Table 3.10: Mean effect of sample position on recovery of fungi and oomycota from tomato roots plated onto PDA and P5ARP (soil crop)

Sample position	Mean % roots with				
	Fusarium	Black dot	Green felty	Pythiaceous	Other
Adjacent	0.7	22.7	13.3	12.4	2
Midway	3.9	27.2	11.9	15.3	3
Significance	<0.05	NS	NS	NS	NS

Black dot- *Collectrichum coccodes*, Green felty- presumed *Trichoderma* spp. NS- no significance

The major colony types obtained from rockwool root samples (sampled on 10th June; Table 3.1) on PDA were white, (pythiaceous and fusarium) pink-red (mostly fusarium) and *Collectotrichum coccodes* colonies. Organisms recovered on P5ARP were generally white and on microscope examination appeared to be pythiaceous or related organism. Similarly to soil sample position data, all major colony types were cultured from all sample positions. ANOVA results with sampling position as a factor found there was no significant difference between the numbers of roots with organism growth between sampling positions (Table 3.11).

These findings suggest that similar levels of organisms are recovered from different positions relative to the propagation cube; therefore more emphasis can be placed upon the practicalities of the removal of root samples at intervals during the growing season at commercial sites. To obtain root samples quickly without affecting yield or plant health, sampling from midway between soil crops and from corner slabs of rockwool crops was noted to be quicker and less likely to affect plant health. Furthermore it was noted that there were higher levels of young/thin root at these sampling positions.

Table 3.11: Mean effect of sample position on recovery of fungi and oomycota from tomato roots plated onto PDA and P5ARP (rockwool crop)

Sample position	Mean % roots			
	White fungus	Pink fungus	Black dot	Other
Cube	60	11.5	8.3	0.96
Midway	90.5	5.8	2.9	1
Corner	100	4.7	1.3	1.01
Significance	NS	NS	NS	NS

Black dot- *Collectrichum coccodes*, White fungus - mostly pythiaceous and fusarium; pink fungus - mostly fusarium. NS- no significance

The range of microorganisms identified by plating onto agar and by T-RFLP was compared from root samples of three different media grown crops. T-RFLP was found to identify more microorganisms on rockwool and NFT crops. All major *taxa* identified by plating onto agar were also detected by T-RFLP analysis (Table 3.12). However, in the case of *Fusarium oxysporum* although matching T-RFs were produced, T-RFLP data alone cannot confirm the presence of this species. This is due to the limitations of using rRNA genes as molecular markers for phylogenetic identification; these regions are highly conserved between genus and as a result *Fusarium oxysporum* is not resolved from certain closely related *Fusarium* species. It has been found that few T-RFs are truly species specific and most are either specific to groups of species within a genus or are genus specific (Dunbar *et al.,* 2001). However, sharing a T-RF (or sharing a restriction site in a gene) usually indicates a close phylogenetic relationship between species; even more so when sharing the same combination of two T-RFs, indicating a very close relationship between organisms, and it has been suggested that such organisms share metabolic capabilities and perform similar community roles (Coleman *et al*., 1993). Clearly, this theory is not applicable in the context of pathogenic compared to non pathogenic organisms of the same species; however such distinctions are not concluded from conventional culturing methods either and to establish this information pathogenicity genes rather than rRNA genes would probably need to be targeted using molecular methods. These results indicate that the

T-RFLP protocol being used is appropriate for studying microbial communities on tomato roots from commercial crops.

Table 3.12: Detection of major fungal/oomycota groups and species, from roots of tomato grown in soil, rockwool and NFT, by conventional and T-RFLP methods

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

3.3.3 Examination of rhizosphere community assemblages of symptomatic and comparative healthy crops using T-RFLP, with the aim of identifying causal agents

To validate optimized T-RFLP and sampling protocols, these methods were used to identify the causal agent of crops with disease symptoms (Table 3.2).

3.3.3.1 Null hypothesis testing

To test the null hypothesis (H_0) that there were no differences in bacterial or eukaryotic communities inhabiting the roots of diseased plants with those inhabiting the roots of visibly healthy plants, the ANOSIM test was carried out on the three T-RFLP datasets under examination (Table 3.13)

Table 3.13: ANOSIM test values and probabilities of null hypothesis tests obtained from comparisons of T-RFLP datasets of healthy roots and the roots of plants with disease symptoms

	ITS2			23S		
	BR	RM	Y	BR	RM	Y
H_0 root health						
R-values	0.68	0.19	0.65	0.67	0.16	0.56
p-values	<0.01	<0.01	<0.01	<0.05	<0.05	<0.01

T-RFLP datasets: BR-brown roots, RM-root mat, Y- Yellowing plant

In all instances the null hypothesis was rejected, suggesting that were differences in microbial community structures detected by ITS2 and 23S rRNA genes, but with varying levels of community overlap. Notably in the root mat dataset the R-values indicate there is barely separation between microbial communities (R=0.19; R=0.16 for eukaryotic and bacterial communities respectively), suggesting that these populations are similar between roots with symptoms and visibly healthy roots. In all T-RFLP datasets ITS2 molecular markers gave the higher R-values than their corresponding 23S rRNA gene R-values, indicating that the eukaryotic populations were more variable between roots of plant with disease symptoms than roots of visibly healthy plants.

3.3.3.2 PCA analysis

Normalized T-RFLP datasets were used for PCA analysis to view transformed microbial community assemblage results in a two dimensional space (Figure 3.3)

Overall, PCA ordination plots show separation on both axis of microbial communities associated with roots of crops with disease symptoms (blue diamonds) and of communities associated with roots of visibly healthy crops (red squares), confirming the hypothesis testing with ANOSIM. In most plots PC1 and PC2 account for over 70% of the total variation in the data, with the exception of the yellowing leaf symptoms data set which provide 69% and 63% coverage of variation for eukaryotic and bacterial datasets respectively.

PC scores for the samples were analysed by one-way ANOVA, with root health as a factor. It was found that PC1 scores in all cases were significant for the grouping of microbial communities associated with healthy plants and diseased plants in PC1 (<0.05). PC2 scores were only significant for groupings of brown root datasets (<0.05). PC2 scores for root mat datasets (p=0.38; p=0.17 for eukaryotic and prokaryotic data respectively) and yellowing datasets (p=0.42; p=0.46 for eukaryotic and prokaryotic data respectively)

were not significant for the grouping of variables relating to the factor under examination.

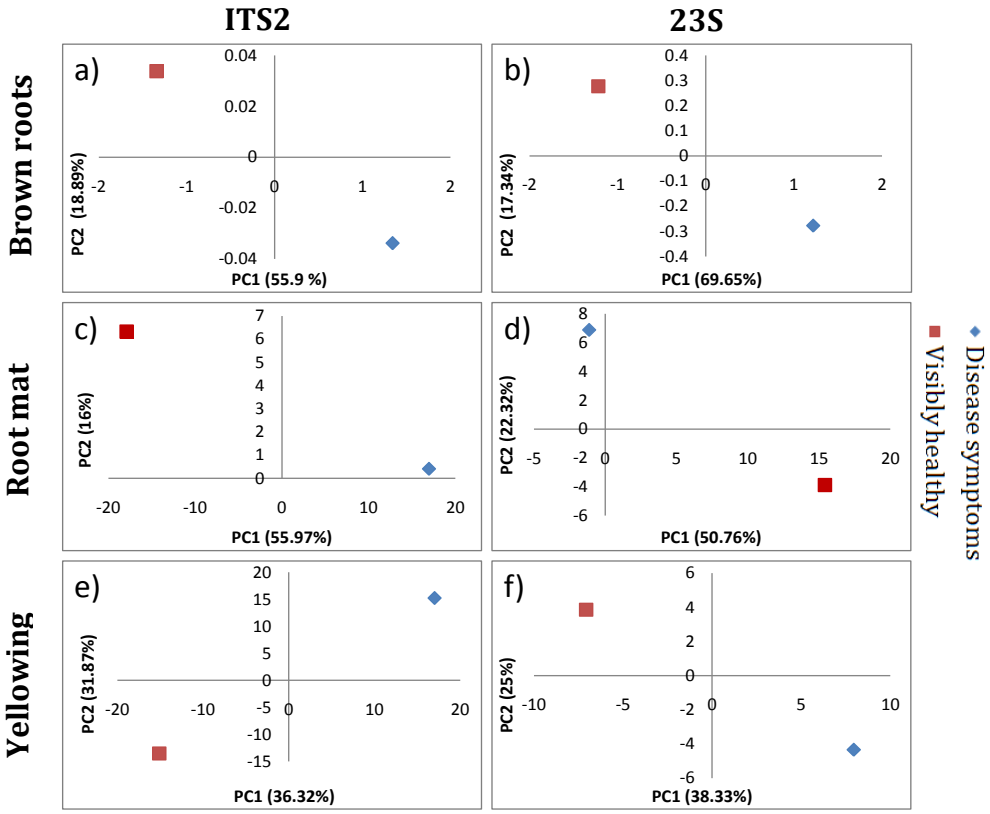

Figure 3.3: PCA ordination plots of microorganisms inhabiting the rhizosphere of healthy roots (red squares) and the roots of plants with disease symptoms (blue diamonds) of brown root (a, b), root mat (c, d) and yellowing leaves (e, f), using T-RFLP profiles of ITS2 (a, c, e) and 23S rRNA genes (b, d, f).

From PCs identified as significant for the grouping of communities based on root health, loading values from which the PCs are computed were further analysed and their significance was established based on the Pio *et al.* (1996) estimation (section 2.9.2). From these significant loading values, the enzyme and T-RF combination can be identified and compared to the output of FRAGSORT (Michel and Sciarini, 2003; section 2.8), which is based on identities of two enzyme combinations giving higher resolution between species and more reliable results (Engebretson and Moyer, 2003), and potential microorganism identities were established (Table 3.14). Based on whether a loading value is positive or negative, organism identities can be

associated with the groupings on PCA ordination plots, suggesting that the presence and/or abundance of the organism in question is significantly contributing to the variation in that grouping.

From Table 3.14, it can be seen that seven potential pathogens (in bold) have been identified by this method of analysis; furthermore, in all of the datasets these pathogens are associated with diseased roots, suggesting that T-RFLP and this method of analysis can be used to identify potential pathogens. Notably from the culturing analysis of these root samples; a *Pythiaceous* sp. (known to cause browning of the root; Blancard, 1994) was isolated from brown roots and *Colletotrichum coccodes* (known to cause leaf yellowing; Blancard, 1994) was isolated from the roots of yellowing crops, confirming the findings from PCA analysis. Furthermore, the causal agent of root mat symptoms *Agrobacterium radiobacter* was identified via this method of analysis; again suggesting that these methods can be used to identify the causal agents of roots disease.

However, as mentioned this method of using PC loadings as a means of identifying organisms significantly contributing to variation in groupings can be as a result of presence and/or abundance and does not detail whether these pathogens are present in other groupings. To establish these aspects, comparisons between FRAGSORT output data and the normalized T-RFLP profiles have been made, with the pathogens identified by PCA analysis (Figure 3.4).

Table 3.14: Microorganisms identified by significant PC loadings (PC1, PC2) contributing to significant PCs, their T-RF and enzyme combination and which factor they are associated with based on their PC loading value.

Data set	Enzyme/T-RF	PC1	PC2	Potential Identity	Associated with
BR	**ITS2**				
	H137	-	-0.40	***Plectosphaerella cucumerina***	BR
	H182	-	0.30	*Paecilomyces lilacinus*	HR
	H241	-	0.30	*Gigaspora rosae*	HR
	H368	-0.35	-	*Sporobolomyces* sp.	HR
	H619	0.37	-	***Pythium ultimum***	BR
	23s				
	M169	-0.33	0.30	*Nitrosomonas* sp.	HR
	M198	0.33	-0.30	*Idiomarina* sp.	BR
	M352	0.26	-	*Rhodospirillum* sp.	BR
	M198	-	0.30	*Pseudomonas* sp.	HR
	M369	-	0.29	*Nitrosospira* sp.	HR
RM	**ITS2**				
	A342	0.32	-	***Plectosphaerella cucumerina***	RM
	H73	0.25	-	***Fusarium oxysporum***	RM
	23S				
	H202	-0.78	-	***Agrobacterium radiobacter 3813***	RM
	H400	-0.33	-	Bacteroidaceae	RM
	M400	-0.36	-	Bacteroidaceae	RM
	M407	0.24	-	Clostridia	HR
Y	**ITS2**				
	A322	-0.42	-	*Cladosporium herbarum*	HR
	H138	0.41	-	***Plectosphaerella cucumerina***	Y
	A189	0.26	-	***Colletotrichum coccodes***	Y
	23S				
	H126	-0.38	-	*Haemophilus* sp.	HR
	H365	-0.45	-	*Acinetobacter* sp.	Y
	M400	-0.41	-	Bacteroidaceae	HR

BR-brown root dataset or associated with brown roots, RM- root mat dataset or associated with root mat roots, Y- Yellowing crop dataset or associated with roots of yellowing plants, HR- associated with visibly healthy roots

Bold identity- known pathogen of tomato roots

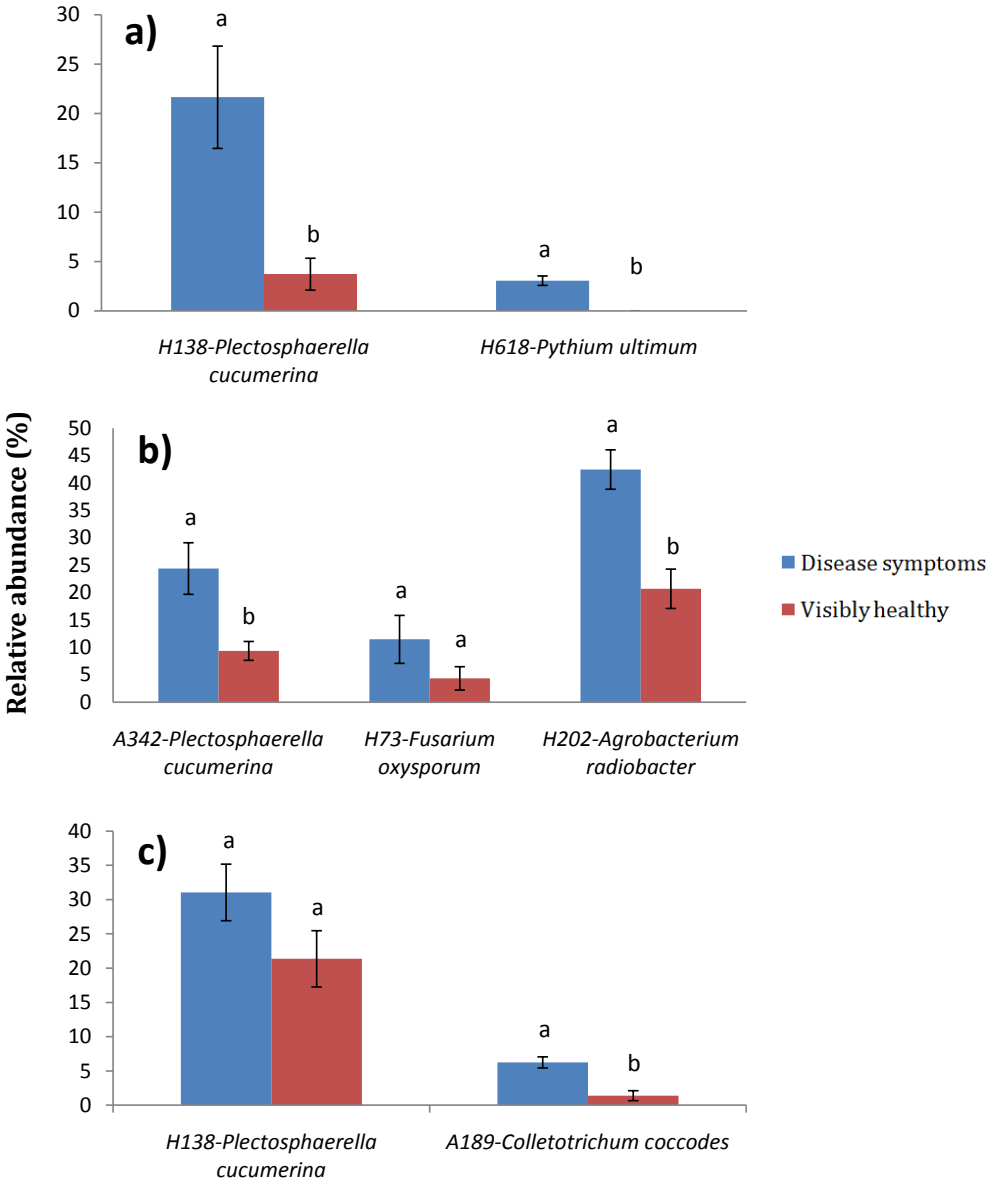

Figure 3.4: Relative abundance of significant T-RFs identified as potential pathogens after PCA analysis of T-RFLP datasets. Brown root dataset (a), root mat dataset (b) and yellowing plant dataset (c). Errors bars represent the standard error of the mean. Different letters represent significant differences in relative abundances of T-RFs from root samples with disease symptoms and their visibly healthy counterparts (p=<0.05)

Figure 3.4 shows that most of the potential pathogens present on crop roots showing symptoms are also present on visibly healthy crop roots. This coincides with the findings from ANOSIM where R-values suggested microbial community overlap between healthy and diseased crops (section 3.3.4.1).

Such findings are not surprising, as all root comparisons were taken from identical crops in the same crop row.

These findings could suggest that the samples of visibly healthy plants are from crops in the early stages of disease. Alternatively it could be that there are potential biocontrol microorganisms present in the community that are preventing pathogens from infecting the visibly healthy plants. Notably, there are some previously reported biocontrol agents identified by PCA analysis (Table 3.14); such as *Sporobolomyces* sp. (Bergstrom and da Luz, 2005) and *Paecilomyces lilacinus* (Kiewnick and Sikora, 2006), as well as plant growth promoting rhizobacteria (PGPR) such as the nitrogen-fixing bacteria *Nitrosomonas* sp*., Nitrosospira* sp. and *Pseudomonas* sp. (Singh *et al*., 2011) which are all associated with the roots of healthy crop comparisons. This is with the exception of an *Acinetobacter* sp. (Singh *et al*., 2011) which is associated with the roots of yellowing plants; however, this is also the dataset with the most microbial population overlap (Table 3.13).

Markedly, in the brown root dataset the pathogen *Pythium ultimum* has been identified by T-RFLP analysis and by culturing methods as the likely causal agent and is only found to be present on disease symptom roots (Figure 3.3, graph (a) H618). Furthermore, a potential biocontrol agent *Pseudomonas* sp. has been identified in this dataset and was found to be associated with healthy roots (Table 3.14); moreover a *Pseudomonas* sp. has been previously shown to reduce levels of *Pythium ultimum* (Warren and Bennett, 1999).

Ultimately, these datasets are snapshots of microbial community assemblages associated with healthy and diseased roots from a single time point, making it difficult to draw clear conclusions from the presence and abundance data. To make significant conclusions about community population effects, communities would need to be studied over time and with more replicates. However, this is not practical in the context of sampling from commercial sites as growers need to treat disease symptoms via chemical or biological control methods quickly, and in severe disease instances removal of the crop may be necessary to prevent further yield loss.

3.3.3.3 Diversity indices and species richness

Species richness and diversity of the species were calculated to compare α-diversity groupings between microbial communities inhabiting the roots of visibly healthy and diseased crops (Table 3.15). In all three datasets, species richness and diversity are higher on diseased roots than healthy root comparisons. This could suggest that higher diversity and species richness in 'plant A' compared to another plant (plant B) from the same environment could indicate pathogenic infection in plant A. These findings do not generally correspond with current theory which indicates higher rhizosphere diversity and species richness being associated with healthy plants (Filion *et al.,* 2004). However, such findings do not account for root damage caused by a pathogen. The increase in diversity could be explained by the colonization of secondary microbes on diseased roots due to the release of utilizable growth substrates from infected damaged tissues (Gardener and Weller, 2001). Notably, the opportunistic weak pathogen *Plectosphaerella cucumerina* is associated with groupings of diseased roots (Table 3.13), suggesting this organism could be taking advantage of higher nutrient levels available around damaged tissues.

Table 3.15: Diversity indices and species richness calculated from T-RFLP datasets for the two rDNA regions under examination, for healthy roots and the roots of plants with disease symptoms.

	ITS2			23S		
Dataset	**S**	**1-D**	**H'**	**S**	**1-D**	**H'**
Brown roots	12.33±1.23	0.8±0.02	2±0.14	14±1.57	0.85±0.01	2.23±0.1
Healthy roots	7±0.63	0.72±0.02	1.56±0.08	8.33±0.21	0.74±0.01	1.7±0.03
Root mat	13.56±1.12	0.86±0.01	2.24±0.08	29.22±2.85	0.91±0.016	2.92±0.14
Healthy roots	11.33±0.9	0.82±0.01	1.98±0.08	25.78±2.85	0.93±0.01	2.92±0.1
Yellowing	18.5±1.76	0.88±0.01	2.47±0.11	39±1.99	0.95±0.01	3.34±0.05
Healthy	18±1.88	0.89±0.02	2.53±0.12	42.38±1.59	0.96±0.01	3.44±0.04

S: Species richness: number of taxa or species present
1-D: Simpson index of diversity: higher values indicating higher diversity
H': Shannon index of diversity: higher numbers indicate higher diversity
±: standard deviation of the average.

3.4 DISCUSSION

Due to the potential biases associated with T-RFLP, and PCR based molecular methods in general, it was important to optimize and test the T-RFLP methods that will be employed throughout this project. Of crucial importance is the establishment of a robust database of T-RFs of known species for accurate phylogenetic identification of community members from T-RFLP profiles.

Once a fairly comprehensive database of fungi and oomycota had been created from previously reported organisms in the tomato rhizosphere and from public sequence databases, it was essential to confirm putative T-RFs. It has been reported that putative fragment length and observed fragment length can differ by 1 to as much as 7bp. Discrepancies between putative and observed fragment length (T-RF drift) have previously affected identification of microbes from environmental samples (Osborn *et al.,* 2000; Kaplan *et al.,* 2001; Kitts, 2001; Kaplan and Kitts, 2003). These biases can be overcome with the creation and validation of robust databases, the use of multiple restriction enzymes to identify potential organisms, multiple technical replicates of community profiles and the study of relative changes in the same ecosystem (Fernández *et al*., 1999).

T-RFLP analysis and sequencing of 40 cultures aided in the confirmation of putative T-RFs of major root pathogens and saprophytes. In addition, the creation of a clone library was useful for the identification of previously undescribed (uncultured) species and for augmenting and improving the T-RFLP database. Notably, from this method it was apparent that the ITS2 primers being used to identify fungi and oomycota were also amplifying the ITS2 regions of other Eukarya in the rhizosphere, namely protozoa and nematodes. Such organisms have an effect on root health, microbial community assemblages and can directly cause root disease; as a key aim of this project is to be able to identify root pathogens, the result of the ITS2 primers being more 'universal' than expected is advantageous in this instance.

From the results establishing appropriate sampling methods, it was found that young roots should be targeted for the examination of tomato rhizosphere microbial communities, as these samples provided good coverage and the major *taxa* present on medium and thick roots were also detectable on thin roots. Furthermore, it has been established that young roots are more likely to be the site of pathogen entry due to higher levels of root exudates and root abrasions caused by active growth (Olivain *et al*., 2006). With regards to sample position, all major organisms detected were present in all sampling positions tested; therefore the chosen sampling method was based on being the quickest method, and having the optimum recovery of young root and least damage to commercial crops. Sampling from midway between soil crops and from corner slabs of rockwool crops was noted to be quicker, less likely to affect plant health and have higher levels of young root.

The optimized sampling methods and T-RFLP analysis protocols were used to detect the presence of causal agents on three crops showing disease symptoms. T-RFLP analysis did detect three pathogens known to cause the disease symptoms observed. Furthermore, plating methods identified the same fungal and oomycete causal agents, further verifying the accuracy of the T-RFLP diagnosis. Notably, some pathogens were also present on healthy crop comparisons, although in lower relative abundances, as well as numerous biocontrol and PGPR associations. However, more data over different time points would be required to establish if the healthy plants developed symptoms or if the presence of the identified biocontrol and PGPR organisms were preventing infection.

In addition to identifying causal agents, T-RFLP protocols identified the same major *taxa* as traditional methods regularly employed to identify root microorganisms, and did so for three common media used for tomato cultivation. This suggests that the optimized methods are suitable for investigating the microbial communities present in the tomato rhizosphere.

4 EFFECT OF GROWTH MEDIA ON RHIZOSPHERE MICROBIAL COMMUNITIES, ROOT HEALTH AND PLANT SURVIVAL

4.1 INTRODUCTION

Production of tomato in temperate regions, such as the United Kingdom, is usually done under greenhouse conditions in soil or hydroponic cultivation systems. Hydroponic systems have become increasingly popular among commercial growers due to increased control over nutrient supply, reduction in soil-borne pathogens and greater comparative yields to soil grown crops (Geraldson, 1982; Jones, 1999).

Hydroponically cultivated crops are grown in nutrient solution, with or without the use of an artificial medium. Due to moves towards carbon-neutral and sustainable methods of crop production, the use of different media in hydroponic systems has become an important issue. The most commonly used medium in such systems throughout Europe is Rockwool (RW; an inert, non-biodegradable substrate), owing to the product's excellent aeration and water-holding properties resulting in consistently high yields. However, because of its high energy production and the high costs involved in its disposal (usually every season), growers are concerned with finding new media that will substitute RW without having negative effects on crop health or yield (Peet and Welles, 2005; Miccolis *et al*., 2007).

Several factors must be considered when selecting media for hydroponic crop cultivation; primarily a medium must provide good aeration, good water-holding capacity but drain freely, be non-toxic and be free from/not encourage disease. Two materials of interest are coir and woodfibre (WF), which are presently waste products of the coconut industry and timber industry respectively. Both media offer a sustainable alternative to RW as they are available in abundance and biodegradable. Furthermore, both media have

been found to offer satisfactory aeration and water-holding capacities (Gruda and Schnitzler, 2004; Mazuela *et al*., 2004; Muro *et al*., 2005).

The media used to cultivate tomato is known to have an effect on the microbial communities which inhabit the rhizosphere and both the media and rhizosphere microbial communities affect plant health and crop yield. In this chapter, the effects of five growth media (soil, RW, nutrient film technique (NFT) solution, coir and WF) are examined throughout the growing season on microbial communities, using T-RFLP and pyrosequencing. In addition, microbial community assemblage data is compared to root health and plant survival assessments.

4.2 METHODS

4.2.1 Routine sampling

Ten commercial crops were examined per growing season in 2009 and 2010. Two crops were sampled from five common media: RW slabs, soil, nutrient film technique (NFT) solution, coir slabs and WF slabs each year using the same commercial nurseries for both seasons. Although it was not possible to use a common variety at all sites, the range of varieties used was kept to a minimum and were all traditional, large-fruited varieties. Root samples from each crop were taken at three time points per year: at 2-4 weeks after rooting into the growing medium (early), around first pick (mid) and in peak production (late). Crop details and dates of root sampling are given in Table 4.1.

At each sampling time point, root samples were collected from three plants in one row. Each sample was split into three sub-samples to provide nine microbial population profiles. Sampled plants were labelled and adjacent plants in the same row were used at sequential sampling time points. Young roots were collected from beneath propagation cubes at early sampling time points in RW, WF, NFT and coir crops and by forking away soil for soil crops. Sample collection methods for mid and late sampling time points are as described in section 2.3. For details of root recovery see section 2.4.

Table 4.1: Details of tomato crops monitored in 2009 and 2010

Growing medium and dataset code	Date planted	Sampling time points			Final assessment
		Early	Mid	Late	
RW					
1	Mid Dec	06-Jan	04-Mar	11-Aug	26-Oct
2	Mid Dec	07-Jan	21-Apr	25-Sep	12-Nov
3	Mid Dec	29-Jan	20-Apr	17-Aug	25-Oct
4	Early Jan	01-Feb	19-Apr	18-Aug	27-Oct
Soil					
1	End Feb	15-Apr	23-Jun	12-Aug	11-Nov
2	End Mar	04-Mar	13-May	12-Aug	11-Nov
3	Early Feb	09-Feb	11-May	10-Aug	15-Oct
4	End Jan	31-Mar	26-May	25-Aug	15-Oct
NFT					
1	Mid Dec	12-Jan	24-Mar	12-Aug	29-Oct
2	End Dec	22-Jan	07-Apr	05-Aug	29-Oct
3	Mid Dec	22-Jan	08-Apr	16-Aug	25-Oct
4	End Dec	15-Apr	06-Jul	05-Oct	25-Oct
Coir					
1	Mid Jan	05-Feb	23-Mar	10-Aug	27-Oct
2	Mid Jan	03-Mar	20-Apr	24-Sep	17-Nov
3	Mid Jan	25-Feb	20-Apr	05-Aug	21-Oct
4	Mid Jan	17-Mar	10-May	09-Sep	09-Nov
WF					
1	Mid Dec	28-Jan	04-Mar	11-Aug	26-Oct
2	Mid Dec	03-Mar	20-Apr	24-Sep	17-Nov
3	Mid Dec	29-Jan	20-Apr	17-Aug	25-Oct
4	End Jan	10-Mar	18-May	17-Aug	15-Oct

1-2: crop samples in 2009, 3-4: crop samples in 2010

4.2.2 DNA extraction and T-RFLP analysis

Total community DNA was extracted from all root samples using the procedures described in section 2.5, followed by PCR amplification of ribosomal DNA (rDNA; section 2.6), restriction digestion (section 2.7) and T-RFLP analysis (section 2.8).

From the resulting T-RFLP profiles, putative taxonomic identities of T-RFs were assigned by importing T-RFLP profile information into FRAGSORT version 5.0 (Michel and Sciarini, 2003). The null hypothesis (H_0) of there being no difference in microbial community assemblages between different crop media and no difference in microbial community assemblages between different

sampling times was tested by Analysis of Similarities (ANOSIM; section 2.9.1). T-RFLP data were also represented in an ordinational space with Principal Component Analysis (PCA; section 2.9.2) and α-diversity and ß-diversity was calculated using species richness and diversity indices (section 2.9.3).

4.2.3 Pyrosequencing analysis

In an attempt to identify unknown T-RFs and to further characterize the microbial ecology of the tomato rhizosphere from different media, DNA extracts from all samples (Table 4.1) were further analysed by pyrosequencing following the procedures discussed in section 2.14. Pyrosequencing data was analysed using the methods described in section 2.15. Two different analyses were conducted, namely phylogenetic analysis (section 2.15.1) for the phylogenetic assignment of pyrosequencing reads, and operation taxonomic unit (OTU) analysis (section 2.15.2) allowing the examination of microbial community assemblages with PCA and estimations of species richness. Furthermore, the use of the same DNA extracts for both T-RFLP and pyrosequencing analysis allowed comparisons to be made between the results obtained by the two molecular methods (section 2.15.3).

4.2.4 Crop assessments

At each sample time point, the three plants from which root samples were taken were examined for leaf yellowing, wilting, stem disease and root appearance. Towards the end of cropping, all nine plants were assessed for plant health (alive or dead), vascular staining in the stem base, and root appearance. Where there was obvious root decay or discolouration, samples of roots were examined by ADAS via microscopy and/or by culturing on agar to determine the identity of fungi associated with different symptoms. Plants in the same row as monitored plants and with symptoms of poor growth attributable to root disease were also examined as above to determine identity of fungi associated with roots. Dates of the final crop assessment are given in Table 4.1.

Data from the occurrence of dead plants, vascular browning in the stem base, root decay and discolouration were used to calculate a plant sickness score (range 0-27; based on numbers of dead plants and vascular staining in the stem) and a root rot score (range 0-12; based on severity scores calculated from numbers of plants with decay or discolouration or corkiness of major and minor roots). Scores were determined by assessing the numbers of plants with different symptoms and using a weighting factor (x2) for the most severe symptoms (dead plants and decay of major roots). Further details of calculations used to determine plant sickness and a root rot scores are available in Appendix II. Data for 2009 and 2010 Simpson diversity scores were combined with plant sickness and root scores and examined by linear regression analysis to provide 20 data sets to determine if there was a link between microbial diversity and plant health. Analysis was performed in GenStat 14th edition (Payne et al., 2011).

4.3 RESULTS

It has been previously documented that different microflora inhabit the root environment of tomato plants grown in soil compared to hydroponic systems (Price, 1976). However, the results from Price's (1976) study are limited to culturable eukaryotic organisms and are applicable to young seedlings only; furthermore, there is little indication of the influence of these microorganisms on diseases or plant health in general.

This section aims to determine whether there are differences in total rhizosphere microbial communities grown in different media over different plant growth stages (sample times), using molecular methods. In addition, it examines microbial community assemblages along with root health and plant survival assessment data to determine if microbial diversity or the presence or abundance of certain microbial constituents affects plant health.

4.3.1 T-RFLP analysis

4.3.1.1 Testing null hypotheses using ANOSIM

ANalysis of SIMilarities (section 2.9.1) were performed on T-RFLP datasets to test the null hypothesis (H_0) that there were no differences in the microbial communities present on the roots in different media and that there were no differences in microbial communities at different sampling times (crop growth stages). H_0 was tested on ITS2 and 23S rDNA T-RFLP datasets as shown in Table 4.2.

From the ANOSIM analysis, both null hypotheses were rejected, indicating that there were significant differences in microbial community structures between communities inhabiting the roots in different media and differences between sampling time points, which is in agreement with findings in other studies (Price, 1976; Menzies *et al*., 2005; Morgan *et al*., 2005; Cavaglieri *et al*., 2009; Baumann *et al*., 2011)

Table 4.2: ANOSIM test values and probabilities of null hypothesis tests obtained from comparisons of T-RFLP datasets of ITS2 and 23S rRNA genes of samples from tomato roots grown in different media (H_0 Media) and taken at different sampling times (H_0 Time).

	ITS2		23S	
	R-values	**p-values**	**R-values**	**p-values**
H_0 Media	0.75	<0.01	0.77	<0.01
H_0 Time	0.60	<0.01	0.22	<0.01

Although microbial communities are significantly different between T-RFLP datasets there are varying levels of community overlap, with 23S rDNA markers giving higher R-values than their corresponding ITS2 R-values in media datasets, indicating that rhizosphere bacterial populations were more variable between different media than eukaryotic populations. However, the opposite is true for sampling time suggesting that few changes occur in bacterial populations over time compared to eukaryotic communities. Furthermore, R-values were lower when comparing microbial assemblages with different time datasets than media datasets indicating that microbial assemblages are more variable between media than over time. According to parameters set by Clarke and Gorley (2001), R-values generated from testing the H_0 time dataset show that the communities are separated but overlapping, for eukaryotic communities and barely separated for bacterial communities. R-values generated from testing the H_0 media dataset can be interpreted as well separated for microbial communities.

4.3.1.2 PCA analysis of media and time T-RFLP datasets

T-RFLP datasets were further analyzed using normalized data (section 2.8) for Principal Component Analysis (PCA; section 2.9.2) to view transformed microbial community assemblages in a two dimensional space (Figure 4.1).

PCA plots show that eukaryotic communities from crops grown in hydroponic systems with solid substrates are grouped together (Figure 4.1: graph a; RW: dark blue diamond; coir: light blue asterisk; WF: purple cross), whereas soil (red square) and NFT (green triangle) communities are grouped relatively

separately. This suggests that hydroponic systems with media have relatively similar eukaryotic community assemblages compared to soil and NFT systems which do not group with any other media.

These results are perhaps not surprising as soil is chemically and physically complex and different to hydroponic systems, ultimately affecting which organisms can thrive in each environment. In the case of NFT systems, it could be explained by the lack of a solid substrate which can act as physical shelter and space for eukaryotic growth. The least expected result was for RW principal component (PC) scores to be similar to the organic substrate (coir and WF) PC scores, as it was reasonable to hypothesize that an inert non-biodegradable substrate such as RW would be inhabited by a different eukaryotic community. However, the beneficial physical properties of RW may counteract the inert nature of the media making this substrate conducive to eukaryotic development. Ultimately, these results suggest that all three hydroponic systems with solid substrates are conducive to relatively similar eukaryotic community development.

Similar results are found for bacterial media dataset PCA plots (Figure 4.1: b), with the exception of soil (red square) mean PC scores being grouped closely to hydroponic systems with media (RW: dark blue diamond; coir: light blue asterisk; WF: purple cross), with NFT (green triangle) hydroponic mean scores being grouped separately. An explanation for this could be that bacteria are heavily reliant on solid substrates for physical shelter and in NFT systems the roots are constantly being washed with nutrient solution, which could make this niche difficult for bacterial community development (Gregory, 2006).

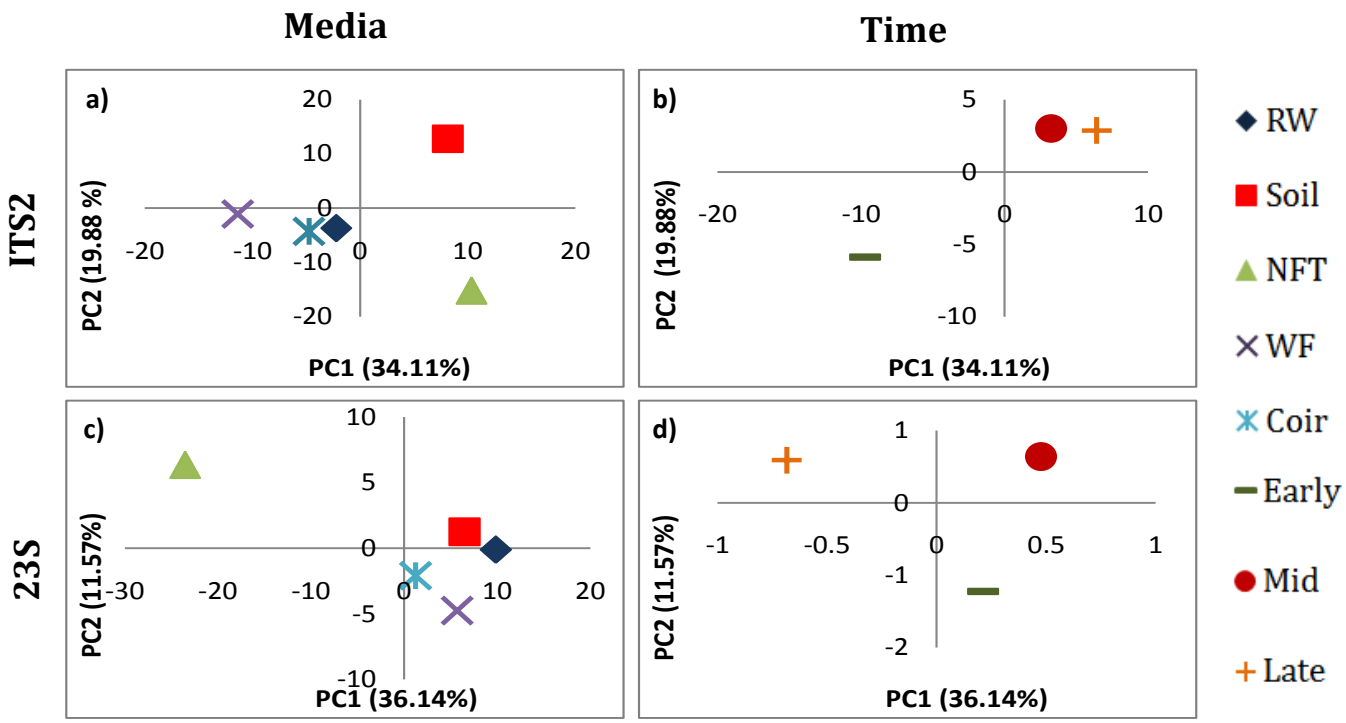

Figure 4.1: PCA ordination plots of microorganisms inhabiting the rhizosphere of tomato crops grown in different media (a, c) and the roots of plants at different sampling times (b, d) using T-RFLP profiles of ITS2 (a, b) and 23S rRNA genes (c , d). Dark blue diamonds represent the mean PC values from RW samples, red squares represent soil samples, green triangles represent NFT samples, purple crosses represent WF samples and light blue asterisks represent coir samples. Dark green dashes represent the mean PC values from early time points, red circles represent mid time points and orange plus signs represent late time points.

With regards to the microbial community assemblages based on time datasets, it can be seen that the eukaryotic community changes more between the early time points (Figure 4.1: graph b; early: dark green dash) than between mid (red circle) and late (orange plus sign) time points. Similar findings have been described in other studies and have been attributed to the initially sterile nature of hydroponic systems rapidly being colonized by eukaryotic communities with the addition of plant material until a stable community is formed, leading to subtle shifts in community structures with time and plant development (Berkelmann *et al.,* 1994; Postma *et al*., 2000; Menzies *et al*., 2005). For soil communities it could be that the tilling and surface sterilization methods used at the start of the growing season disrupt the natural microflora resulting in rapid microbial community stabilization and structural changes with the addition of plants between early time points and mid time points and again more subtle shifts in the population between mid and late time points.

For the PCA plot of bacterial community assemblages over time (Figure 4.1:d), it would seem that the bacterial community changes at all sampling time points, which does not agree with the ANOSIM result of there being barely any separation between bacterial communities over time. However, ANOVA results of PC scores imply that the groupings visualized in Figure 4.1 graph d are not significant with time as a factor (PC1 $p = 0.84$; PC2 $p= 0.50$), implying that the groupings are not significant or are the result of other variables not under examination. All other PC scores (both PC1 and PC2) with time and media as factors were significant ($p=<0.05$), suggesting that the visualized groupings in Figure 1 graphs a, b and c are significant microbial community changes with media and time.

PCs identified as significant with media and time as factors were further analyzed by determining which loading values were significantly contributing to groupings (section 2.9.2). From these significant loading values, the enzyme and T-RF combinations were identified and compared to the output of FRAGSORT (section 2.8), resulting in a likely organism identity. Based on

whether a loading value is positive or negative, organism identities can be associated with the groupings on PCA ordination plots, suggesting that the presence and/or relative abundance of the organism in question is significantly contributing to groupings on that PC.

For eukaryotic community data PC1 and PC2 identified six enzyme and T-RF combinations that were significantly contributing towards PC groups (Table 4.3). From Fragsort output data, incorporating clone library results and pyrosequencing analysis results; four potential organisms were identified as matching significant T-RFs; these were *Penicillium* sp. (A324), *Plectosphaerella cucumerina* (A341/H138), *Gliocladium* sp. (A341/H154) and *Pythium* sp. (A384/H205).

Based on loading values (Table 4.3) it can be seen that *Pythium* sp. and *Gliocladium* sp. are associated with hydroponically grown roots and early time points, *Plectosphaerella cucumerina* is associated with all factors and *Penicillium* sp. are associated with mid and late time points in soil, coir and NFT systems. These results are confirmed by mean abundances of these peaks from T-RFLP normalized datasets (Figure 4.2), whereby higher relative abundances are associated with the appropriate groupings on PCA plots.

Table 4.3: Eukaryotic organisms identified by significant PC loadings (PC1, PC2) contributing to significant PCs, their T-RF and enzyme combination and which factor (media or time point) they are associated with based on their PC loading value.

Enzyme/T-RF	PC1	PC2	Potential Identity	Media	Time
A324	-	-0.41	*Penicillium* sp.	R/C/W	E
A341	0.32	-0.27	*Plectosphaerella cucumerina/ Gliocladium* sp.	ALL	ALL
A384	-0.75	-	*Pythium* sp.	R/N/C/W	E
H154	-	-2.6	*Gliocladium* sp	R/C/W	E
H138	0.25	-0.45	*Plectosphaerella cucumerina*	ALL	ALL
H205	-0.39	-0.32	*Pythium* sp.	R/N/C/W	E

R= rockwool; S= soil; N=NFT; C=coir; W= woodfibre; E=early; M=mid; L=late

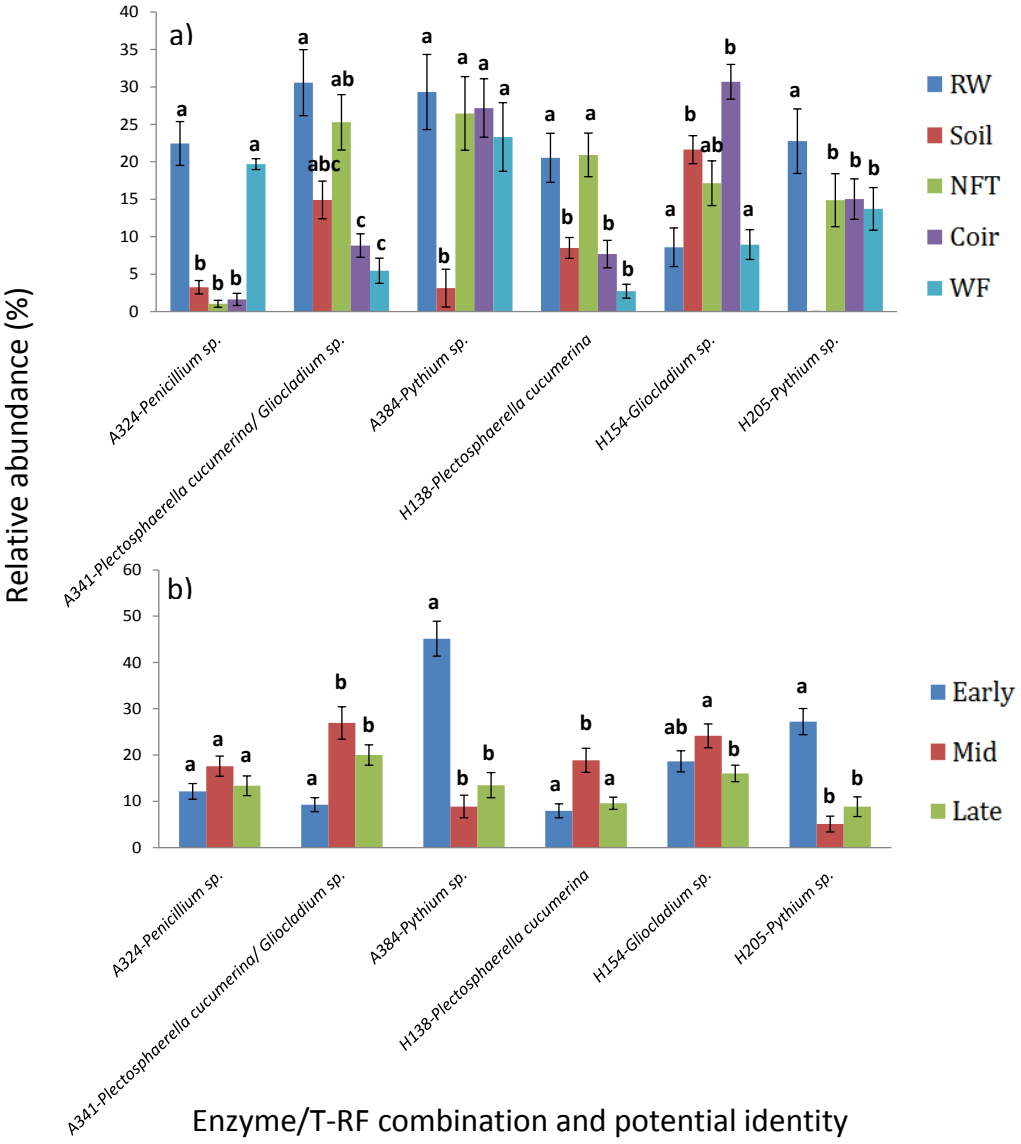

Figure 4.2: Relative abundance of enzyme and T-RF combinations that had a significant effect on PC groupings from PCA analysis of T-RFLP ITS2 media and time datasets. Graph a) shows media dataset and b) represents the time dataset. Error bars represent the standard error of the mean. Different letters represent significant differences in relative abundances of T-RFs (p=<0.05). A= *Alu*I; H= *Hae*III.

PC1 and PC2 identified five enzyme and T-RF combinations that were significantly contributing towards PC groups (Table 4.4) from bacterial community data. From Fragsort output data four potential organisms were identified as matching significant T-RFs; these were Alphaproteobacteria (H168), *Agrobacterium radiobacter* (H201), Gammaproteobacteria sp. (H375/M375) and *Rhodobacter sphaeroides* (M312).

Based on loading values (Table 4.3) it can be seen that an Alphaproteobacteria sp. is associated with RW, soil and NFT, confirmed by higher relative abundances in Figure 4.3. Gammaproteobacteria was the class identified from the in silico database for the combination H375/M375 which are uncut by either enzyme. However, the organism is associated with NFT for one enzyme/T-RF combination and all media for the other, suggesting that this is perhaps not the correct identification of the organism contributing to these T-RFs. Notably on Figure 4.3 H375 and M312 give similar abundance profiles between media, perhaps indicating that the organism contributing to PC groups is an unidentified prokaryote with H375/M312 combination. Only *Rhodobacter sphaeroides* gives a peak at around M312; however if the above assumption is true this organism may also be wrongly identified. *Agrobacterium radiobacter* (H201) was identified as being associated with Coir and WF crops; however it is present in all media and only significantly higher in coir crops (Figure 4.3).

Bacterial species identification was more difficult and was often not specific to a genus or species level as many organisms gave similar enzyme T-RF combinations from the same class. Furthermore, with there being no clone library or pyrosequencing data for bacterial peaks, all identities were based on *in silico* digestion of previously published sequences resulting in a less refined taxonomic identification.

Table 4.4: Bacterial organisms identified by significant PC loadings (PC1, PC2) contributing to significant PCs, their T-RF and enzyme combination and which media they are associated with based on their PC loading value.

Enzyme/T-RF	PC1	PC2	Potential Identity	Media
H168	0.42		Alphaproteobacteria	R/S/N
H201		-0.51	*Agrobacterium radiobacter*	C/W
H375	-0.6		Gammaproteobacteria	N
M312	-0.28		*Rhodobacter sphaeroides*	N
M375	-0.55	0.72	Gammaproteobacteria	ALL

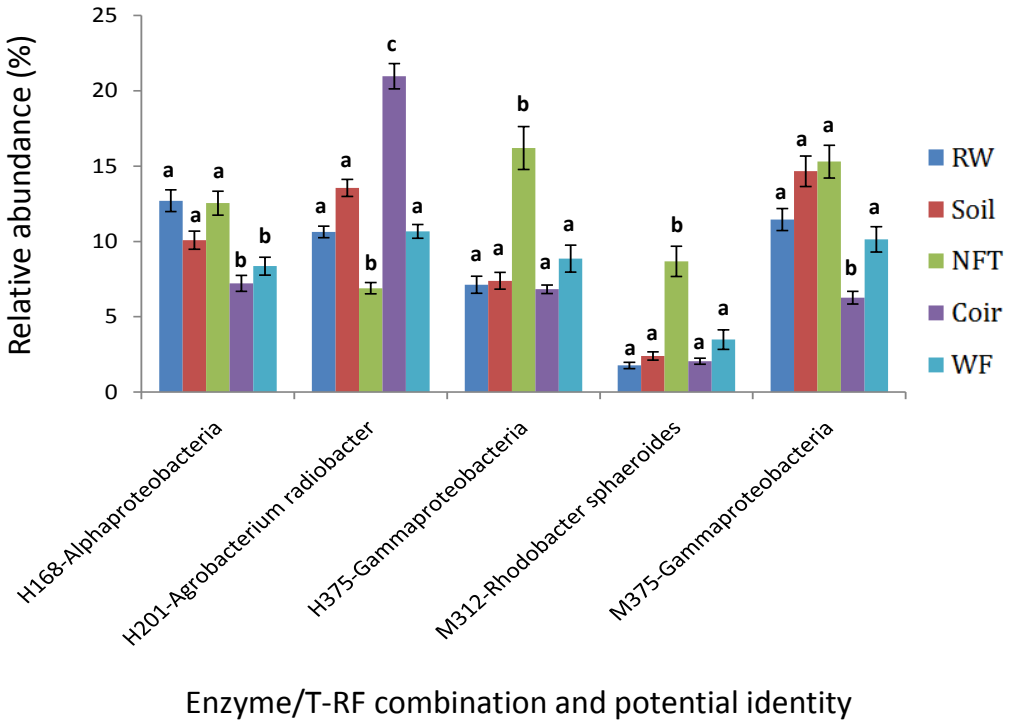

Figure 4.3: Relative abundance of enzyme and T-RF combinations that had a significant effect on PC groupings from PCA analysis of T-RFLP 23S rDNA media datasets. Error bars represent the standard error of the mean. Different letters represent significant differences in relative abundances of T-RFs (p=<0.05). H= *Hae*III; M= *Mse*I.

From Figure 4.2 and Figure 4.3 it is clear that organisms responsible for microbial groupings are often present in all media but with different relative abundances. This finding does not fit in with the parameters set by Clarke and Gorley (2001) for ANOSIM results, that suggest that R-values >0.75 can be interpreted as well separated. Clearly there is some level of microbial community overlap with certain T-RFs being present in all media. Despite this overlap, differences in microbial communities associated with the root from

different media have been found to be significantly different with ANOSIM and PCA analyses.

4.3.1.3 Diversity of microbial communities using media and time T-RFLP datasets

Species richness and diversity of microbial communities were calculated using the number of taxa and the diversity indices Simpson index (1-D) and Shannon index (H'), to compare α-diversity and ß-diversity between microbial communities inhabiting the roots of crops grown in different media and at different crop growth stages (Table 4.5).

NFT has the least diversity compared to other media in both the eukaryotic and prokaryotic communities, further implying that the lack of solid matrices that provide physical protection and space for microbial growth makes this medium less conducive for microbial community development.

For both communities, soil has the highest diversity scores and number of taxa present; this result is in part in agreement with Price's (1976) and Menzies *et al*. (2005) who found higher numbers of species present in soil than hydroponic culture. The Menzies *et al*. (2005) study also concluded that there was a significant increase in fungal diversity among root substrates over time; this result was also established in this study. Higher eukaryotic community diversity with time can be partly attributed to the sterile nature of most media at the start of the growing season and the rapid colonization that occurs soon after planting (Postma *et al*., 2000). Furthermore, the diversity may in part be due to potential increases in sloughed off cells and changes in root exudation with plant age, potentially creating more conducive conditions for fungal growth (Halmen *et al*., 1972; Jaeger *et al*., 1999).

A similar result was expected for bacterial communities and indeed there was an increase in the number of taxa present at all time points and an increase in diversity between early and mid time points. However, these changes were not found to be significant when tested by ANOVA. This is in agreement with the findings from PCA analysis, yet it is difficult to hypothesize why bacterial

community structures seem to be less responsive to crop growth stage or sample time. This method of analysis seems to imply that once a stable bacterial community is established in the rhizosphere, presumably within the first 2-4 weeks (early sampling time point), the community does not change significantly in diversity or species richness during the three crop growth stages examined.

Table 4.5: Mean diversity indices and species richness calculated from T-RFLP datasets for the two rDNA regions under examination, for all media and time points under examination

	ITS2			23S		
	S	**1-D**	**H'**	**S**	**1-D**	**H'**
Media						
RW	5.14±0.35	0.51±0.02	1.08±0.06	12.34±0.59	0.83±0.01	2.10±0.06
Soil	5.81±0.25	0.66±0.01	1.34±0.04	14.41±0.81	0.85±0.02	2.21±0.08
NFT	4.39±0.23	0.50±0.02	0.98±0.05	10.65±0.85	0.76±0.03	1.86±0.11
Coir	5.78±0.43	0.54±0.02	1.16±0.07	13.85±0.64	0.85±0.01	2.22±0.05
WF	5.15±0.25	0.51±0.02	1.15±0.05	14.31±0.95	0.84±0.02	2.24±0.1
p-values	NS	<0.01	<0.01	<0.01	<0.01	<0.01
Time						
Early	4.86±0.24	0.50±0.02	1.04±0.04	12.97±0.3	0.82±0.01	2.12±0.03
Mid	5.40±0.24	0.55±0.02	1.12±0.04	13.29±0.33	0.83±0.01	2.13±0.03
Late	5.88±0.26	0.57±0.02	1.16±0.04	13.31±0.34	0.83±0.01	2.13±0.03
p-values	<0.05	<0.01	<0.01	NS	NS	NS

S: Species richness: number of taxa or species present
1-D: Simpson index of diversity: higher values indicating higher diversity
H': Shannon index of diversity: higher numbers indicate higher diversity
±: Standard deviation of the average.
NS: No significance

4.3.1.4 Potential pathogens associated with the roots of tomato, grown in different media identified by T-RFLP

The identification of species from T-RFs using the database of *in silico* digestions found potential eukaryotic pathogens in all growth media, with different potential pathogens being associated with different media types (Table 4.6). However, *Plectosphaerella cucumerina*, a known cause of root and stem rot, and *Colletotrichum coccodes*, the cause of black dot, were found in all media, and in certain samples appeared to be the most abundant

organisms in eukaryotic populations (Table 4.12). These organisms are considered weak pathogens that primarily affect plants near the end of cropping (Blancard, 1994), but the presence of either of these organisms did not generally result in disease symptoms in the end of season crop assessments (discussed in section 4.4). Due to the common occurrence of these organisms in all media types, their constant presence warrants further investigation to establish how different relative abundances impact root health and to determine what conditions trigger pathogenesis.

Table 4.6: Potential fungal pathogens found associated with roots of 20 commercial tomato crops in UK in 2009 and/or 2010 using T-RFLP

Potential fungal species	Disease common name	Crops detected in:	
		No. (of 20)	Growing Medium
Botrytis cinerea	Grey mould	1	coir
Colletotrichum coccodes	Black dot	9	all
Fusarium sp.	-	6	RW, soil, NFT, WF
Humicola fuscoatra	-	1	coir
Plectosphaerella cucumerina	-	17	all
Phytophthora sp.	-	4	RW, coir, WF
Pyrenochaeta lycopersici	Corky root rot	4	soil , WF
Pythium sp.	-	7	RW, NFT, coir, WF
Spongospora subterranea	Powdery scab	1	coir
Thielaviopsis basicola	Black root rot	1	NFT, WF
Verticillium sp.	-	2	soil

Species of *Pythium* and *Fusarium* were found in many of the crops tested (seven and six crops out of twenty, respectively), sometimes at relatively high abundance levels (Table 4.12). Pythium root rot and fusarium crown and root rot symptoms were present in some crops where these fungi were found but not in others; surprisingly there was no link between relative abundance and disease and not enough data to establish what other factors could be affecting disease occurrence (discussed further in section 4.4). However, there have been reports of non-pathogenic *Fusarium* species protecting plants from pathogenic species of *Fusarium* (Olivain and Alabouvette, 1997), and T-RFLP based on the rDNA does not distinguish between pathogenic and non-pathogenic species. Furthermore, symptomless infection with *Pythium* has been previously reported, where it was noted to affect plant growth without the expression of symptoms on host roots (Stranghellini and Kronland, 1986).

Again, further investigation with these pathogens in more controlled experimental environments may help establish why disease develops in some crops and not in others.

Interestingly, there were differences in the pathogens identified in soil and hydroponic systems, with soil-borne fungal pathogens such as *Fusarium* sp. and *Verticillium* sp. being more common in soil, and pathogenic Oomycota species being more common in hydroponic systems. These findings are likely to be due to evolutionary adaptations allowing certain pathogens to thrive in different environments. For instance, Oomycota that produce zoospores, like *Pythium* sp. and *Phytophthora* sp., are adapted well to liquid environments, whereby they can actively swim towards their hosts and can cause an epidemic in favourable conditions (Stanghellini and Rasmussen, 1994).

4.3.2 Pyrosequencing analysis

To further characterize and compare the microbial communities of the rhizosphere in different media, all samples in Table 4.1 were analyzed using pyrosequencing technology. A total of 58,373 PCR amplicons that span the ITS1 region were sequenced, of which only 1454 qualified for further analysis after trimming of low quality reads, and of those 1397 were found to be unique sequences (Table 4.7). Trimmed reads were selected for further analysis using *de novo* OTU analysis (section 2.15.2) and phylogenetic analysis (section 2.15.1) methods.

Table 4.7: Data summary of total reads from pyrosequencing analysis. Values under trimmed tags are the numbers of reads remaining after the removal of primers and low-quality data. Values under unique tags are the numbers of distinct sequences within a set of trimmed tags.

Media	Total Reads	Trim	Unique
Coir	13101	189	186
NFT	13947	423	385
RW	9787	166	165
Soil	6974	200	192
WF	14928	476	469

4.3.2.1 OTU analysis

To determine the number of species found in each media and to evaluate sampling methods, each trimmed read was clustered using sequence tags into groups of defined sequence variation that ranged from unique sequences (no variation) to 10% differences by using Mothur (Schloss *et al*., 2009). These clusters served as OTUs for generating rarefaction curves (Figure 4.4) and for making calculations with the abundance-based coverage estimator (ACE) and the Chao1 estimator of species diversity (Table 4.8).

The rarefaction curves in Figure 4.4 were used to evaluate species richness by plotting OTUs versus the number of tags. The rarefaction curves predict that additional sampling will lead to significantly increased estimates of total diversity, as in each dataset curves did not reach a plateau even when relatively large genetic distances (5% or 10% difference) defined similarity groups.

Results from ACE and Chao1 species richness estimators suggest similar findings to rarefaction analysis, with these indices showing that the species richness estimations were often one order of magnitude higher than the number of OTUs (Table 4.8). As these indices constantly estimated a higher number of OTUs, it can be concluded that further sampling is needed for full identification of the number of species present in each medium.

However, such high estimates of diversity for the tag sequences have been suggested to be affected by the small size of PCR amplicons and by *de novo* protocols that do not require the comparisons with a database (Sogin *et al*., 2006). Nevertheless, it can be assumed that an increased number of samples would correspond to a higher number of sequences, resulting in higher species richness detected in all five media.

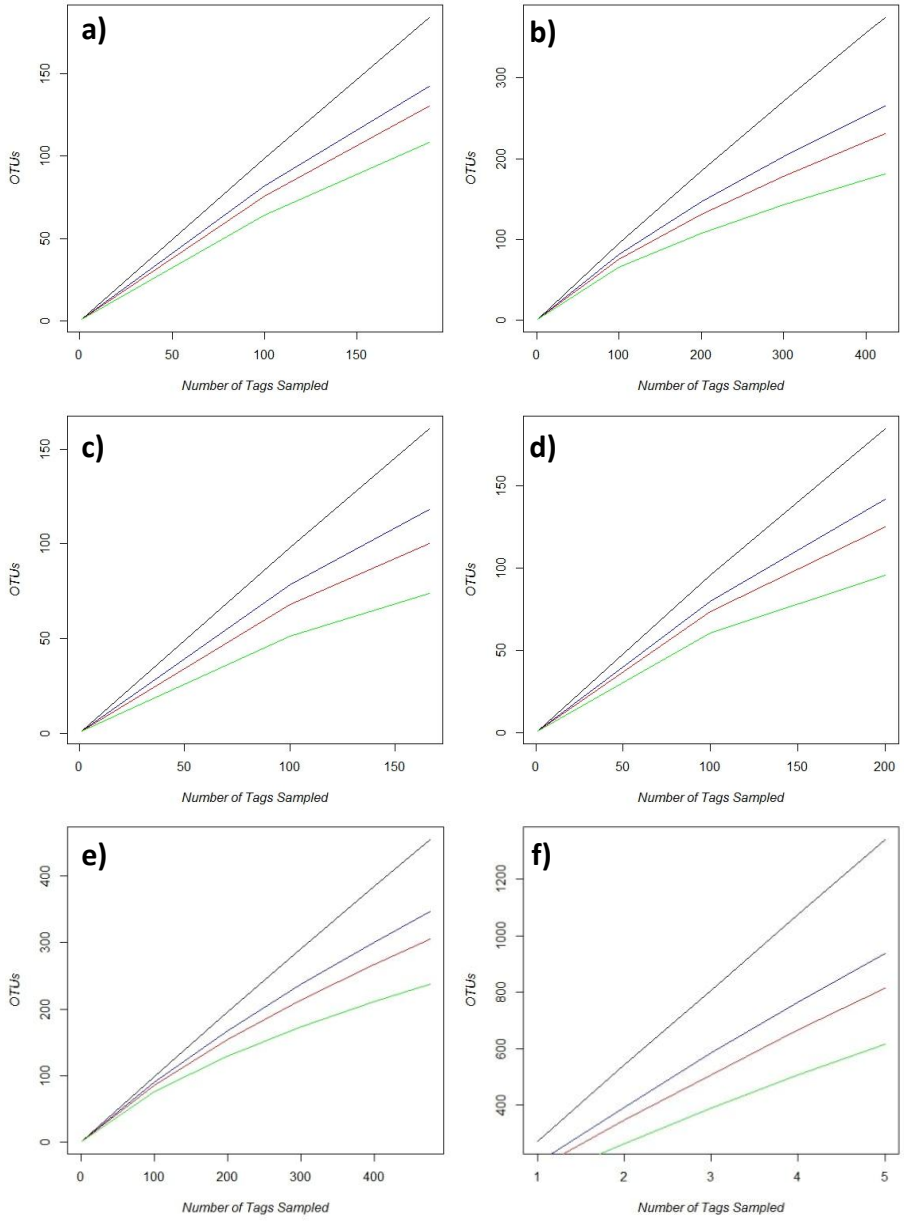

Figure 4.4: Rarefaction curves of pyrosequencing data obtained from the amplification of the ITS1 region of eukaryotes associated with the rhizosphere of tomato crops grown in a) coir b) NFT systems c) RW d) soil and e) WF. Graph f) represents reads generated from all media. Curves indicate the relationship between reads (x axes) and number of OTUs (y axes). Black represent unique sequences, blue are clusters with 0.03 dissimilarity, red 0.05 dissimilarity and green 0.10 dissimilarity.

Table 4.8: Similarity-based OTUs and species richness estimates for OTUs with differences that do not exceed 3%, 5%, or 10%.

		0.03			0.05			0.10		
Media	**Reads**	**OTUs**	**ACE**	**chao1**	**OTUs**	**ACE**	**chao1**	**OTUs**	**ACE**	**chao1**
COIR	189	138	1891	802	126	1262	665	105	1339	655
NFT	423	257	1729	799	228	1637	776	174	979	550
RW	166	113	642	447	95	710	313	72	444	195
SOIL	200	141	903	521	124	518	316	93	292	207
WF	476	342	2300	1359	299	1878	1049	229	848	528

4.3.2.2 Phylogenetic analysis

To compare samples and to identify which groups of Eukarya are associated with roots grown in each medium, pyrosequencing reads were identified by alignment against ITS1 reference sequences based upon the fungal phylogenetic tree developed by James *et al*. (2006) and best BLAST matches. Best matches of the BLAST searches were likely to be closely related species, and to confirm these relationships and to group unidentified Eukarya from BLAST results, a phylogenetic tree was constructed (Figure 4.5) in MEGA5 employing the maximum likelihood method (Felsenstein, 1981) based on the Tamura-Nei model (Tamura and Nei, 1993) following the methods in section 2.15.1.

In Figure 4.5; all reads can be identified by green filled (previously identified taxa) or unfilled (unidentified taxa) dots. A total of 58 reads were uniquely grouped on the tree (7 coir; 15 NFT; 8 RW; 8 Soil; 20 WF), with 35 sequences matching taxonomically classified eukaryotes and 23 matching unclassified eukaryotic clones or not matching any previously published sequences on NCBI database. This suggests that 40% of unique pyrosequencing reads belong to undescribed eukaryotic taxa.

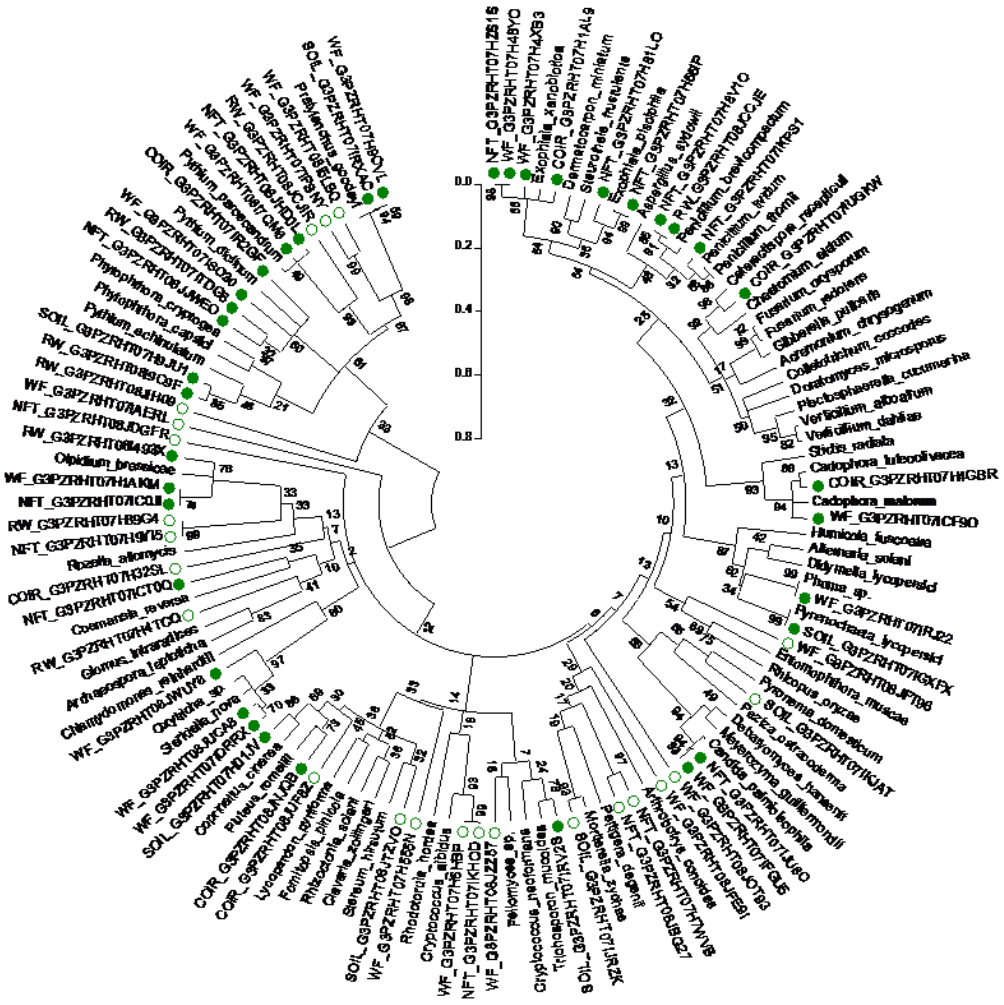

Figure 4.5: Phylogenetic tree of 58 unique sequences (green filled and no fill dots) selected from pyrosequencing data and 67 references sequences based on James *et al*. (2001) fungal tree of life using Maximum Likelihood method on the Tamura-Nei model (Tamura and Nei, 1993). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). Filled green dots represent sequences with known taxa (best BLAST matches are closest reference sequence) and green dots with no fill represent sequences of undescribed taxa (no best BLAST matches or uncultured best BLAST matches). The media each sequence is associated with is stated in the sequence name.

The number of unique sequences identified using these methods (section 2.15.1) are significantly less than those identified in Table 4.8 from the OTU analysis. This is in agreement with Sogin *et al*. (2006) who suggested that using tag sequences in cluster analysis can over-estimate species richness and this was found when certain unique clusters had identical best BLAST matches and were found to group together in phylogenetic trees. However, the results for the phylogenetic analysis will underestimate species richness as sequences which did not cluster with other sequences were not incorporated into the analysis.

From the ITS1 references on the phylogenetic tree (Figure 4.5) it can be seen that eukaryotic reads belong to four kingdoms, namely Fungi, Chromista, Protozoa and Anamalia. There are more species of Fungi present than any other kingdom followed by Chromista belonging to the Phyla Oomycota, both of which are present in the root environment of all five media. From the kingdom Protozoa, two unique species belonging to the Phyla Ciliophora are found in WF crops. One species from Anamalia was identified belonging to the Phyla Nematoda and was present in soil and WF crops.

Fungi identified on the roots belong to the Phyla Ascomycota, Basidiomycota, Zygomycota, and Chytridiomycota, some of which vary in which media they are associated with, detailed in Table 4.9. Notably not all unique reads in all media are accounted for in Table 4.9 as not all reads had a best BLAST match or grouped with reference sequences. Phylogenetic identities to the genus and species level and relative abundances in each media are discussed in section 4.3.3 in comparison to T-RFLP results.

Table 4.9: Kingdom and Phyla associated with the tomato roots grown and the number of unique sequences from each Phylum in each medium

Kingdom/Phylum	Fungi				Chromista	Protozoa	Animalia
	A	B	C	Z	Oomycota	Ciliophora	Nematoda
Coir	3	2			1		
NFT	8	1			2		
RW	1		1	1	3		
Soil	2	3		1	1		1
WF	8	3			4	2	1

A: Ascomycota; B: Basidiomycota; C: Chytridiomycota; Z: Zygomycota

4.3.3 Comparison of results obtained from T-RFLP and Pyrosequencing analysis

The fingerprinting method T-RFLP and the sequence-based method pyrosequencing were both used in this chapter to study eukaryotic populations associated with roots grown in each medium. Results from both analyses were compared using the number of OTUs obtained from each medium (Table 4.10), media groupings on PCA plots (Figure 4.6) and also a comparison was made from phylogenetic identity and abundance data for each medium (Figure 4.7).

Table 4.10 shows the number of OTUs obtained from pyrosequencing cluster analysis and the total number of taxa identified by T-RFLP analysis. T-RFLP and pyrosequencing data were very similar and both found that over the whole season a higher number of eukaryotic taxa were associated with WF and NFT roots and the least number of different taxa were associated with RW crops. These results could imply that more taxa can utilise the roots as substrates in the WF and NFT systems. However, it is more likely that the eukaryotic communities in these systems were less stable and prone to greater shifts in community constituents with time. Notably, the mean number of taxa present in each sample is not highest in NFT or WF crops (Table 4.5), which would support the latter theory of greater community shifting in WF and NFT crops as opposed to the two media being more conducive to eukaryotic growth.

Overall, both pyrosequencing and T-RFLP OTU data give similar results, with pyrosequencing data estimating approximately three times more species than T-RFLP, even at larger genetic distances. This suggests that pyrosequencing is a more sensitive method allowing more in depth examination of the number of eukaryotic taxa associated with tomato roots. Notably OTU data for pyrosequencing analysis does not involve the removal of data contributing to <1% of the community, as is the case for T-RFLP analysis, which will account for some of the increase in sensitivity.

Table 4.10: Total number of OTUs obtained from T-RFLP and pyrosequencing analysis of eukaryotic communities

	T-RFLP	Pyrosequencing cluster distances		
		0.03	0.05	0.10
COIR	55	138	126	105
NFT	61	257	228	174
RW	47	113	95	72
SOIL	58	141	124	93
WF	63	342	299	229

Pyrosequencing clustered reads and normalized T-RFLP data were analyzed by PCA to examine overall patterns of variation in microbial community assemblages and the groupings were visually compared. Ordination plots (Figure 4.6) show similar groupings between pyrosequencing data (graph b) and T-RFLP data (graph a), with eukaryotic communities from crops grown in hydroponic systems with solid substrates grouping relatively closely together (Figure 4.6; RW: dark blue diamond; coir: light blue asterisk; WF: purple cross). However, soil (red square) and NFT (green triangle) communities are grouped relatively separately, implying that hydroponic systems with media have more similar eukaryotic community assemblages compared to soil and NFT systems which are relatively different to any other media. This result suggests that even though T-RFLP gives less coverage of the eukaryotic community, the method still provides reliable information about community assemblages and their patterns of relative abundance.

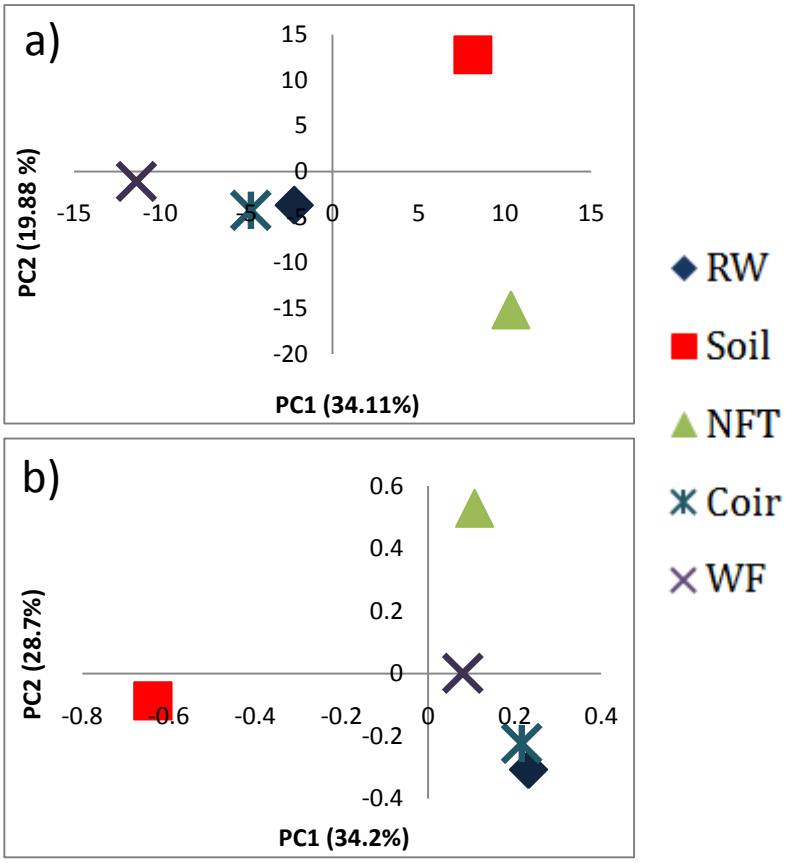

Figure 4.6: PCA ordination plots of eukaryotic organisms inhabiting the rhizosphere of tomato crops grown in different media from a) T-RFLP data and b) pyrosequencing data. Dark blue diamonds represent the mean PC values from RW samples, red squares represent soil samples, green triangles represent NFT samples, light blue asterisks represent coir samples and purple crosses represent WF samples.

Figure 4.7 shows which eukaryotic taxa contributed to >1% of total community populations and their relative abundance levels for pyrosequencing data (graph a) and T-RFLP data (graph b). There are similarities in the organisms identified by both methods and in some cases their relative abundances between media; notably *Penicillium* sp. are most abundant in RW and WF and *Pythium* sp. are most abundant in RW crops for both molecular methods.

However, there are clearly large differences in the taxa identified as contributing to >1% of the total community by the two molecular methods. In some instances, differences in the taxa identified are as a result of the

limitations of T-RFLP analysis affecting the identification of abundant organisms. For example, *Olpidium brassicae* is found at abundant levels in RW and WF crops (Figure 4.7: graph a) and is present at lower levels in NFT crops (Figure 4.5) in pyrosequencing data, but this organism was not identified from T-RFLP analysis methods. Furthermore, this organism has been cloned from crop sites (Table 3.7) and gives the restriction profile *Alu*I-39/*Hae*III-424, but peaks <50bp are not detectable in the T-RFLP analysis due to sensitivity of the method, and for this reason were not considered in the analysis (section 2.8). A peak at 424bp was found at abundant levels when digestion was with *Hae*III but could not be identified.

In addition, there are more similarities in the groups of taxa identified between the molecular methods than Figure 4.5 suggests. For instance, *Exophila* sp. are identified as being present in WF crops from pyrosequencing data but are not shown to be present in Figure 4.7 from T-RFLP analysis (graph b). However, *Exophila* sp. were identified as major taxa contributing to eukaryotic communities in some of the WF crops under T-RFLP analysis but did not account for 1% of total community populations for all 36 WF crops examined (Table 4.12).

Despite fewer differences in taxa identified than suggested by Figure 4.5, there are clear differences in the relative abundance of many taxa identified by the two methods, as well as differences in taxa identified. Notably, the genera *Gliocladium*, *Fusarium*, *Collectotrichum* and *Plectosphaerella* are not picked up by pyrosequencing methods. These findings could be attributed to the choice of target region being different between the two methods (pyrosequencing and T-RFLP analysis targeted ITS1 and ITS2 respectively) with different efficacies of the universal primer pairs used potentially having an effect on the abundance data and on which organisms are preferentially amplified.

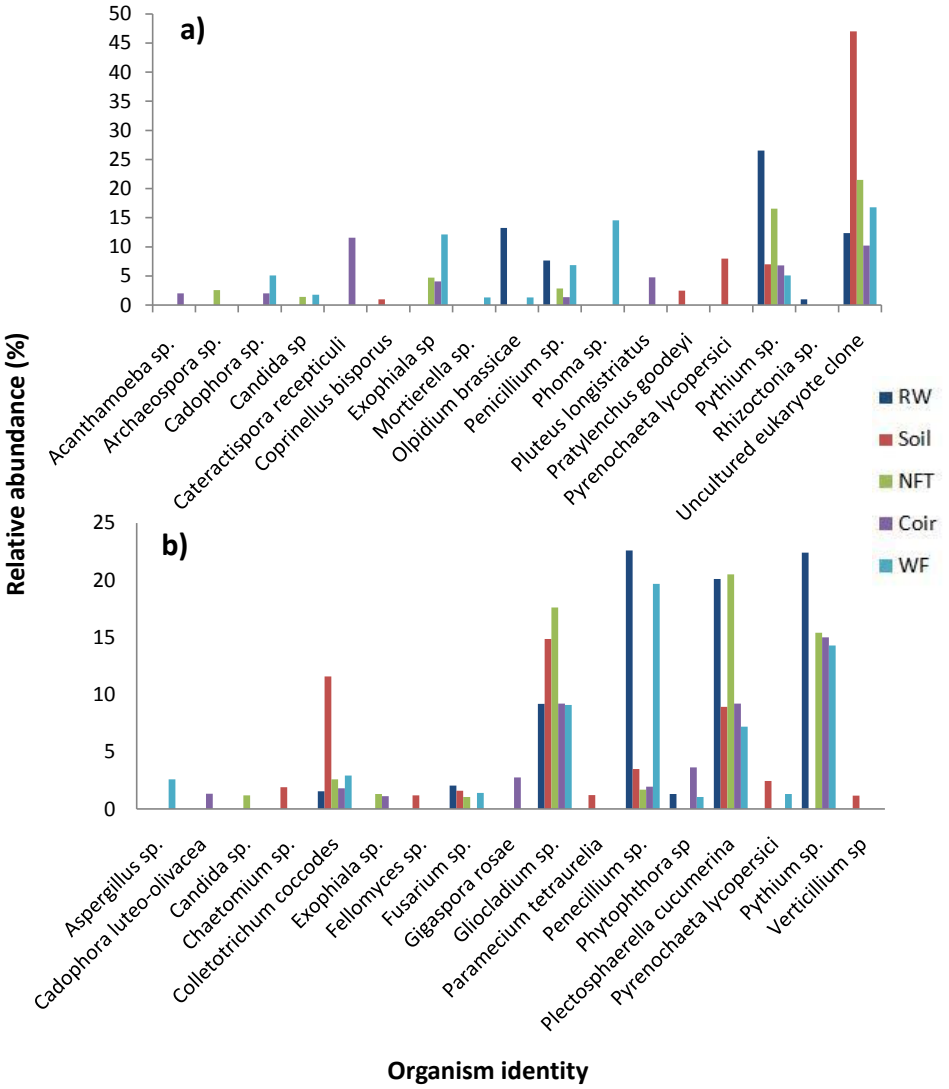

Figure 4.7: Relative abundance of eukaryotic organisms contributing to >1% of total community assemblages associated with tomato roots grown in five different media as identified by a) pyrosequencing and b)T-RFLP.

With *Gliocladium*, *Fusarium*, *Collectotrichum* and *Plectosphaerella* not being present in pyrosequencing data but often being the most abundant organisms present in T-RFLP data, could suggest that the T-RFLP identification is incorrect; however, these organisms have been cloned and cultured from certain crops sites (Table 3.8; Table 3.7; Table 4.13). Furthermore, preliminary results with a microarray based detection system have identified these organism at corresponding crop sites to T-RFLP results with the same samples (Devine, G., School of Biosciences, University of Nottingham, personal

communication). Therefore it could be that the primer pair used to amplify the ITS1 region bind less preferentially to the organisms in question than the primers employed to amplify the ITS2 region. However, the primers used to amplify the ITS1 region are in relatively common usage for the amplification of fungal communities and have been reported to amplify a wide range of fungal organisms; thus it would be expected to find differences in abundance, but perhaps not such discrepancies in the taxa identified (Buée *et al*., 2009).

With this is mind, the most likely factor contributing towards the discrepancies between the two methods is the low level of quality reads obtained from pyrosequencing analysis, with approximately 97% of the reads produced during pyrosequencing analysis being discarded due to low quality sequences (Table 4.7). It could well be that organisms identified as abundant in T-RFLP analysis were amplified by the pyrosequencing primers employed but the resulting sequences were discarded during the trimming process. Further experiments would be required to establish the cause of high levels of low quality reads and to determine if this is the reason organisms found at high levels from T-RFLP analysis were not identified in this instance.

Although differences are present in some of the taxa identified and their relative abundances between T-RFLP and pyrosequencing analysis, both methods reveal that Ascomycota were the dominant fungal phylum followed by Basidiomycota. Furthermore, both methods identified the Phyla Oomycota, Ciliophora and Nematoda (Cliophora and Nematoda sp. were found by T-RFLP but at <1% of the population; data not shown). Moreover, where there were agreements in the identity of taxa, the relative abundances between media were similar. In addition both methods revealed similar species richness levels between media and similar community assemblage patterns.

Ultimately, pyrosequencing should allow for better coverage of the community than T-RFLP analysis (Table 4.10) and more accurate examination of the community constituents due to the sequence based nature of the analysis. However, to remove bias associated with *de novo* OTU analysis resulting in over estimations of species richness and to improve the

phylogenetic analysis (i.e. not excluding unclustered reads), it would be better to use open-reference OTU analysis methods (where clusters are defined by the best database matches; Sogin *et al.,* 2006). To assign phylogeny to this data, online tools and a robust defined database would be required; however this method is more difficult for eukaryotic studies, given the limited number of eukaryotic database resources (Bik *et al*., 2012).

4.3.4 Crop health assessments

Very few of the 180 sampled plants showed symptoms of poor growth or poor health during either season (Appendix II). The exceptions were: two soil crops (soil 1; soil 4) which showed wilting in 2009 and leaf yellowing and necrosis in 2010; three NFT crops (NFT 1; NFT2; NFT 3) which showed leaf yellowing and discoloured roots in one crop and discoloured roots in the other in 2009; in 2010 one crop had severely discoloured roots; two coir crops (coir 2; coir 3) which showed leaf yellowing or wilting and discoloured roots in 2009 and 2010. Notably most symptoms of poor health were visible in the late sample time, with the exception of NFT 2 which occurred mid season. T-RFLP did identify potential pathogens on crops with signs of poor health during the season, however such organisms were also present and relatively abundant on visibly healthy plants (Section 4.3.1.4). This suggests that the signs of poor health observed are not related to the presence or abundance or root pathogens in these instances and may be due to numerous other environmental factors.

At the end of cropping, all monitored plants in 2009 were alive in the two RW crops, the two NFT crops and one WF crop (Table 4.11). In contrast, four plants in each of the soil crops (caused by fusarium wilt or stem rot) and four plants in one of the coir crops had died (due to verticillium wilt). In addition, a single plant died in the second coir crop and the second WF crop (both due to verticillium wilt). At the end of cropping in 2010, all monitored plants were alive except for one plant in a rockwool crop (fusarium wilt or stem rot), one plant in an NFT crop (due to fusarium crown and root rot) and one plant in a

coir crop (due to verticillium wilt; Table 4.11). In all instances, *Fusarium* sp. were identified by T-RFLP in affected plants; however, *Verticillium* sp. were not identified in any of the late sampling time points in diseased plant (Tablw 4.13) . This suggests that T-RFLP is affective in identifying fusarium disease but not verticillium wilt. However, in all the crops where one or more monitored plants died, plant deaths occurred after late sampling and as such could have become infected after the late sampling time point. Notably, there appears to be no obvious link between root health assessment results throughout the season (Appendix II) and crop death at the end of cropping assessment (Table 4.11).

Table 4.11: Summary of stem base and root assessments on monitored plants at end of cropping

Growing medium and dataset	Number of plants (of 9):				Mean severity on roots (0-3)		
	Alive	Verticillium sporing	Fusarium sporing	Root mat	black dot	decayed roots	Corky roots
RW							
1	9	0	0	0	0	1	0
2	9	0	0	0	0	0	0
3	9	0	0	0	1	2	0
4	8	0	3	0	1	2	0
Soil							
1	5	0	3	0	0	3	2
2	5	0	1	0	0	3	2
3	9	0	0	0	2	2	1
4	9	0	0	0	1	1	1
NFT							
1	9	0	0	0	3	2	0
2	9	0	0	0	3	2	0
3	9	0	0	0	2	0	0
4	8	1	1	0	2	0	0
Coir							
1	5	4	0	0	1	2	0
2	8	1	1	0	1	0	0
3	8	2	0	0	0	1	0
4	9	0	0	0	0	1	0
WF							
1	9	0	0	0	1	1	0
2	8	1	1	0	0	1	0
3	9	0	0	0	0	1	0
4	9	0	0	0	0	1	0

1-2: crop samples in 2009, 3-4: crop samples in 2010

Most of the plants from which roots were sampled remained healthy at the end of cropping but a few were affected by verticillium wilt, fusarium wilt, fusarium crown and root rot or vascular staining. Black dot and black root rot were observed quite commonly on roots, especially in NFT solution and soil (Table 4.11). Root blackening was obvious on the mass of fine roots in all NFT crops over both years, and *Colletotrichum coccodes* and *Thielaviopsis basicola* were confirmed to be associated with these symptoms. No root mat symptoms were seen on any plants over the two seasons. All pathogens were identified by T-RFLP on relevant diseased crops with exception of some plants with verticillium wilt, again suggesting that T-RFLP is ineffective at identifying *Verticillium* sp. However, T-RFLP has been optimized to identify this pathogen (Chapter 3) and it has been picked up on a soil crop with disease symptoms (Table 4.13). In addition, end of cropping assessments took place approximately 10-12 weeks after the late sampling for T-RFLP analysis (Table 4.1) which could account for differences in end of cropping results and T-RFLP findings.

T-RFLP identified many likely saprophytic fungi which have previously been reported on tomato roots (section 3.3.1) including species of *Aspergillus*, *Candida, Chaetomium, Cladosporium, Epiccocum, Exophiala*, *Gliocladium, Penicillium* and *Trichoderma*; furthermore the mycorrhizal fungus *Gigaspora* sp. was found in all substrates (Rasmann *et al*., 2009). *Aspergillus, Penicillium, Gliocladium* sp. and *Trichoderma* sp. (potential antagonists) were found in most or all substrates (Whipps, 2001; Table 4.12).

Potential fungal pathogens detected by T-RFLP, which did not result in visible disease in all cases, were *Humicola fuscoatra*, *Phytophthora* sp., *Plectosphaerella* sp., *Spongospora* sp., *Macrophomina* sp., *Colletotrichum coccodes, Fusarium* sp. and *Pythium* sp. (Table 4.6; Table 4.12). There are many cases reported of pathogens inhabiting the rhizosphere environment without causing disease (discussed in section 4.3.1.4). There was no obvious link between relative abundance data of the pathogen or any of the saprophytes mentioned that would suggest a direct link to disease

occurrence. It is likely that other factors played a part in the outcome of disease; there could be a number of abiotic factors including temperature, moisture levels, pH and nutrient levels affecting microbial interactions and these would need to be monitored and controlled to establish their effects. The common occurrence of *Colletotrichum coccodes, Plectosphaerella* sp., *Fusarium* sp. and *Pythium* sp. warrants further investigation with some of the saphrophytes found under more controlled conditions to establish their effects and ecological roles.

Table 4.12: Common pathogenic and saprophytic eukaryotes identified in five growing media and their relative abundance (%) between 36 crops per medium

Organism identity	RW	Soil	NFT	Coir	WF
Saprophytes					
Aspergillus sp.		0.12		0.15	2.16
Candida sp.	0.59		0.14	0.64	0.72
Chaetomium sp.		1.90		0.11	0.12
Cladosporium sp.	0.04		0.14	0.03	0.13
Epicoccum sp.	0.59	0.04	0.45		
Exophiala sp.	0.13	0.42	1.31	1.11	0.23
Gigaspora sp.	0.91	0.19	0.83	0.24	2.75
Gliocladium sp.	9.18	14.87	17.60	9.20	9.10
Penecillium sp.	22.60	3.50	1.70	1.96	19.68
Trichoderma sp.		0.15			
Pathogens					
Botrytis cinerea				0.15	
Colletotrichum coccodes	1.54	11.58	2.59	1.80	2.92
Fusarium sp.	2.04	1.60	1.05		1.40
Humicola fuscoatra				0.17	
Phytophthora sp	1.30			3.63	1.06
Plectosphaerella cucumerina	20.11	8.93	20.50	9.20	7.20
Pyrenochaeta lycopersici		2.46			1.30
Pythium sp.	22.40		15.40	15.00	14.30
Spongospora subterranea				0.23	
Thielaviopsis basicola		0.97			0.45
Verticillium sp.		1.16			

In most cases T-RFLP matched findings from culturing and microscopy end of crop health assessments (Table 4.13). In a few cases T-RFLP did not detect the fungi which were found to be causing disease in a crop, notably, verticillium wilt in coir and WF crops which resulted in crop death. However as mentioned, disease symptoms resulting in crop death occurred after the last sample was taken with root health assessments taking place between 10-12 weeks later than the last sampling. It is reasonable that pathogens could have entered such systems from an unknown source and caused disease within that time frame. Due to the time differences between late sampling and end of crop assessment any differences between culturing and microscopy and molecular methods cannot be attributed to limitations or advantages in either methods.

Table 4.13: Summary of eukaryotic pathogens found associated with plants during routine root monitoring or at the end of cropping by isolation onto agar and/or microscopy and whether their presence was identified by molecular methods

Growing medium	*Colletotrichum coccodes*	*Fusarium* sp.	*Pyrenochaeta lycopersici*	*Pythium* sp.	*Thielaviopsis basicola*	*Verticillium* sp.
RW	CT	CT		CTP		
Soil	CT	CT	CTP	CP		CT
NFT	CT	CT		CTP	CT	
Coir	T	C		CTP		C
WF	CT	CT	TP	CTP	T	C

C: Identified using classical plating methods; T: Identified by T-RFLP; P: Identified by pyrosequencing

Data for 2009 and 2010 diversity scores were combined with plant sickness and root scores providing 20 data sets and examined by regression analysis (Table 4.14). There was no obvious association between either 'plant sickness' or 'root rot' scores and fungal diversity or bacterial diversity (as measured by the Simpson diversity index) at any of the sampling times (Table 4.15). There appeared to be an association between plant sickness and bacterial diversity at the T2 sample time, but this was likely due to one low bacterial diversity value (0.475) which corresponded to a zero in plant sickness score. This was

for the NFT data set which had the extremes for bacterial diversity and influenced the result at this time point.

Overall, there was no obvious association between either 'plant sickness' or 'root rot' scores and eukaryotic diversity or bacterial diversity, this may be due to the limited data set, the difficulty in objectively determining root health, diversity may not be a good indicator of plant health, the use of different varieties and growing media, and the complexity of potential microbial interactions and abiotic factors on roots. Ideally this aspect of the work should have focussed on one variety in order to reduce confounding variation and in more controlled environments. Work elsewhere has shown that plant variety can influence rhizosphere microorganisms and plant health as well as many abiotic factors (Jones *et al*., 1991; Blancard, 1994; Tucci *et al*., 2011). However, the project was to determine effects of all the main growing media at commercial sites, and there were inevitably different varieties being grown at different nurseries using different crop management practices. Future work seeking to relate microbial diversity with root health should, wherever possible, focus on one variety and under controlled conditions.

Table 4.14: Crop and root appearance and Eukarya (E) and bacterial (B) diversity at three time points (T1: early, T2: mid, T3: late) during crop production in 20 tomato crops

Growing medium and dataset	Plant sickness (0-27)	Root rot (0-12)	Microbial diversity (0-1)					
			ET1	ET2	ET3	BT1	BT2	BT3
RW								
1	9	3	0.50	0.64	0.58	0.85	0.84	0.81
2	5	0	0.41	0.31	0.53	0.82	0.85	0.82
3	1	5	0.65	0.50	0.71	0.88	0.77	0.85
4	3	5	0.50	0.68	0.81	0.86	0.86	0.85
Soil								
1	14	9	0.74	0.70	0.70	0.89	0.83	0.85
2	13	11	0.78	0.77	0.75	0.83	0.82	0.86
3	2	7	0.75	0.78	0.62	0.88	0.84	0.86
4	3	4	0.69	0.80	0.70	0.84	0.88	0.76
NFT								
1	3	2	0.39	0.69	0.74	0.87	0.65	0.89
2	7	2	0.09	0.02	0.32	0.70	0.91	0.85
3	0	2	0.63	0.57	0.47	0.83	0.48	0.82
4	8	2	0.75	0.64	0.73	0.80	0.78	0.73
Coir								
1	14	6	0.34	0.37	0.42	0.83	0.79	0.81
2	10	0	0.48	0.64	0.61	0.83	0.77	0.85
3	2	2	0.70	0.70	0.79	0.85	0.90	0.89
4	0	2	0.74	0.62	0.84	0.87	0.89	0.85
WF								
1	9	5	0.55	0.62	0.32	0.66	0.90	0.81
2	10	1	0.49	0.53	0.55	0.85	0.86	0.89
3	1	2	0.69	0.79	0.73	0.88	0.81	0.90
4	0	2	0.71	0.75	0.77	0.91	0.87	0.85

Table 4.15: Association of microbial diversity on tomato roots at three time points during crop production with crop and root appearance at the end of cropping (data for 2009 and 2010 combined; n=20)

Crop assessment	% variance accounted for in relation of Eukarya (E) and bacterial (B) diversity with crop appearance at three sample times (T1, Early; T2 Mid; T3 Late)					
	ET1	ET2	ET3	BT1	BT2	BT3
Plant sickness	13	0	22	0	72	0
Root rot	0	3	0	29	0	0

4.4 DISCUSSION

Significant differences were found in eukaryotic and prokaryotic microbial communities associated with the rhizosphere of tomato plants grown in different media, as identified by ANOSIM, PCA and diversity indices using T-RFLP datasets. This result was expected as tomato rhizosphere microbial communities have been previously found to differ between crop growth media by culturing methods (Price, 1976). However, the study of differences in total rhizosphere microbial communities (unculturable organisms) has not previously been evaluated in the tomato crop between different media.

In general, our results indicate that microbial community populations and assemblage patterns throughout the tomato growing season are similar between roots grown in hydroponic systems with media compared to crops grown in soil systems and NFT systems. Both soil systems and NFT systems were found to have relatively distinctive communities associated with their roots. Furthermore, species richness and diversity were highest in the root environment of soil grown crop and lowest in NFT grown crops. These results are not surprising as it is well known that the organic matter in soil is an important source of nutrients for microorganisms and contains higher levels of fungal and bacterial propagules than hydroponic systems (Postma *et al.,* 2008). Soilless systems without organic components such as NFT and RW are considered poor for microbial growth and so it was surprising to find RW giving similar result to WF and coir crops. However, there is evidence that once plants are introduced to such systems, exudates from the plant and sloughed root cells provide organic substrates conducive for microbial growth (Postma *et al*., 2000; Calvo-Bado *et al*., 2006). Similar results were not found in NFT crops, as well as lower levels of microbial species richness and diversity suggesting that NFT is less conducive for microbial community development, probably due to the lack of solid substrate acting as physical protection and space for microbial growth (Menzies *et al*., 2005).

Furthermore, results suggested that media had an effect on which potential pathogens were present, with soil-borne pathogens being predominantly

found in soil or organic media hydroponic systems (WF and coir) and oomycota being more commonly associated with hydroponically grown crops (Adams *et al.,* 1989; Stanghellini and Rasmussen, 1994). Notably, the presence of potential pathogens did not always result in disease symptoms at the end of cropping assessment. Parallel results have been found in other studies and have been partially attributed to the microbiological properties of growing systems suppressing pathogens by direct antagonism (Raaijmakers *et al.,* 1997; Howell, 1998; Hultberg *et al.,* 2000), niche exclusion (Baker, 1991; Eparvier and Alabouvette, 1994) and stimulation of systemic plant defence responses (Paulitz, 1997; Duijff *et al*., 1998). However, there was no data to support a link between relative abundance data or community diversity data to disease occurrence. Similar findings have been reported for certain species whereby pathogenic and non-pathogenic *Fusarium oxysporum* populations exhibited independent growth and nutritional competence, which were not related to their ability to grow in the rhizosphere and were not connected to their ability to infect roots of tomato (Steinberg *et al*., 1999a, b). These results suggest that many factors control the rhizosphere competence of different species and the outcome of disease. Further studies under more controlled conditions are required to clarify which factors direct rhizosphere competence and affect the likelihood of disease.

Pyrosequencing data provided accurate taxonomic information regarding which eukaryotic species were present in the rhizosphere of each medium and this was instrumental in confirming putative T-RFs and improving the eukaryotic databases. Furthermore, the pyrosequencing provided useful information regarding the coverage of the microbial community from samples collected and implied that further sampling is required to fully characterize communities present. Ultimately, pyrosequencing data was limited, potentially due to the primers employed, the lack of quality reads, by biases associated with the methods used to conduct OTU analysis, and phylogenetic analysis. It would be more accurate to use open-reference OTU analysis methods (where clusters are defined by the best database matches; Sogin et

al., 2006) and to assign phylogeny to this type of data using online tools and a robust defined database. RDP classifier is a popular tool for this type of analysis, which matches eight-base sequence 'words' to the RDP database, returning confidence scores for each taxonomic assignment (Cole *et al.,* 2009). However, RDP classifier and many other tools available to deal with pyrosequencing data have been initially optimized for research on prokaryotes and as such, are not yet compatible with eukaryotic community data (Bik et al., 2012). However, as of December 2011 RDP classifier has incorporated a robust 18S large subunit rRNA gene database allowing for rapid taxonomic classification which is computationally over 460 fold faster than BLAST methods and provides equal or superior accuracy (Liu *et al*., 2012). In light of the development of online tools for the study of eukaryotic communities, it would be sensible to take advantage of these resources and target the 18S SSU region for further investigations of eukaryotic communities of the tomato rhizosphere when employing pyrosequencing.

In general, the comparison of molecular methods showed that T-RFLP results correlated well with the pyrosequencing results regarding comparable species richness levels associated with each medium, community assemblage patterns and Phyla identified in the root environment. Pyrosequencing provided detailed data on the taxonomic identity of eukaryotic community constituents to a genus and species level, their relative abundance levels and community assemblage patterns, as well as information regarding the level of community coverage and sampling requirements. T-RFLP also provided detailed information of community assemblage patterns, although it gave limited detail of the taxonomic identity of Eukarya, their abundance levels and provided less coverage of the community present. Furthermore, clone libraries, culturing data and pyrosequencing data was necessary to verify the identity of T-RFs. On the other hand, T-RFLP was found to be a reliable method that, at a fraction of the cost, provided an overview of the eukaryotic communities present in the rhizosphere and allowed comparisons to be made regarding community constituents, their relative abundance, microbial

diversity of each environment and whether these differences were statistically significant. Notably, it has been found that when T-RFLP methods are used to compare and study changes in communities, most of the biases associated with this method are minimized (Fernández *et al*. 1999).

5 EFFECT OF SOIL AMENDENTS AND ROOTSTOCK VARIETY ON ORGANIC TOMATO RHIZOSPHERE MICROBIAL COMMUNITIES, ROOT HEALTH AND PLANT HEALTH

5.1 INTRODUCTION

Concerns over the need for increased crop yields, groundwater contamination, chemical residues on foods and chemical resistance among pests and plant pathogens have stimulated debates about the sustainability of conventional chemical-intensive agriculture and have increased consumer and grower demand for sustainable, reliable and affordable alternatives. Subsequently, organic farming practices that remove the use of chemical fertilizers and pesticides and rely on soil amendments, biological control agents (BCAs) and cultural methods to maintain soil fertility and control pests and pathogens are becoming more popular amongst tomato growers (Nicholson, 1986; Drinkwater *et al*., 1995; Dixon and Margerison, 2009).

Organic tomato growers often use composts and bioactive plant products (predominantly Brassicaceae residues) as soil amendments to improve soil fertility by increasing the levels of organic matter for plant nutrition and stimulating indigenous microbial activity. If applied well ahead of planting, these amendments have been recognized to facilitate the biological control of soil-borne pathogens via the activities of BCAs within the indigenous microbial community (Hoitink and Boehm 1999; Cohen *et al*., 2005).

Amendments that increase the fertility of soil can facilitate the introduction of BCAs as well as stimulate resident microbial communities. A large number of diverse soil microorganisms have been characterized as BCAs of soil-borne plant pathogens and many have been incorporated into soil amendments for commercial use, involving the introduction of a single organism or mixtures of organisms (Handelsman and Stabb 1996; Whipps, 2000). However, the

unreliable nature of this disease control approach, whereby certain introduced organisms may control disease, provide only partial disease control or fail to establish themselves in the root environment resulting in no disease control, has prevented this approach from being widely adopted in conventional systems (Weller, 1988).

Another cultural method commonly employed by organic growers to improve crop health and disease resistance is to graft plants onto disease resistant rootstocks. There are not many tomato cultivars with excellent stable disease resistance combined with desired fruit qualities, so the use of available resistant cultivars as rootstocks to control disease is an invaluable resource (Lee *et al*., 1994). Grafted plants are particularly suited to crop production in organic systems due to their higher stress tolerance, more efficient fertilizer use, and soil borne disease resistance. Proposed theories for improved disease control in grafted crops are that they provide inherent resistance and improved plant nutrient uptake. However, increasing evidence indicates that induction of plant systemic defence mechanisms and differences in rhizosphere microbial communities also play a part in disease resistance (Rumberger *et al*., 2007; Guan *et al*., 2012).

This chapter aims to determine the effects of five commonly used soil amendment treatments, some of which primarily increase soil fertility and indigenous microbial activity (composted green waste, composted bark and Biofence) and others that are primarily used to introduce BCAs (Trianum-P and Compete Plus in alternation with Colonize AG), on organic tomato root health, plant health and rhizosphere microbial populations. Furthermore, it aims to determine whether introduced BCAs can establish themselves in the root environment. In addition, the effects of six commonly used rootstocks with excellent but differing resistances were compared to determine their effects on organic tomato on plant health and rhizosphere microbial populations.

5.2 METHODS

Two trials took place at an organic nursery in glasshouses with a history of root disease problems, a continuous cultivation of tomato crops in soil and no soil disinfestation treatment between crops.

5.2.1 Soil amendment trial

To determine the effect of three pre-plant soil amendments (composted green waste, composted bark and Biofence) and two microbial drench treatments (Compete Plus in alternation with Colonize AG, and Trianum-P) on rhizosphere microbial communities, plant health and root health in an organic crop, a season long experiment was conducted in a glasshouse where organic tomatoes had been grown for five consecutive years. Amendment specifications are detailed in Table 5.1.

Table 5.1: Details of soil amendments

Product	Specification
PHC Compete Plus (CP)	*Bacillus, Pseudomonas, Streptomyces, Trichoderma* formulated with vitamins, humic acids and seaweed extract.
PHC Colonize AG	A plant flavonoid.
Trianum-P	*Trichoderma harzianum* strain T-22
Composted Green Waste (CGW)	Primarily composted tomato crop waste, produced on site.
Melcourt Composted Fine Bark (CFB)	A soil conditioner consisting of matured (at least 12 weeks) British conifer bark with a particle size distribution of 1-10 mm and <5% wood content. Bulk density 390-440 kg/m^3, dry matter 55%, organic matter 85%, pH 4.5-5.5, low in N, P, Mg; medium level K, electrical conductivity 150 µS/cm.
Biofence	Pellets of Caliente mustard seed meal (*Brassica juncea* cv. Carinata) a soil fertiliser.

Soil was amended in winter 2009 prior to planting cv. Piccolo on Beaufort rootstock in February 2010. Details of amendment application are described

in Table 2.1 in section 2.2.1. There were also plots with no amendments added acting as untreated controls (T1). The crop was grown to commercial standards according to normal practice of the host nursery.

The trial was a randomised block designs with six fold replication, detailed in Figure 5.1. Root samples were collected (section 2.3) on three occasions from each plot (36 samples) during the growing season; 19th April (8 weeks after planting; early), 28th May (around peak fruit load; mid) and 26th July (main season) to determine microbial populations by T-RFLP.

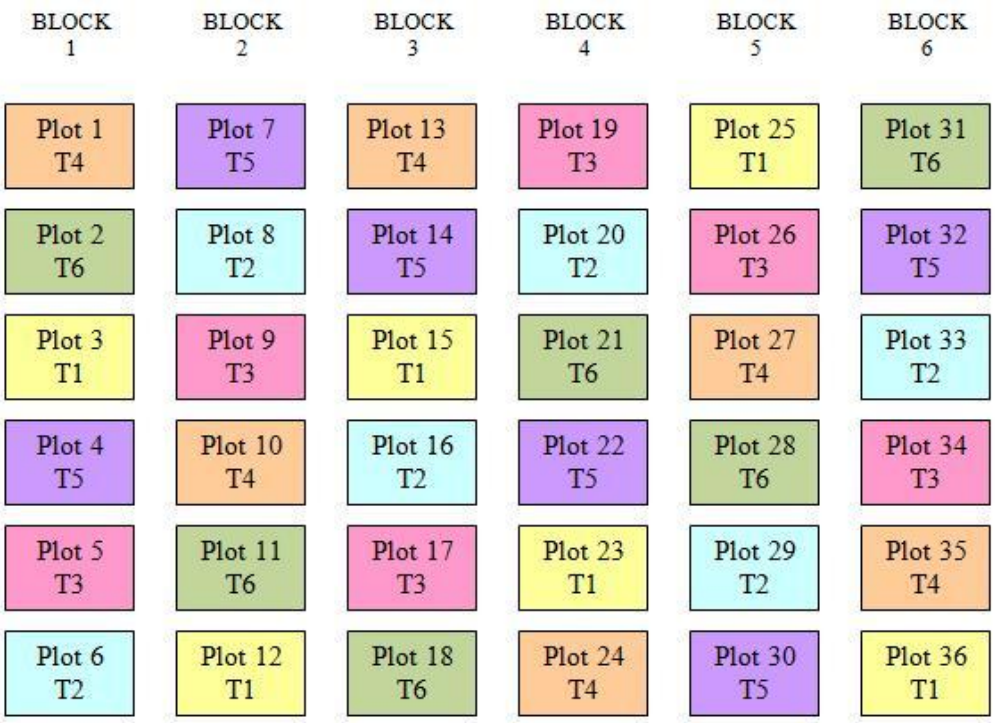

Figure 5.1: Diagram of the randomized soil amendment trial plot design: consisting of six blocks containing six plots. Individual plots consisted of an island bed of 18 planting pots (2 plants per pot) spaced at 50 cm (plot dimension was 9.5 m x 0.8 m). The six plots in a block were arranged along one row. The six blocks were arranged in adjacent bays of crop. T1: Untreated; T2: CP/Colonize; T3: Trianum; T4: CGW; T5: CFB; T6: Biofence.

5.2.2 Rootstock trial

The rootstock trial was conducted using an organic tomato crop cv. Roterno grafted onto six different rootstocks with different resistances (Table 2.2). Organic tomatoes had been grown in the house for at least 10 years. The

experiment was located in an area where leaf yellowing and poor growth occurred in 2009. The crop was grown according to normal nursery practice which included incorporation of green waste compost prior to planting and monthly drench treatment with PHC Compete Plus and PHC Colonize AG in alternation (product specifications are detailed in Table 5.1). The crop was planted in February 2010.

The experiment was a randomised block design with six-fold replication detailed in Figure 5.2. Root samples were collected (section 2.3) on two occasions from each plot (36 samples) during the growing season; 19th April (8 weeks after planting; early), 28th May (around peak fruit load; mid) to determine microbial populations by T-RFLP.

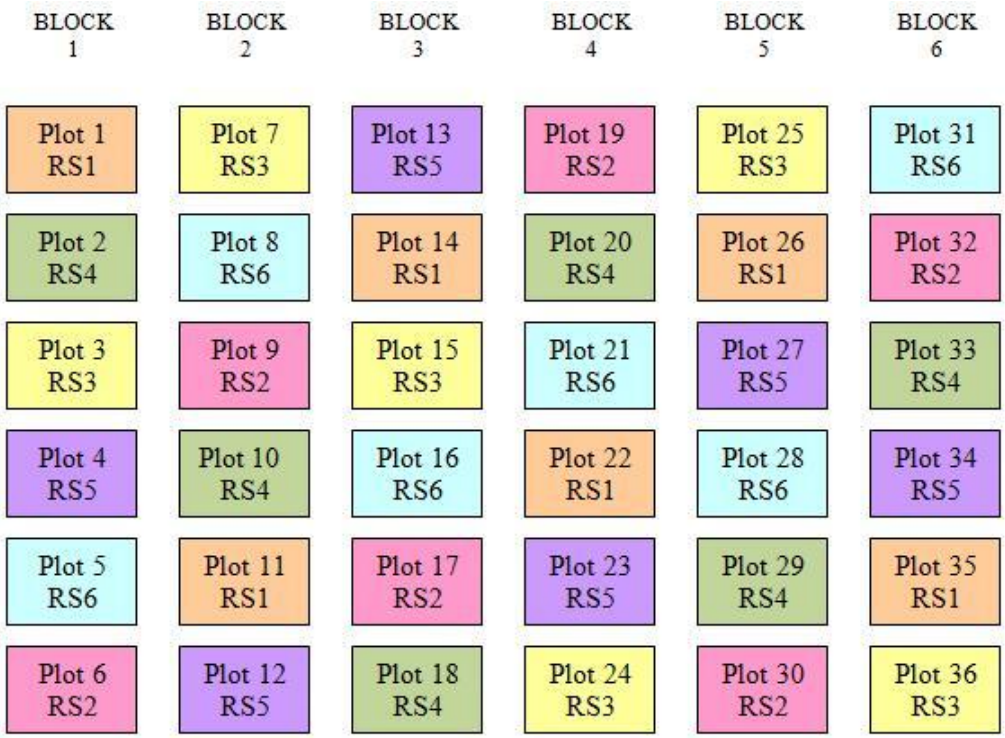

Figure 5.2: Diagram of the rootstock trial randomized plot design: consisting of six blocks containing six plots. Individual plots consisted of an island bed of 18 planting pots (2 plants per pot) spaced at 50 cm (plot dimension was 9.5 m x 0.8 m). The six plots in a block were arranged along one row. The six blocks were arranged in adjacent bays of crop. RS1: Beaufort; RS2: Efialto; RS3: Emporador; RS4: Optifort; RS5: Stallone; RS6: Unifort

5.2.3 DNA extraction and T-RFLP analysis

Total community DNA was extracted from all root samples using the procedures described in section 2.5, followed by PCR amplification of ribosomal DNA (rDNA; section 2.6), restriction digestion (section 2.7) and T-RFLP analysis (section 2.8). From the resulting T-RFLP profiles, putative taxonomic identities of T-RFs were assigned by importing T-RFLP profile information into FRAGSORT version 5.0 (Michel and Sciarini, 2003). The null hypotheses (H_0) were tested by Analysis of Similarities (ANOSIM; section 2.9.1). T-RFLP data were also represented in an ordinational space with Principal Component Analysis (PCA; section 2.9.2) and α-diversity and ß-diversity was calculated using species richness and diversity indices (section 2.9.3).

5.2.4 Crop and soil assessments

Plants were assessed on one side of a row at sampling times to determine the number of yellowing and wilting or dead heads. At the end of cropping on 5th November, the number of green stem bases was assessed. Twenty plants in each plot were examined for vascular staining in the stem base. These plants were also forked up in the first three replicates (block 1-3), and the health of roots were estimated. Roots showing different symptoms were collected at the final assessment and tested for possible causal fungi by isolation onto agar at ADAS (section 3.3.2). Results were examined by analysis of variance (ANOVA) with treatment as a factor; p-values <0.05 were considered to be significant. No assessment was possible at the end of cropping for the rootstock trial as the crop was pulled out early due to severe damage from an aerial pest problem.

As the soil amendment trial was located in a glasshouse with previous *Verticillium* root disease problems, soil samples from the whole trial area were taken at the end of cropping and tested for *Verticillium dahliae* by ADAS (using methods previously described by Harris *et al*., 1997), and for *V. dahliae* and *V. albo-atrum* by PCR molecular tests at Fera and by T-RFLP (section 2.8).

5.2.5 Establishment of Trichoderma in the root environment.

Root samples were taken from three crops on two occasions from the soil amendment trial, treatments T1 and T3 (blocks 1 and 4), to determine the effect of Trianum-P treatment on establishment of *Trichoderma* sp. Root samples were taken on 25th February 2010, one week after planting (to determine effect of propagation treatment), and on 6th April 2010, two weeks after a second application of Trianum-P on the nursery (to determine effect of production nursery treatments). On 25th February, root samples were taken from the side at the bottom half of the propagation cube and from the soil. On 6th April, young roots were taken as described in section 2.3. Samples were tested by culturing at ADAS (section 3.3.2) and T-RFLP analysis (section 2.8); furthermore, they were posted to Koppert BV for determination of the relative levels of *Trichoderma* populations as number of colony forming units per gram of dried root (cfu/g; Table 5.2).

Table 5.2: Interpretation of relative levels of *Trichoderma* species associated with root and soil samples determined by Koppert BV.

Relative level	Density of *Trichoderma* sp. (cfu/g)	Interpretation
0	$0 - 1 \times 10^3$	Not present or barely present
1	$1 \times 10^3 - 1 \times 10^4$	Moderately present
2	$1 \times 10^4 - 1 \times 10^5$	Well present
3	$> 1 \times 10^5$	Very well present

5.3 RESULTS

5.3.1 Soil amendment trial

5.3.1.1 Null hypothesis

To test the null hypothesis (H_0) that there were no differences in bacterial or eukaryotic communities inhabiting the roots grown in different soil amendments plus an untreated control and that there were no differences in microbial communities at different sampling times, the ANOSIM test was carried out on ITS2 and 23S rDNA T-RFLP datasets as shown in Table 5.3.

Table 5.3: ANOSIM test values and probabilities of null hypothesis tests obtained from comparisons of T-RFLP datasets of ITS2 and 23S rDNA of samples from organic tomato roots grown in different soil amendments (H_0 Treatment) and taken at different sampling times (H_0 Time).

	ITS2		23S	
	R-values	**p-values**	**R-values**	**p-values**
H_0 Treatment	0.01	NS	0.02	NS
H_0 Time	0.648	<0.01	0.6303	<0.01

NS: no significance

For the treatment datasets, the H_0 is accepted indicating that there were no significant differences in microbial communities between treatments. This suggests that the soil amendments used to improve soil fertility did not alter the indigenous microbial communities; furthermore, amendments used to introduce BCAs failed to establish themselves in the root environment. However, the H_0 was rejected for H_0 time datasets; significant probabilities obtained with ANOSIM for the H_0 time indicate that there were significant shifts in the microbial populations between the time points examined. These shifts can be interpreted as well separated with a level of community overlap (Clarke and Gorley, 2001). This also indicates that although changes in microbial communities occur, they were similar between treatments over time.

5.3.1.2 PCA analysis

Normalized T-RFLP datasets were analyzed by PCA to visually compare microbial community patterns between organic crops grown in different amendments over time. Figure 5.3 shows ordination plots generated from this analysis for ITS2 rDNA data (a and b) and 23S rDNA data (graphs c and d), which accounted for >60% of variance and >50% of variance in the data respectively.

Principal component (PC) scores were analyzed by ANOVA with treatments and time as factors, and it was found that PC scores were not significant with treatment as a factor (eukaryotic: PC1 p=0.094, PC2 p=0.07; bacterial: PC1 p=0.06, PC2 p=0.06), indicating that community groupings on Figure 5.3 (a and b) are not significantly different or are significantly grouping as a result of factors not under examination. However, PC scores were found to be significant with time as a factor (p=<0.05) implying that groupings on Figure 5.3 (b and d) are significantly influenced by time. These findings are in agreement with ANOSIM results. Eukaryotic communities appear to change at all time points, whereas bacterial communities appear to be more similar between mid and late time points than the early time point.

PCs identified as significant with time as a factor were further analyzed by determining which loading values were significantly contributing to groupings (section 2.9.2). From these significant loading values, the enzyme and T-RF combinations were identified and compared to the output of FRAGSORT (section 2.8), resulting in a likely organism identity. Based on whether a loading value is positive or negative, organism identities can be associated with the groupings on PCA ordination plots, suggesting that the presence or relative abundance of the organism in question is significantly contributing to groupings on that PC.

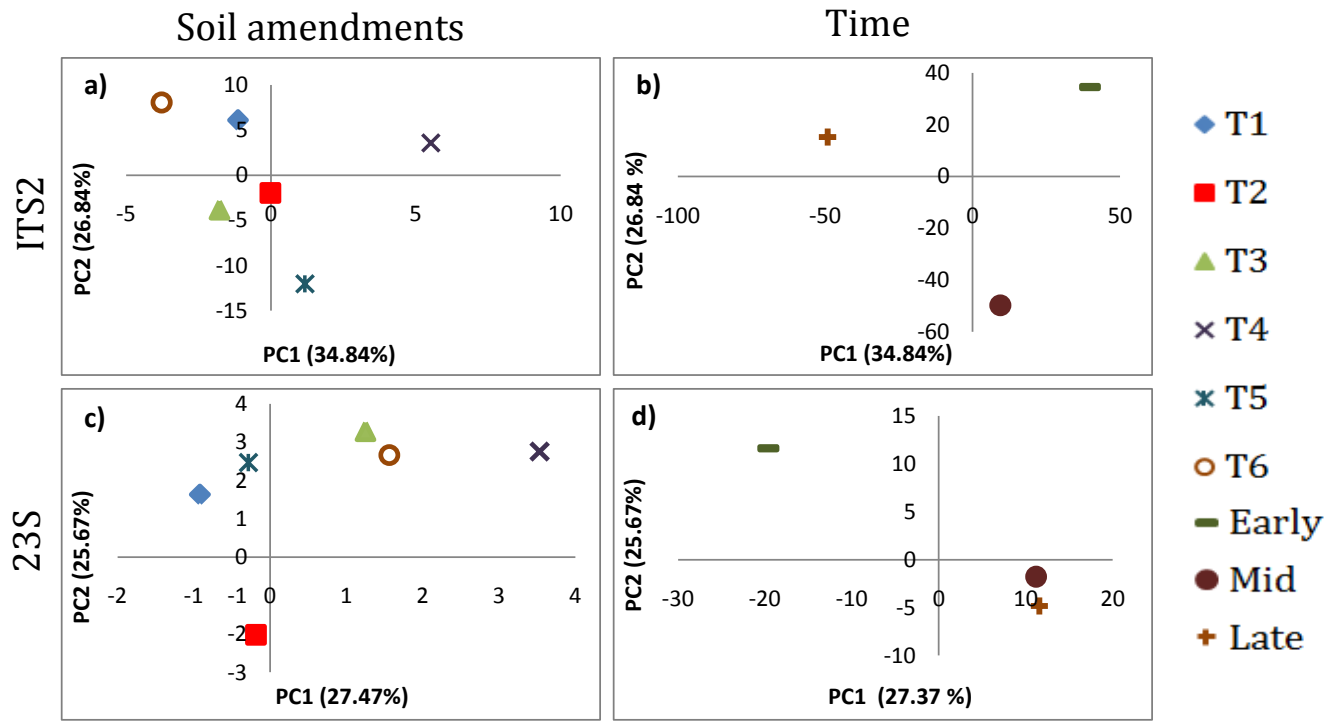

Figure 5.3: PCA ordination plots of microorganisms inhabiting the rhizosphere of organic tomato crops grown with different soil amendments (a, c) and the roots of plants at different sampling times (b, d) using T-RFLP profiles of ITS2 (a, b) and 23S rDNA (c, d). Blue diamonds represent the mean PC values from T1, red squares represent T2 samples, green triangles represent T3 samples, turquoise asterisks represent T4 samples, purple crosses represent T5 samples and unfilled orange circles represent T6. Dark green dashes represent the mean PC values from early time points, Dark red circles represent mid time points and orange plus signs represent late time points.

For eukaryotic community data PC1 and PC2 identified three enzyme and T-RF combinations that were significantly contributing towards PC groups. From FRAGSORT output data, three potential organisms were identified as matching significant T-RFs; these were *Penicillium* sp. (A323), *Coprinellus* sp. (A384) and *Colletotrichum coccodes* (H152) (Table 5.4). *Penicillium* sp. and *C. coccodes* were associated with late sampling time and *Coprinellus* sp. was associated with mid sampling time. In all cases, relative abundance data corresponded to significant T-RFs identified by PCA and which sample time they are associated with (Figure 5.4).

Table 5.4: Eukaryotic organisms identified by significant PC loadings (PC1, PC2) contributing to significant PCs, their T-RF and enzyme combination and which time point they are associated with based on their PC loading value.

Enzyme/T-RF	PC1	PC2	Potential Identity	Associated with:
A323	-0.59		*Penicillium* sp.	Late
A384		-0.87	*Coprinellus* sp.	Mid
H152	-0.69		*Colletotrichum coccodes*	Late

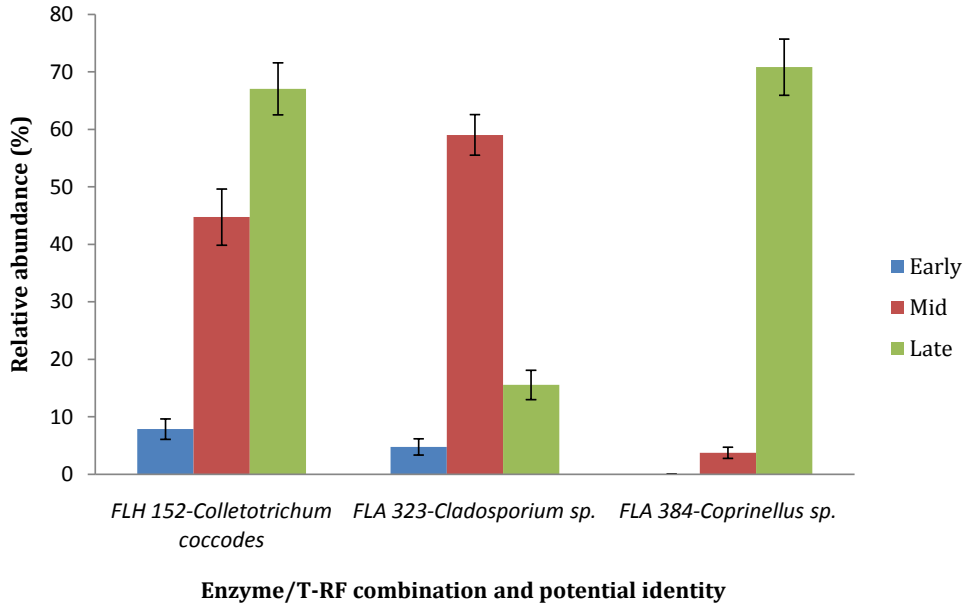

Figure 5.4: Relative abundance of enzyme and T-RF combinations that had a significant effect on PC groupings from PCA analysis of T-RFLP ITS2 time datasets. Error bars represent the standard error of the mean. All means were found to be significantly different between time points (p=<0.05). A= *Alu*I; H= *Hae*III.

For bacterial community data PC1 and PC2 identified five enzyme and T-RF combinations that were significantly contributing towards PC groups. From FRAGSORT output data, three potential organisms were identified as matching significant T-RFs; these were *Agrobacterium radiobacter* (H202), Gammaproteobacteria (H375/M374) and *Clostridium* sp. (H400/M400) (Table 5.5). *Agrobacterium radiobacter* and Gammaproteobacteria were associated with mid and late sampling times and *Clostridium* sp. was associated with early sampling time.

Table 5.5: Bacterial organisms identified by significant PC loadings (PC1, PC2) contributing to significant PCs, their T-RF and enzyme combination and which time point they are associated with based on their PC loading value.

Enzyme/T-RF	PC1	PC2	Potential Identity	Associated with:
H202	0.31		*Agrobacterium radiobacter*	Mid/Late
H375		-0.66	Gammaproteobacteria	Mid/Late
H400	-0.29		*Clostridium* sp.	Early
M374		-0.55	Gammaproteobacteria	Mid/Late
M400	-0.46	0.32	*Clostridium* sp.	Early

5.3.1.3 Diversity indices

For further comparison of the eukaryotic and bacterial populations present in the rhizosphere of tomato grown with different soil amendments and a control treatment over time, diversity estimations were provided from normalized T-RFLP datasets using the number of OTUs as a species richness estimator and the diversity indices Shannon index (H') and Simpson index (1-D), mean values are shown in Table 5.6. Outputs from species richness and diversity indices were tested by ANOVA and it was found that there were no significant differences in microbial populations associated with the roots between different soil amendments or the control treatment. These results are in agreement with ANOSIM and PCA analyses.

Table 5.6: Diversity indices and species richness calculated from T-RFLP datasets for the two rDNA regions under examination, for all treatments and time points under examination

	ITS2			23S		
	S	**1-D**	**H'**	**S**	**1-D**	**H'**
Treatment						
T1	6.08±0.7	0.62±0.05	1.31±0.13	13.64±1	0.82±0.04	2.2±0.14
T2	4.64±0.49	0.53±0.05	1.06±0.11	15.11±0.68	0.88±0.01	2.41±0.06
T3	4.58±0.59	0.5±0.06	1.02±0.14	15.17±0.77	0.88±0.01	2.38±0.06
T4	4.94±0.55	0.53±0.05	1.06±0.12	15.39±0.91	0.87±0.02	2.38±0.09
T5	5.11±0.79	0.47±0.07	1.01±0.17	15.58±0.57	0.89±0.01	2.45±0.05
T6	4.86±0.54	0.51±0.05	1.02±0.11	16.33±0.5	0.9±0.01	2.51±0.04
p-values	NS	NS	NS	NS	NS	NS
Time						
Early	6.68±0.46	0.66±0.03	1.42±0.08	15.33±0.44	0.87±0.01	2.42±0.04
Mid	4.03±0.36	0.45±0.04	0.87±0.08	14.63±0.057	0.87±0.01	2.32±0.05
Late	4.4±0.33	0.47±0.04	0.95±0.08	15.65±0.58	0.88±0.02	2.44±0.08
p-values	<0.01	<0.01	<0.01	<0.01	NS	<0.01

S: Species richness: number of taxa or species present
1-D: Simpson index of diversity: higher values indicating higher diversity
H': Shannon index of diversity: higher numbers indicate higher diversity
±: Standard deviation of the average.
NS: No significance

Microbial populations were found to change significantly between time points using the species richness and diversity indices with the exception of 1-D using 23S rDNA datasets. Although the H' and 1-D indices show similar trends in microbial diversity between samples, for example in bacterial communities diversity is highest at the late sampling time point, they were found to differ in their ability to determine statistically significant differences. This is because both indices are influenced by the data in different ways, whereby the 1-D is more influenced by the abundances of the most common species, whilst the H' is more influenced by species evenness (Magurran, 1988). This highlights the importance of using more than one diversity index to examine the diversity of microbial communities and the potential issues of limiting complex assemblages into a single value.

Eukaryotic and bacterial communities were found to reduce in species richness and diversity between early and mid time points and then increased at late time points. This could be due to the introduction of plants and high

levels of organic matter present at the start of the season causing diversity to be relatively high and then as communities stabilize and potential reductions in organic matter due to microbial respiration occur, this causes the diversity levels to reduce (Postma *et al*., 2000). Similarly as in Chapter 4, towards the end of the season, diversity was highest and could be attributed to potential increases in sloughed off cells and changes in root exudates, potentially creating more conducive conditions for microbial growth (Halmen *et al*., 1972; Jaeger *et al*., 1999).

5.3.1.4 Crop and soil assessments

Plants were assessed at sampling times to determine the number of yellowing and wilted or dead heads. At the first assessment (Early) there were significant differences between treatments; leaf yellowing was significantly higher in the CGW treatment and was absent in most other treatments. The incidence of wilting or dead heads at the early time point was also low with no significant differences between treatments (Table 5.7).

At mid and late sampling times there was no significant difference between the incidences of leaf yellowing, wilting or dead heads between treatments (data not shown). At the end of cropping assessment, there was no difference between treatments in the mean percent of green stem bases, and no differences in the occurrence of vascular staining (Table 5.8). These results indicate that there was no visible difference in plant health (for variables under examination) between mid and late sampling times and at the end of cropping. Differences between GCW and other composts comprised of different materials have been previously reported and attributed to the fact that GCW is predominantly composed of plant material, being decomposted with difficulty in the short term and acting as a long term source of nutrients (Pérez-Piqueres *et al*., 2006). This could be an explanation for differences in plant health early on in the season.

Table 5.7: Effect of soil amendments on plant health of soil grown organic tomato, cv. Piccolo on Beaufort rootstock at the early time point as assessed by leaf yellowing and wilted or dead heads.

Treatment	Mean % heads affected by	
	Leaf yellowing	Wilted or dead
T1 Untreated	0	0.5 ± 0.3
T2 CP/Colonize	0	1.4 ± 0.5
T3 Trianum	0	0
T4 CGW	9.0 ± 0.7	0.9 ± 0.4
T5 CFB	0.4 ± 0.1	0.5 ± 0.3
T6 Biofence	0	0.5 ± 0.3
p-value	<0.001	NS

±: Standard deviation of the average; NS: No significance

Table 5.8: Effect of soil amendments on plant health in tomato cv. Piccolo on Beaufort rootstock at end of cropping as assessed by mean percent of green stem bases and vascular staining.

Treatment	Mean % green stem bases	Mean % stem bases with vascular staining
T1 Untreated	94 ± 1.7	14 ±3.3
T2 CP/Colonize	91 ± 2.0	19 ± 3.8
T3 Trianum	94 ± 1.7	19 ± 3.8
T4 CGW	90 ± 2.1	21 ± 4.0
T5 CFB	94 ± 1.8	23 ± 4.5
T6 Biofence	91 ± 2.1	15 ± 3.4
p-value	NS	NS

±: Standard deviation of the average; NS: No significance

From the root health assessment at the end of cropping from the first three replicates black dot and corky symptoms were found on crops from all treatments. There were no significant differences between treatments in percentage of root affected by black dot or corkiness symptoms (Table 5.9). Isolation tests on root samples collected at the final assessment confirmed *C. coccodes,* a known cause of black dot, was associated with the black dot symptom. A *Fusarium* sp. was isolated quite consistently from the corkiness symptom, suggesting that this is the likely cause of those symptoms. No *P. lycopersici*, a known cause of corky root rot, was isolated. Notably, *C. coccodes* and *Fusarium* sp. were identified by T-RFLP; furthermore, *C. coccodes* was found in relatively high abundance particularly later on in the growing season (late sampling time; Table 5.4; Figure 5.4). No root mat symptoms were found

despite *Agrobacterium radiobacter* being identified at mid and late time points by T-RFLP (Table 5.4; Figure 5.4); notably, T-RFLP cannot determine whether the isolates present are pathogenic or non pathogenic.

Table 5.9: Effect of soil amendments on extent and appearance of roots of tomato cv. Piccolo on Beaufort rootstock

Treatment	Mean % root length affected by		
	Corkiness	Black dot	Corkiness + black dot
T1 Untreated	5.2	11.8	17
T2 CP/Colonize	9	12.5	21.5
T3 Trianum	4.4	10.6	15.1
T4 CGW	8.6	20.2	28.8
T5 CFB	5.5	11.4	16.9
T6 Biofence	3.6	29.3	22.9
p-value	NS	NS	NS

Results from soil sample analysis at the end of cropping indicated that *Verticillium dahliae* was present from culturing and microscopy methods conducted by ADAS. However there were no verticillium root rot symptoms on crop roots. Furthermore, *V. dahliae* and *V. albo-atrum* were not identified by PCR molecular tests at Fera or by T-RFLP analysis. *Verticillium nigrescens* was identified by T-RFLP analysis from comparisons to databases containing information of *in silico* restriction profiles. It could be that this organism identified by culturing methods was *V. nigrescens,* and further tests would be required to establish if the isolate was indeed *V. nigrescens*.

5.3.1.5 Effect on Trianum-P treatment on soil Trichoderma populations

From the analysis by Koppert BV of roots treated with Trianum-P and roots from an untreated control crop, it was found that *Trichoderma* sp. were present at high levels on root samples collected on 25th February (one week after planting) and were moderately present or at high levels on root samples collected on 6th April (two weeks after second treatment) on both treated and untreated controls. There was no consistent difference in *Trichoderma* sp. levels between plants treated with Trianum-P and untreated plants during

propagation treatment or at the nursery treatment (Table 5.10). In addition, none of the isolates examined by culturing methods were identified as the BCA present in Trianum-P, *T. harzianum*; furthermore, *T. harzianum* was not identified by T-RFLP methods. These results indicate that there were high levels of indigenous *Trichoderma* sp. that were able to become established on roots either during propagation or within a few days of planting. It also suggests that the BCA *T. harzianum* when applied as a soil amendment at the propagation stage or at the nursery was not able to become successfully established on the roots during this trial. These results further back ANOSIM, PCA and diversity scores from T-RFLP analysis whereby no difference was found between soil amendment treatments or the untreated control and suggests that one reason for these results may be the inability for applied BCAs to establish themselves in the root environment.

Table 5.10: Detection of *Trichoderma* species on roots of soil-grown tomato plants treated with Trianum-P and an untreated control.

Samples from block 1 and 4	Density of *Trichoderma sp.* as cfu/g on samples collected:	
	25-Feb	06-Apr
T1 plot 3	6.1×10^5 (3)	9.1×10^4 (2)
T1 plot 23	4.3×10^5 (3)	6.1×10^4 (2)
T3 plot 5	5.2×10^5 (3)	9.0×10^4 (2)
T3 plot 19	1.8×10^5 (3)	1.5×10^5 (3)

T1 = control; T3 =Trianum-P treatment; numbers in brackets are relative levels of *Trichoderma* as determined by Koppert BV (Table 5.2).

5.3.2 Rootstock trial

5.3.2.1 Null hypothesis

Analysis of similarities was performed on T-RFLP datasets for 23S and ITS2 rDNA, in order to test the null hypotheses that:

i) there were no differences in microbial communities associated with the rhizosphere of different rootstocks;

ii) there were no differences in microbial communities between time points examined.

The hypothesis testing was carried out comparing T-RFLP datasets from the rootstock trial using rootstock and time as factors in the statistical test (Table 5.11).

Table 5.11: ANOSIM test values and probabilities of null hypothesis tests obtained from comparisons of T-RFLP datasets of ITS2 and 23S rDNA of samples from organic tomato roots grown on different rootstocks (H_0 Rootstock) and taken at different sampling times (H_0 Time).

	ITS2		23S	
	R-values	p-values	R-values	p-values
H_0 Rootstock	0.09	<0.05	0.29	<0.01
H_0 Time	0.16	<0.01	0.52	<0.01

Both null hypotheses were rejected, indicating that the communities between different rootstocks and sampling time points were significantly different, matching findings from previous studies (Rumberger *et al*., 2007; Cavaglieri *et al*., 2009). However, although the ITS2 rDNA showed significant probabilities for both rootstock and time factors, R-values generated were very low (R =0.09; R= 0.16 respectively), indicating there was barely any separation between eukaryotic communities. R values generated for bacterial communities can be considered as separated but overlapping (Clarke and Gorley, 2001)

5.3.2.2 PCA analysis

Normalized T-RFLP datasets were further analyzed by PCA to view community assemblage patterns on an ordination plot and to determine which organisms were significantly contributing to different groupings among samples. Figure 5.5 shows ordination plots generated from this analysis for ITS2 rDNA data (a and b) and 23S rDNA data (c and d), which accounted for >50% of variance in the data.

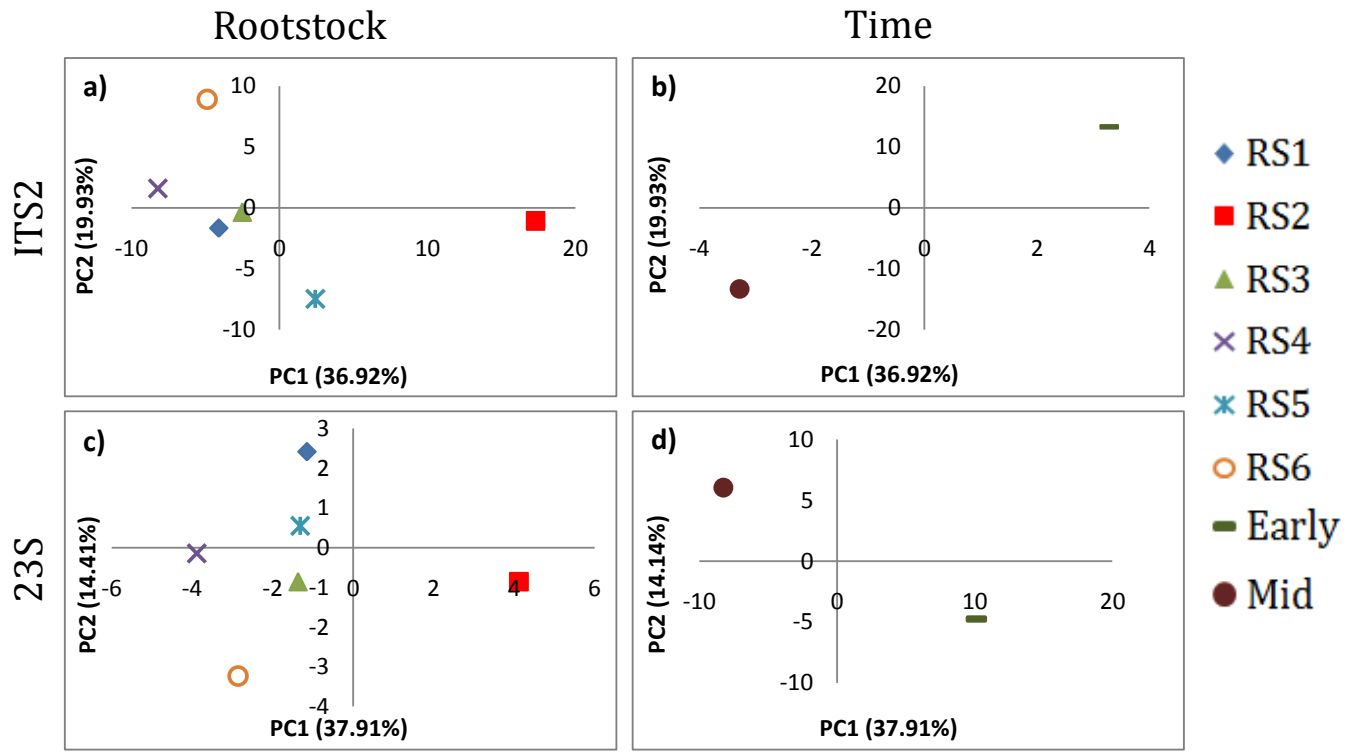

Figure 5.5: PCA ordination plots of microorganisms inhabiting the rhizosphere of tomato crops grown with different rootstock varieties (a, c) and the roots of plants at different sampling times (b, d) using T-RFLP profiles of ITS2 (a, b) and 23S rDNA (c, d). Blue diamonds represent the mean PC values from RS1, red squares represent RS2 samples, green triangles represent RS3 samples, turquoise asterisks represent RS4 samples, purple crosses represent RS5 samples and unfilled orange circles represent RS6. Dark green dashes represent the mean PC values from time point one, Dark red circles represent time points two.

Principal component (PC) scores were analyzed by ANOVA with rootstock and time as factors. For eukaryotic communities PC1 was not significant for either factor (p=0.4 for both factors) and PC2 was significant for time (P=<0.01) but not for rootstock (P=0.7). This could be due to their being barely any separation in eukaryotic communities as implied by ANOSIM and consequently there were no significant loadings with rootstock as a factor and only significance in PC2 for time, which accounts for <20% of the variation. For bacterial communities PC scores were not significant for separating rootstocks (p=0.8), implying that other factors were contributing to variation in the data such as time, which was found to be significant for PC scores. However PCs were significant with times as a factor (p=<0.01).

Significant loading values contributing to groupings of significant PCs were identified and their corresponding enzyme and T-RF combinations were compared to the output of FRAGSORT (section 2.8), resulting in a likely organism identity. Based on whether a loading value is positive or negative, organism identities were associated with the groupings on PCA ordination plots, suggesting that the presence or relative abundance of the organism in question is significantly contributing to groupings on that axis, see Tables 5.12 and 5.13.

For eukaryotic community data PC2 identified four enzyme and T-RF combinations that were significantly contributing towards PC groups. From FRAGSORT output data, four potential organisms were identified as matching significant T-RFs; these were *Colletotrichum coccodes* (A189), *Plectosphaerella cucumerina* (A341), *Coprinellus* sp. (A385) and *Phytophthora cinnamomi* (H182) (Table 5.12). As with other results the potential pathogens *C. coccodes* and *P. cucumerina* were highly abundant and contributing to later time points, furthermore a potential BCA *Coprinellus* sp. was associated with earlier sampling times.

Table 5.12: Eukaryotic organisms identified by significant PC loadings contributing to PC2, their T-RF and enzyme combination and which time point they are associated with based on their PC loading value.

Enzyme/T-RF	PC2	Potential Identity	Time
A189	-0.37	*Colletotrichum coccodes*	TP2
A341	-0.4	*Plectosphaerella cucumerina*	TP2
A385	0.55	*Coprinellus* sp.	TP1
H182	-0.56	*Phytophthora cinnamomi*	TP2

For bacterial community data significant PCs identified seven enzyme and T-RF combinations that were significantly contributing towards PC groups. From FRAGSORT output data, five potential organisms listed in Table 5.13 were identified.

Table 5.13: Bacterial organisms identified by significant PC loadings contributing to PC2, their T-RF and enzyme combination and which time point they are associated with based on their PC loading value.

Enzyme/T-RF	PC1	PC2	Potential Identity	Associated with:
H201	-0.36	-	*Agrobacterium radiobacter*	TP2
H202	-0.36	-	*Agrobacterium radiobacter*	TP2
H365	0.31	-0.36	*Acinetobacter* sp.	TP1
M201	-	-0.30	*Acinetobacter* sp.	TP1
M340	-0.68	-0.64	*Methylobacterium* sp.	TP2
M362	-	0.25	*Sulfurimonas* sp.	TP2
M365	0.27	-	*Leptothrix* sp.	TP1

5.3.2.3 Diversity indices

Species richness and diversity of the species were calculated to compare α-diversity groupings between microbial communities inhabiting the roots of organic crops with different rootstocks over time (Table 3.14). Species richness and diversity scores were tested for significance by ANOVA with rootstock and time as factors.

Microbial communities were found not to have significant differences in the number of taxa present or their diversity between different rootstocks. Furthermore, communities were found to have higher numbers of taxa and higher diversities associated with early time point than the mid time point, as

found with the soil amendment trial. However diversity indices did not detect significance for ITS2 rDNA, possibly due to there being barely any separation between communities as identified by ANOSIM.

Table 5.14: Diversity indices and species richness calculated from T-RFLP datasets for the two rDNA regions under examination, for all rootstocks and time points under examination

	ITS2			23S		
	S	**1-D**	**H'**	**S**	**1-D**	**H'**
Rootstock						
RS1	5±0.69	0.62±0.04	1.23±0.13	16±0.84	0.88±0.01	2.43±0.07
RS2	5.92±0.62	0.65±0.05	1.37±0.13	16.29±0.61	0.89±0.01	2.46±0.05
RS3	5.96±0.91	0.67±0.05	1.39±0.15	15.41±0.72	0.87±0.01	2.35±0.05
RS4	5.67±0.77	0.63±0.06	1.32±0.15	15.75±0.6	0.87±0.01	2.39±0.05
RS5	5.21±0.82	0.57±0.08	1.19±0.19	15.04±0.94	0.86±0.02	2.3±0.1
RS6	7.21±1.32	0.63±0.08	1.43±0.22	14.96±1.22	0.84±0.04	2.3±0.15
p-values	NS	NS	NS	NS	NS	NS
Time						
Early	6.38±0.58	0.66±0.04	1.4±0.1	16.23±0.42	0.89±0.01	2.45±0.03
Mid	5.28±0.4	0.6±0.04	1.24±0.1	14.93±0.5	0.85±0.02	2.3±0.06
p-values	<0.01	NS	NS	<0.01	<0.01	<0.01

S: Species richness: number of taxa or species present
1-D: Simpson index of diversity: higher values indicating higher diversity
H': Shannon index of diversity: higher numbers indicate higher diversity
±: Standard deviation of the average.
NS: No significance

5.3.2.4 Potential pathogens identified by T-RFLP

The PCA method did not determine significant differences in community assemblages between different rootstocks. However, from T-RFLP relative abundance data and FRAGSORT data, potential pathogens were identified on the different rootstocks examined shown in Table 5.15.

From this there appeared to be some possible rootstock effects worth further investigation, notably: lower levels of *C. coccodes* on Beaufort, Emperador, Stallone and Unifort than Efialto and Optifort; lower level of *P. cucumerina* on Optifort than other varieties; and potential susceptibility of Emperador and Unifort to phytophthora root rot, compared with the other varieties.

With different pathogens being more abundant on certain rootstocks, such as *C. coccodes* being found with greatest abundance on Efialto and Optifort and a *Phytophthora* sp. found only on Emperador and Unifort, there may be a

benefit in rotating rootstocks for use in organic soils to delay build up of pathogens in soil. Further work would need to be conducted using some of these rootstocks in the same plots over several seasons in conjunction with rootstock rotations to confirm whether there is a build up of potential pathogens and disease problems as a result of using the same rootstock. Complementary experiments could establish if pathogen build up in the soil and disease can be overcome by rootstock rotations.

Table 5.15: Potential fungal pathogens found associated with roots of organic commercial tomato crops grafted with different rootstock varieties using T-RFLP

Likely fungal pathogen	T1 Bea	T2 Efi	T3 Emp	T4 Opt	T5 Sta	T6 Uni
Colletotrichum coccodes	3.6	13.6	4.8	14.6	7.2	5.7
Cylindrocarpon destructans	-	0.6	-	-	-	-
Fusarium solani	-	-	-	-	0.4	-
Fusarium sp.	-	0.7	-	-	-	-
Macrophomina phaseolina	7.2	5.6	7.6	2.2	7.2	3.6
Phytophthora cinnamomi	-	-	34.2	-	-	30.9
Plectosphaerella cucumerina	14.1	19.9	15.3	2.1	22.3	10.4
Pyrenochaeta lycopersici	5.7	5.6	7.6	2.2	7.2	3.6

Previous work in other crops has found that different rootstocks do have an effects on microbial communities. However, such studies have generally compared varieties with good resistance to particular pathogens against varieties with no resistance (Rumberger *et al*., 2007). It may well be that rootstocks with good resistance to common pathogens give similar community profiles when grown in identical conditions and grafted onto the same tomato cultivar. However, it would not have been appropriate to use rootstocks with low resistance to pathogens in the commercial environment used for our studies.

5.3.2.5 Crop assessments

At the early assessment, there were significant differences between rootstocks in leaf yellowing and wilted or dead heads (Table 4.2). Leaf yellowing was relatively common (30-40% of plants) in rootstocks Stallone and Optifort, and significantly less in rootstocks Efialto, Beaufort and Unifort (9-14%). Wilted or dead heads occurred at a high incidence in cv. Unifort (47% of plants) and affected less than 29% of plants on all other rootstocks.

At the final assessment at the mid sampling time, there were no significant differences between rootstocks in the incidence of plants with leaf yellowing or the wilted or dead heads per plot (data not shown).

Table 5.16: Effect of rootstock on plant health of soil grown organic tomato, cv. Piccolo on Beaufort rootstock at the early time point as assessed by leaf yellowing and wilted or dead heads.

Rootstock	Mean % heads affected by	
	Leaf yellowing	Wilted or dead
RS1 Beaufort	10.7 ± 4.7	28.9 ± 8.2
RS2 Efialto	8.9 ± 4.2	11.2 ± 6.4
RS3 Emperador	29.9 ± 6.7	19.5 ± 7.2
RS4 Optifort	39.8 ± 6.9	9.3 ± 5.3
RS5 Stallone	43.2 ± 6.9	22.7 ± 7.6
RS6 Unifort	14.1 ± 5.2	46.6 ± 8.8
p-value	<0.01	<0.05

±: Standard deviation of the average; NS: No significance

Unfortunately no further assessments were possible due to a severe attack of russet mite which resulted in the crop being pulled out early. Based on these results, there is some evidence that an Efialto rootstock results in less leaf yellowing and wilted or dead heads than some other rootstocks early in the season in a crop of cv. Roterno grown in soil.

5.4 DISCUSSION

Composts have been commonly used on organic farms for increased soil fertility and disease suppression ever since the benefits of compost were suggested in 1988 (Garibaldi, 1988). Many composts have been reported to be suppressive against several soil-borne pathogens in various cropping systems (Noble and Coventry, 2005). However, no effect on disease suppression, and even an increase in disease symptoms due to compost usage have also been demonstrated (Termorshuizen *et al*., 2006). In general our findings suggest that the compost amendments used did not significantly affect plant health, root health or microbial community assemblages associated with the roots when compared to an untreated control on a commercial organic tomato nursery. Furthermore, the levels of the pathogens *C. coccodes* and *Fusarium* sp. were not suppressed by the composts used, giving similar disease symptom levels as the untreated control crops (Table 5.9).

It has been reported that the level and reproducibility of suppressive properties of compost can be increased by the addition of BCAs (Postma *et al*., 2006); however, the introduction of BCAs has also been found to vary in disease suppression success. Some BCAs have been reported to control disease, provide only partial disease control or fail to establish themselves in the root environment resulting in no disease control (Weller, 1988). Many studies suggest that the early application of BCAs, preferably at the propagation stage, is necessary for reliable establishment in the root environment (Van Os *et al*. 2004b; Calvo-Bado *et al*., 2006). However, our results from T-RFLP, plant and crop health methods suggest that BCAs do not necessarily alter microbial community assemblages or affect plant or root health even with early BCA application at the propagation stage. Furthermore, cultivation results suggested BCAs used in this study did not control levels of pathogens (notably *C. coccodes* or *Fusarium* sp.). This could have been due to the inability of BCAs to establish themselves in the root environment; this hypothesis was confirmed for the Trianum-P amendment whereby the BCA *T.*

harzianum was not identified by T-RFLP or culturing and microscopy analysis. This was presumably due to the high levels of indigenous species of *Trichoderma* outcompeting them as implied from Koppert BV results (Table 5.10). However, another theory may be that crop cultivar is important in the establishment of *Trichoderma* sp. Tucci *et al*. (2011) found significant differences in tomato crop response to *Trichoderma* sp. largely due to differences in crop genotype, suggesting that the response to certain BCAs is under genetic control.

Ultimately results from the soil amendment trial indicate that the rhizosphere microbial population structure in soil grown tomato is not easily altered by the treatments we used. Furthermore, the finding that microbial communities did shift with time but with community shifting being similar between treatments implies that other factors including time and those not under investigation had more control over microbial community composition. Bossio *et al*. (1998) have suggested that the factors influencing community structure can be ranked by importance with soil type and time being the most important factors for governing the composition of microbial communities, and this could explain why communities were found to change over time but not between treatments.

Certain publications suggest that the addition of both compost and BCAs at an early stage is required for successful disease suppression via increased activity of indigenous microbial communities and incorporation of BCAs (Hoitlink and Boehm, 1999; Pugliese *et al*., 2011). Notably in this experiment BCAs and composts were examined separately and future experiments could compare the singular use of BCAs and composts with a combination of the two treatments to establish if this is true for organic soil grown tomatoes. However, other studies suggest that established microbial communities are resistant to perturbation and changes in community constituents are predominantly plant-driven (Van Os *et al*. 2004a; Tucci *et al*., 2011). This would suggest that for the successful establishment of BCAs, microbial inoculants need to be ecologically competent and compatible with the host

plant and the environment to become established and to have beneficial effects. Thus, even if BCAs were applied early with compost, they may still not become established in the root environment or be able to suppress disease.

Similar results were obtained from the rootstock trial whereby PCA and species richness and diversity indices could not identify significant differences in microbial communities between treatments (rootstocks) and plant health assessments later in the season did not find significant differences between treatments. Although significant differences in microbial communities were identified by ANOSIM, R-values were very low implying that communities were not very well separated (R=<0.3; Table 5.11), which could explain why no significance was found by the other methods of analysis. Previous work in other crops has found that different rootstocks do have effects on microbial communities. However, such studies have generally compared varieties with good resistance to particular pathogens against varieties with no resistance (Rumberger *et al*., 2007). It may well be that rootstocks with good resistance to common pathogens give similar community profiles when grown in identical conditions and grafted onto the same tomato cultivar. However, it would not have been sensible to use rootstocks with low resistance to pathogens in the commercial environment used for our experiments. Once again it could be that other factors play more important roles in influencing microbial community structure and plant health than rootstock variety such as soil type, time and plant genotype (Bossio *et al*., 1998; Tucci *et al*., 2011).

In conclusion, both trials suggest that microbial community assemblages and their effect on plant health are not only difficult to examine with regards to determining their ecological roles due to complex interactions with the plant and the environment affecting microbial metabolic pathway expression and ultimately their ecological roles, but community structures are also difficult to perturb and control via organic methods in a soil environment. This is probably due to complexity of the soil environment which varies considerably in chemical, physical and biotic composition, and, consequently, also in ability to suppress disease and to facilitate the introduction of certain BCAs (Hoitink

and Boehm, 1999; Tiedje *et al*., 2001). It may be easier to alter and examine microbial communities is less complex environments, such as water, to establish which factors affect microbial function and ecological role before scaling up to the soil environment (Kent and Triplett., 2002). Ultimately, more understanding of microbial community function and plant effects and environmental effects are needed before the application of soil amendments and rootstock variety can be a reliable method for disease suppression and control. It is very likely that these disease suppression methods will need to be specific for a given environment, farming method and crop.

6 EFFECT OF RECYCLED NUTRIENT SOLUTION WATER PURIFICATION TREATMENTS ON TOMATO RHIZOSPHERE MICROBIAL COMMUNITIES AND ROOT PATHOGENS

6.1 INTRODUCTION

Hydroponic systems are an increasingly popular method of crop production as an alternative to soil production, due to more control over the plant growth environment, often resulting in higher yields and a reduction in soilborne pathogens. These systems were originally developed as open (run-to-waste) systems, whereby runoff nutrient solutions are disposed of into the environment. However, due to more strict government regulations concerning the discharge of spent nutrient solution and increased pressure to reduce water usage, closed (recirculating) systems, whereby nutrient solutions are replenished and recycled, have been developed to reduce pollution and the consumption of freshwater (Jensen 1997; Waechter-Kristensen, 1997; Vallance *et al*., 2011).

Although the use of recycled nutrient solutions does reduce water usage and alleviate nutrient runoff from crop production sites, dispersal of root pathogens via the recycled water is a major concern and is thought to be the main source of pathogens in closed systems (Hong and Moorman, 2005; Pagliaccia *et al*., 2008). It has been noted that while hydroponic systems avoid some soilborne pathogens, recirculating solutions are potentially more conducive to outbreaks of other plant pathogens, predominantly *Phytophthora* and *Pythium* species (Stanghellini and Rasmussen, 1994; Calvo-bado *et al*., 2003; Vallance *et al*., 2011). Consequently, several water disinfection methods have been investigated and developed for use in closed hydroponic cultivation (Runia, 1995; Ehret et al., 2001).

Two approaches for water disinfection are available, known as active methods and passive methods (Vallance *et al*., 2011). Active methods involve chemical or physical procedures, such as heat treatment (van Os *et al*., 1988; Ehret *et al*., 2001), ultraviolet (UV) irradiation (Runia, 1995; Zhang and Tu, 2000) and ozonisation (Runia, 1995; Yamamoto *et al*., 2003) which have germicidal effects on target pathogens as well as non target microorganisms and have been documented to eliminate up to 99% of the microflora colonising nutrient solutions (Vallance *et al*., 2011). Alternatively, passive methods involve biological strategies such as biofiltration (Collins and Graham, 1994; Calvo-Bado *et al*., 2003) and biocontrol methods (Paulitz and Berlanger 2001; Guetsky *et al*., 2002). Notably, passive methods do not result in complete sterilization or total removal of the natural microflora of nutrient solutions (Vallance *et al*., 2011) but have been reported to reduce or eliminate plant pathogens via mechanical and biological factors (Deniel *et al*., 2006; Vallance *et al*., 2009).

Although there are differences between microbial communities colonizing nutrient solutions and those colonizing the rhizosphere, the two communities can affect one another (Vanpeer and Schippers, 1989; Koohakan et al., 2004). The presence of plant pathogens and low microbial population levels in nutrient solutions are thought to eventually affect their counterparts in the rhizosphere (Zhang and Tu, 2000); furthermore, microflora on the roots can either grow and reproduce in nutrient solutions or survive and move through the water matrix (Hong and Moorman, 2005).

This chapter aims to determine the effect of an active water disinfection method (UV irradiation) and a passive water disinfection method (slow sand filtration; SSF) on tomato rhizosphere microbial communities and relative abundance levels of plant pathogens. Both UV irradiation and SSF disinfection methods have been recognized to reduce root rot in horticultural crops (Tu *et al*., 1999; Calvo-bado *et al*., 2003). However, both these active and passive methods have also been suggested to eliminate or suppress some pathogens while fail to effectively control others (Runia, 1993; Zhang and Tu, 2000). The

effects these methods have on pathogen populations and rhizosphere communities are of primary importance and are poorly understood particularly in commercial environments (Zhang and Tu, 2000; Hong and Moorman, 2005).

6.2 METHODS

6.2.1 UV irradiation of recirculated water

UV irradiation involves electromagnetic radiation with a wavelength between 100nm and 400nm, with optimal germicidal effects at 250mJ cm^{-2} obtained with a wavelength of 258nm in commercial greenhouses (Runia, 1994).

A nutrient film technique (NFT) tomato crop cv. Aranka was grown at a commercial nursery, whereby part of the crop was supplied with recirculated nutrient solution treated with a low-pressure UV lamp (UV dose 258nm; Figure 6.1) and the other part with untreated recirculated nutrient solution. Both UV treated and untreated crops were grown in the same glasshouse, under identical conditions and grown to commercial standards according to normal practice of the host nursery.

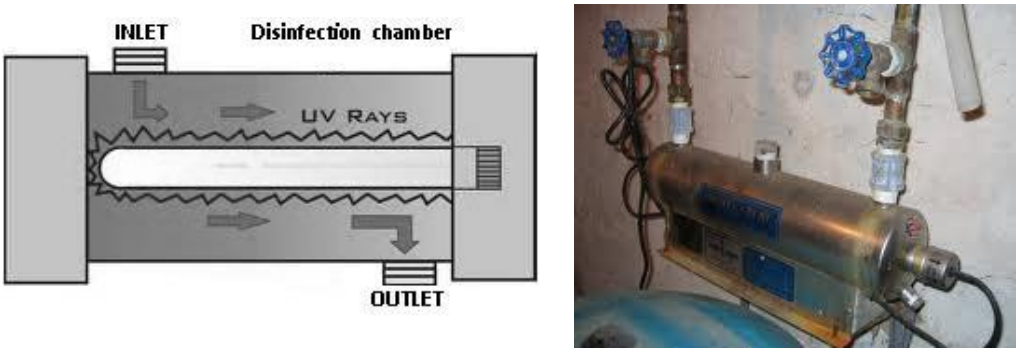

Figure 6.1: Diagram (left) and original picture (left) of the low-pressure ultraviolet lamp used in this chapter. The arrows on the diagram indicate the direction of the flow of recycled irrigation water.

Root samples were collected (section 2.3) in July 2010 when poor growth and root browning symptoms occurred in the crop. Roots were examined by T-RFLP (section 2.8) and culture onto agar, involving eight root samples (1 cm long) being cut from all plants and plated on PDA. Half the root samples were surface sterilized in sodium hypochlorite (1% for 1 minute) and the other half were untreated. Individual cultures were sub-cultured onto new plates; ITS2 regions were amplified (section 2.6), purified (section 2.10) and sequenced (section 2.12).

6.2.2 Slow sand filtration of recirculated water

SSF involves the slow passage of water through a column of sand and results in a reduction or removal of root pathogens and alterations in microflora of nutrient solutions via mechanical filtration through sand and biological effects of the *schmutzdecke*. The *schmutzdecke* is a bioactive layer that develops in the top layer of sand (Figure 6.2) and is generally considered as a biofilm that contains a diverse microbiota which alters and enriches the microbial communities present in recirculating solutions (Joupert and Pillay, 2008).

The experimental NFT systems (described in section 2.2), one connected to the slow sand filter (SSF) and one control system without SSF (Co), were employed in parallel over three replicate runs (Table 6.1). *Fusarium oxysporum* f. sp. *radicis lycopersici* (collection from the University of Nottingham; 5 ml of inoculum 10^6 spores ml^{-1}) was added to the nutrient solutions the day plants were transferred into the systems and after 14 days during each run in both the SSF and control NFT systems.

Root samples were collected (section 2.2) and examined by T-RFLP (section 2.8) and samples from run SSF-Co1 by culturing onto agar, involving eight root samples (1 cm long) being cut from all plants and plated on PDA. Half the root samples were surface sterilized in sodium hypochlorite (1% for 1 minute) and the other half were untreated. Individual cultures were sub-cultured onto new plates; ITS2 regions were amplified (section 2.6), purified (section 2.10) and sequenced (section 2.12).

Table 6.1: NFT runs, time of the year, sampling time points and temperatures of irrigation water during each replicate.

Dataset code	Time of year	Sampling time points	Water temperature (°C)
NFT Run			
SSF-Co1	Aug-10	14; 28 days	24-34
SSF-Co2	Oct-10	14; 28 days	18-25
SSF-Co3	May-11	14; 28 days	18-28

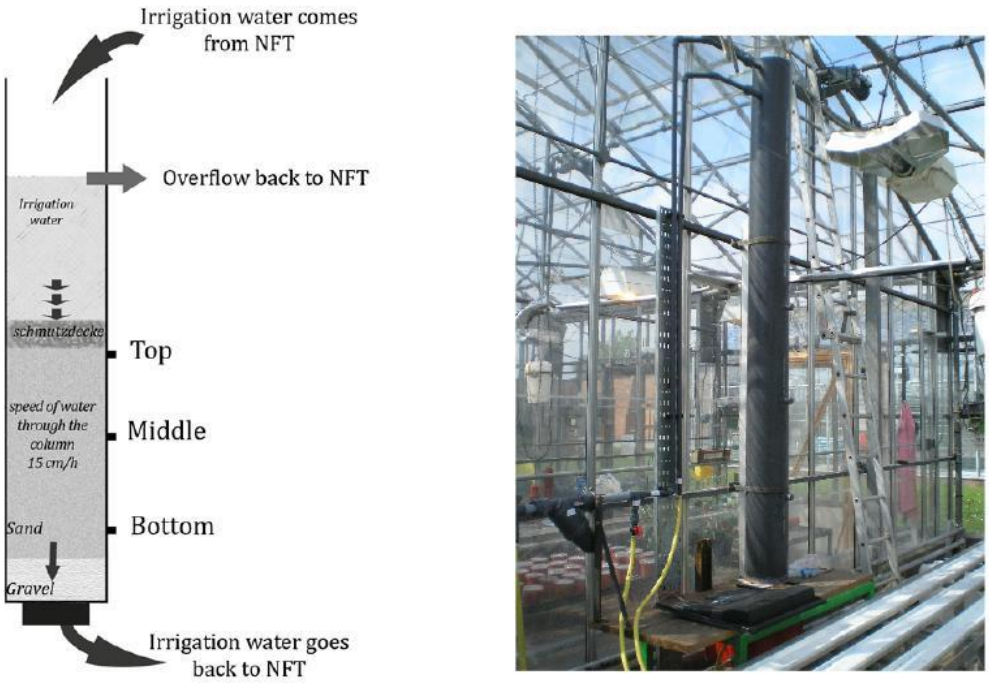

Figure 6.2: Diagram (left) and original picture (right) of the slow sand filter used in this chapter. The arrows on the diagram indicate the direction of the flow of recycled irrigation water (Cafá, 2012).

6.2.3 DNA extraction and T-RFLP analysis

Total community DNA was extracted from all root samples using the procedures described in section 2.5, followed by PCR amplification of rDNA (section 2.6), restriction digestion (section 2.7) and T-RFLP analysis (section 2.8). From the resulting T-RFLP profiles, putative taxonomic identities of T-RFs were assigned by importing T-RFLP profile information into FRAGSORT version 5.0 (Michel and Sciarini, 2003). The null hypotheses (H_0) were tested by Analysis of Similarities (ANOSIM; section 2.9.1). T-RFLP data were also represented in an ordinational space with Principal Component Analysis (PCA; section 2.9.2) and α-diversity was calculated using species richness and diversity indices (section 2.9.3).

6.3 RESULTS

6.3.1 Effect of an active method of nutrient solution disinfection on rhizosphere microbial communities and root pathogens

6.3.1.1 ANOSIM

To test the null hypothesis (H_0) that there were no differences between bacterial or eukaryotic communities inhabiting the roots supplied with UV light treated recirculated nutrient solution and roots from an untreated control, the ANOSIM test was carried out on ITS2 and 23S rDNA T-RFLP datasets as shown in Table 6.2

Table 6.2: ANOSIM test values and probabilities of null hypothesis tests obtained from comparisons of T-RFLP datasets of roots supplied with nutrients solutions treated with UV irradiation and untreated control roots.

	ITS2		23S	
	R-values	**p-values**	**R-values**	**p-values**
H_0 UV vs. Co	0.14	<0.05	0.19	<0.01

In both instances the null hypothesis was rejected, suggesting that were differences in microbial community assemblages between roots supplied with UV treated recycled solution and those supplied with untreated recirculating solution, detected by ITS2 and 23S rDNA. However, the R- values generated for both eukaryotic and bacterial communities are very low implying that there is barely any separation between microbial communities (Clarke and Gorley, 2001).

6.3.1.2 PCA analysis

Normalized T-RFLP datasets were used for PCA analysis to view transformed microbial community assemblage results in a two dimensional space. Figure 6.3 shows ordination plots generated from this analysis for ITS2 rDNA data (graph a) and 23S rDNA data (graph b). PC1 in both plots accounted for 100% of the variation in the data. PC1 scores were analyzed by one-way ANOVA

with treatment as a factor, and was found not to be significant between UV treatment and untreated crops (ITS2 p=0.1; 23S p=0.08).

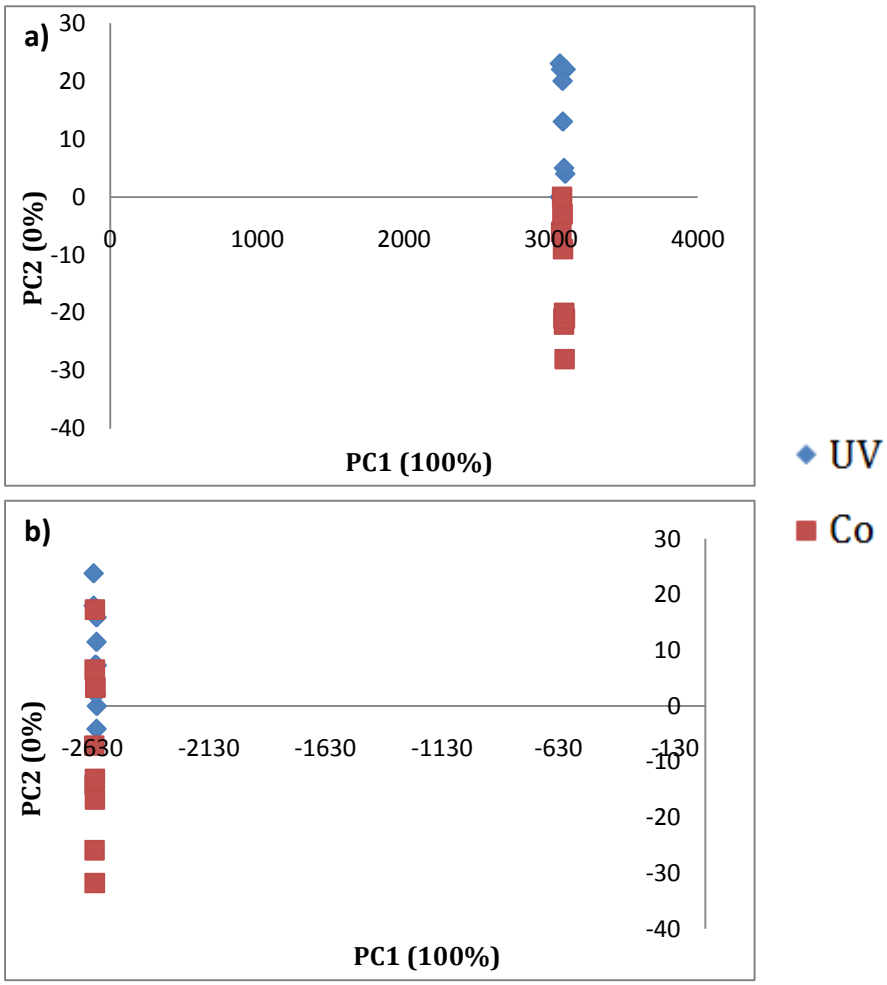

Figure 6.3: PCA ordination plots of microorganisms inhabiting the rhizosphere of roots supplied with UV light treated recirculating solution (blue diamonds) and the roots of control plants supplied with untreated recirculating solutions (red squares) using T-RFLP profiles of ITS2 (a) and 23S rDNA (b).

This result is confirmed by no separation of UV treated and untreated control crops on PC1 axes in ordination plots. Notably on Figure 6.3 (a) groups appear to be separated by PC2; however, this component accounted for no variation in the data and was excluded from the analysis. These results imply that there are no significant differences in microbial community assemblages between the UV treated crops and untreated control crops when analyzed with PCA. These results can be considered as consistent with ANOSIM which found barely any separation in microbial communities between the two treatments.

6.3.1.3 Species richness and diversity indices

Further comparisons of the eukaryotic and bacterial populations were made by determining species richness and diversity estimations with normalized T-RFLP datasets using the number of OTUs as a species richness estimator and the diversity indices Shannon index (H') and Simpson index (1-D), mean values are shown in Table 6.3.

Table 6.3: Diversity indices and species richness calculated from T-RFLP datasets for the two rDNA regions under examination, for roots in UV light treated solutions (UV) and untreated controls (Co).

	ITS2			23S		
	S	**1-D**	**H'**	**S**	**1-D**	**H'**
UV	3.55±0.19	2.38±0.13	1.08±0.17	5.56±0.48	3.14±0.21	3.45±0.25
Co	3.68±0.3	2.42±0.22	1.11±0.11	6.17±0.54	3.08±0.24	3.47±0.27
p-values	NS	NS	NS	NS	NS	NS

S: Species richness: number of taxa or species present
1-D: Simpson index of diversity: higher values indicating higher diversity
H': Shannon index of diversity: higher numbers indicate higher diversity
±: Standard deviation of the average.
NS: No significance

ANOVA was conducted on species richness and diversity scores and found no significant differences between rhizosphere microbial communities in UV irradiation treated recirculating solution and the control plants (Table 6.3). Van Os *et al.* (2004b) reported a reduction in microbial diversity levels of nutrient solution after UV treatment but found that differences often disappeared once solutions met plant material and this could partially explain why no significant differences were found in the rhizosphere environment. Nutrient solutions had already come in to contact with plant material before reaching sampled plants and it could be that there was little difference in the diversity of solutions between treatment and control plants resulting in little or no effect on rhizosphere microbial communities.

6.3.1.4 Pathogens associated with roots supplied with UV treated recirculated nutrient solution and an untreated control

T-RFLP *in silico* analysis revealed three potential root pathogens from both treatment and control roots: *Pythium dissocotum*, *Colletotrichum coccodes* and *Plectosphaerella cucumerina.* Fungal root pathogens *Py. dissocotum* and *C. coccodes* were isolated from both UV treated and control roots via culturing methods, while *Pl. cucumerina* could only be confirmed on UV treated roots (Table 6.3). Notably, *C. coccodes* was isolated from surface sterilized roots and *Py. dissocotum* from non-surface sterilized roots which implies that *C. coccodes* successfully infected roots and was the causal agent of the poor root health symptoms observed.

The relative dominance of *Py. dissocotum* has been documented in previous studies conducted in soilless hydroponic systems (Moulin *et al*., 1994; Moorman *et al* 2002; Herrero *et al*., 2003; Le Floch *et al*., 2007). Moreover, problems with *Pythium* sp. and *C. coccodes* in nutrient solutions treated with UV irradiation have been recognized whereby the thick cell walls of oospores produced by *Pythium* sp. and the dark pigmented fungal structures of *C. coccodes* such as pycnidia and acervulus are thought to make these species less susceptible to UV light treatment and thus are retained in solutions, multiply and accumulate in the rhizosphere (Zhang and Tu, 2000; Vallance *et al*., 2011). It has been suggested that the accumulation of survived propagules can cause minimal effects at high UV doses over a short period of time (Stanghellini *et al*., 1984), but can build up sufficiently enough to cause root rot over the growing season (Buyanovsky *et al.,* 1981).

Table 6.4: Recovery of fungi by isolation from tomato roots in UV treated water and untreated control roots, their best BLAST match and sequence homology.

Water	Sample disinfection	Best BLAST match	Homology (%)
UV	SS	*Colletotrichum coccodes*	98
UV	NSS	*Plectosphaerella cucumerina*	99
UV	NSS	*Pythium dissocotum*	97
Co	SS	*Colletotrichum coccodes*	99
Co	NSS	*Pythium dissocotum*	98

From T-RFLP normalized data, tomato plants grown with UV treated solution had significantly higher levels of *C. coccodes* than the untreated control crops ($p<0.01$) (Fig 6.4). *Pythium dissocotum* was identified by T-RFLP in both UV treated and untreated roots; however, there was no significant difference in relative abundance levels ($p=0.2$). Higher levels of *C. coccodes* in UV treatment crops could be attributed to the potential high availability of ecological niches after disinfection, possibly allowing retained *C. coccodes* to occupy them. Other environmental factors not under examination could also be causing the significant differences in levels of *C. coccodes* between treatment and control crops; for example positional effects could have influenced the development of disease, as crops were located in different areas of the glasshouse.

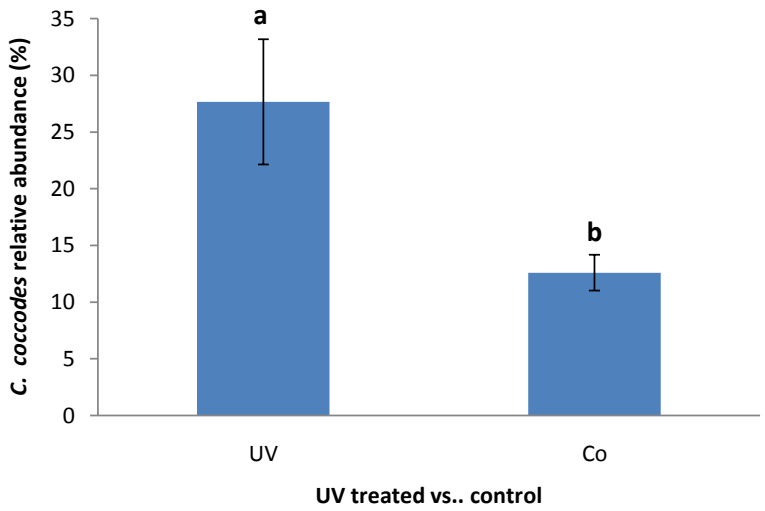

Figure 6.4: Mean relative percentage abundance levels of *C. coccodes* present on roots from UV treated water and untreated water as determined by T-RFLP output data. Error bars represent the standard error of the mean. Different letters represent significant differences.

In spite of different relative abundance levels of *C. coccodes*, both UV treated and control plants had disease symptoms. The level of sampling in this experiment was low and taken on one occasion; more informative results could be obtained by examining a similar experimental design with sampling taken over time, whilst monitoring nutrient solution microbial communities and analysis of the chemical composition of the nutrient solution.

Furthermore, examination of solution from the outlet of UV irradiation filters could confirm whether *Pythium* oospores and *C. coccodes* fungal structures can survive high disinfection levels in a commercial nursery.

6.3.2 Effect of a passive method of nutrient solution disinfection on rhizosphere microbial communities and root pathogens

6.3.2.1 ANOSIM

ANOSIM was performed for the comparison of T-RFLP datasets to test the null hypotheses (H_0) that there were no differences in the rhizosphere microbial community between roots in filtered recirculating water and roots in control recirculating water (H_0 NFT system) and there were no differences between time points replicates (H_0 time). H_0 was tested for 23S and ITS2 rDNA T-RFLP datasets, as shown in Table 6.5.

Table 6.5: ANOSIM test values and probabilities of null hypotheses (H0) tests obtained comparing T-RFLP datasets of 23S rDNA and ITS2 rRNA genes. H0 was tested between SSF and Co NFT systems (H0 NFT systems) and between time points (H0 time).

	ITS2		23S	
	R-values	p-values	R-values	p-values
H_0 NFT systems	0.41	<0.01	0.30	<0.01
H_0 time	0.64	<0.01	0.61	<0.01

The two null hypotheses (H_0) were rejected, indicating that the rhizosphere microbial communities were different between the two NFT systems and different between time points. Significant probabilities obtained with ANOSIM for the H_0 NFT systems imply that the effects of the SSF significantly alter the microbial constituents associated with the rhizosphere. Significant probabilities obtained for the H_0 time suggest that a shift in the microbial population was identified between 14 and 28 days of monitoring.

R-values generated from the analysis indicate that rhizosphere microbial communities are different but overlapping between NFT systems (SSF vs. Co)

over time. ITS2 rDNA markers give higher R-values than their corresponding 23S R-values in NFT datasets, indicating that rhizosphere eukaryotic populations were more variable between the two NFT systems than bacterial populations, implying that Eukarya are affected more by the SSF. This was also true for time datasets suggesting that few changes occur in bacterial populations over time compared to eukaryotic communities. The overall result suggests that differences were detected, but that the level of dissimilarity was relatively low.

6.3.2.2 PCA analysis

T-RFLP datasets comparing NFT systems were further compared using PCA analysis to view transformed microbial community assemblage results in a two dimensional space (Figure 6.5). Overall, PCA ordination plots show separation on both axis (PC1 and PC2) of microbial communities associated with roots supplied with SSF treated water (blue diamonds) and of communities associated with roots in untreated control water (red squares), confirming the hypothesis testing with ANOSIM. PC1 and PC2 account for 56.13% of the total variation in the eukaryotic dataset (Figure 6.5: a) and 47.18% in the bacterial dataset.

PC scores for the samples were analysed by one-way ANOVA, with NFT system as a factor. It was found that PC1 scores were significant for the grouping of eukaryotic communities ($p=<0.05$) but not for bacterial communities ($p= 0.62$). The opposite was found for PC2 scores whereby these scores were significant for groupings of bacterial communities ($p=<0.05$) but not for eukaryotic communities ($p= 0.67$). Factor loadings describe which T-RFs contribute the most variation in PCA and were further analyzed to establish which were making a significant contribution to PC1 for eukaryotic communities and PC2 for bacterial communities.

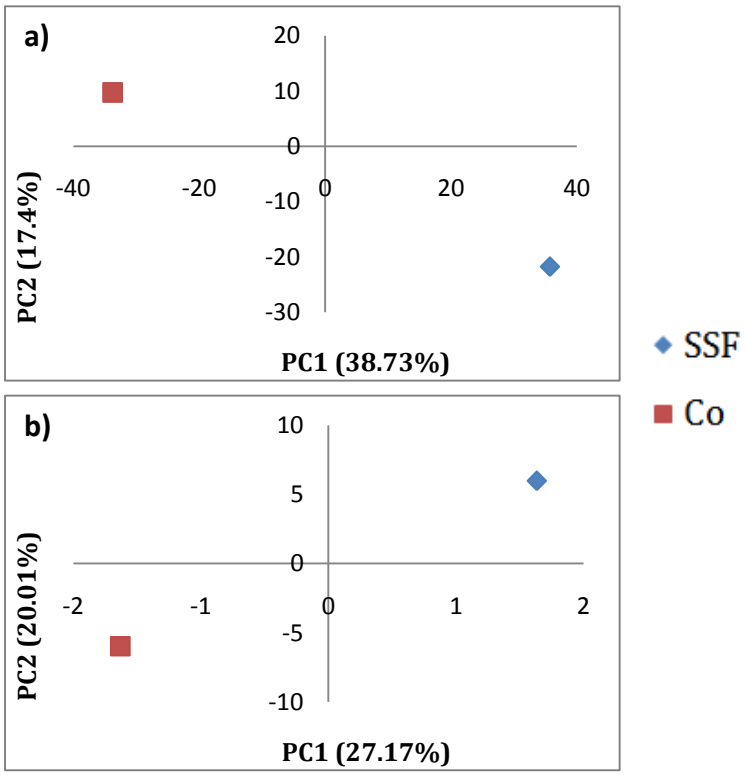

Figure 6.5: PCA ordination plots of microorganisms inhabiting the rhizosphere of tomato plant in SSF treated recirculating solution (blue diamonds) and tomato plants in untreated control recirculating water (red squares), using T-RFLP profiles of ITS2 (a) and 23S rDNA data (b).

For eukaryotic community data PC1 identified three enzyme and T-RF combinations that were significantly contributing towards PC groups (Table 6.6). From FRAGSORT output data, three potential organisms were identified as matching significant T-RFs; these were *Coprinellus* sp. (A384), *Fusarium oxysporum* (H73) and *Rhodotorula* sp. (H212).

Table 6.6: Eukaryotic organisms identified by significant PC1 loadings, their T-RF and enzyme combination and which NFT system they are associated with based on their PC loading value.

Enzyme/T-RF	PC1	Potential Identity	NFT system
A384	0.24282	*Coprinellus* sp.	SSF
H73	-0.25268	*Fusarium oxysporum*	Co
H212	0.46588	*Rhodotorula* sp.	SSF

Based on loading values (Table 6.6) it can be seen that *Coprinellus* sp. and *Rhodotorula* sp. are associated with roots in SSF treated water, suggesting that the SSF positively affect the rhizosphere competence of these organisms. Conversely, the potential pathogen *Fusarium oxysporum* (potentially the inoculated strain) is associated with roots in control water, implying the SSF successfully removes *F. oxysporum* via mechanical filtration or reduces the rhizosphere competence of *F. oxysporum* via biological activity of the *schmutzdecke*. These results are confirmed by mean abundances of these peaks from T-RFLP normalized datasets (Figure 6.6), whereby higher relative abundances are associated with the corresponding groupings on PCA plots. From Figure 6.6, it can be noted that all three organism are present in both treated and untreated roots and only the relative levels of *F. oxysporum* are significantly different when tested by ANOVA. This is in agreement with ANOSIM which identified differences between treatments but at a relatively low level of dissimilarity.

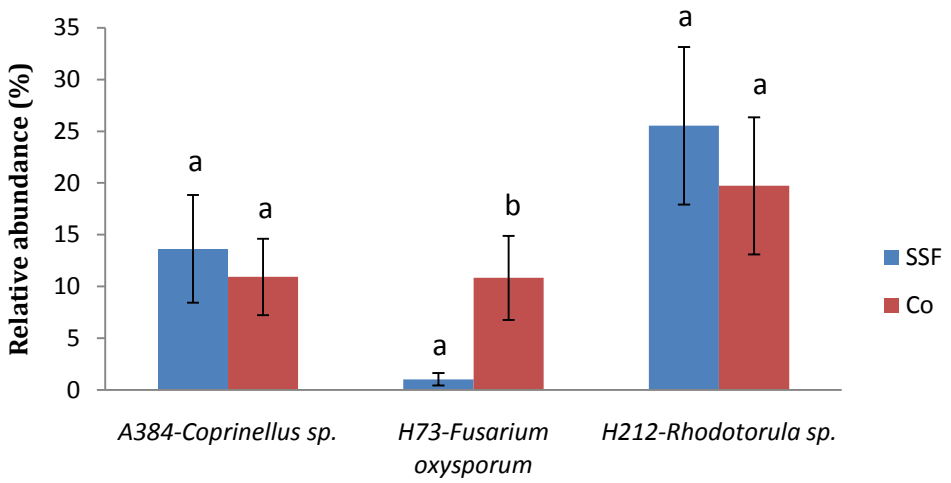

Figure 6.6: Relative abundance of enzyme and T-RF combinations that had a significant effect on PC1 groupings from PCA analysis of T-RFLP ITS2 NFT system datasets. Error bars represent the standard error of the mean. Different letters represent significant differences in relative abundances of T-RFs (p=<0.05). A= *Alu*I; H= *Hae*III.

Further to the effects of SSF potentially reducing *F. oxysporum* levels via mechanical and biological means, *Coprinellus* sp. (identified by *in silico* analysis in the rhizosphere; Table 6.6) have been previously reported to be antagonistic towards *F. oxysporum* via hyphal interference (Nakasaki *et al*., 2007) and could perhaps be playing a part in the control of *F. oxysporum*. However, the basidiomycetous fungi *Coprinellus* sp. prefer soil environments (Suhara *et al*., 2011) and have not been previously isolated from the tomato rhizosphere. Interestingly, the presence of this fungus was confirmed by pyrosequencing in the tomato rhizosphere in the media trial, but only in soil grown crops (Figure 4.5; 4.7). No fruiting bodies associated with *Coprinellus* sp. were identified during the SSF trials and further studies would need to be conducted to confirm if this organism can survive in hydroponic systems and if so, can it interfere with the establishment of *F. oxysporum* in the tomato rhizosphere?

For bacterial community data PC2 identified three enzyme and T-RF combinations that were significantly contributing towards PC groups. From FRAGSORT output data, the three potential organisms identified as matching significant T-RFs were *Pseudomonas* sp. (H160), *Agrobacterium radiobacter* (M361) and *Azoarcus aromaticum* (M373) (Table 5.7). *Pseudomonas* sp. (H160) and *Ag. radiobacter* were associated with roots in SSF treated water and *Az. aromaticum* was associated with control water.

Table 6.7: Bacterial organisms identified by significant PC2 loadings, their T-RF and enzyme combination and which NFT system they are associated with based on their PC loading value.

Enzyme/T-RF	PC2	Potential Identity	NFT system
H160	-0.42046	*Pseudomonas* sp.	SSF
M361	-0.62639	*Agrobacterium radiobacter*	SSF
M373	0.36529	*Azoarcus aromaticum*	Co

PC loading values indicates that *Pseudomonas* sp. and *Ag. radiobacter* are associated with SSF treatment rhizosphere communities and *Az. aromaticum* with control plants. These results are confirmed by mean relative abundances

of these peaks from T-RFLP normalized datasets (Figure 6.7), whereby higher relative abundances are associated with the corresponding groupings on PCA plots. All three peaks are present in both treated and control crops; furthermore, none of T-RF relative abundances are significantly different between treatment and control roots when analyzed by ANOVA (Figure 6.7). This once again confirms ANOSIM which identified differences between treatments but at a low level of dissimilarity.

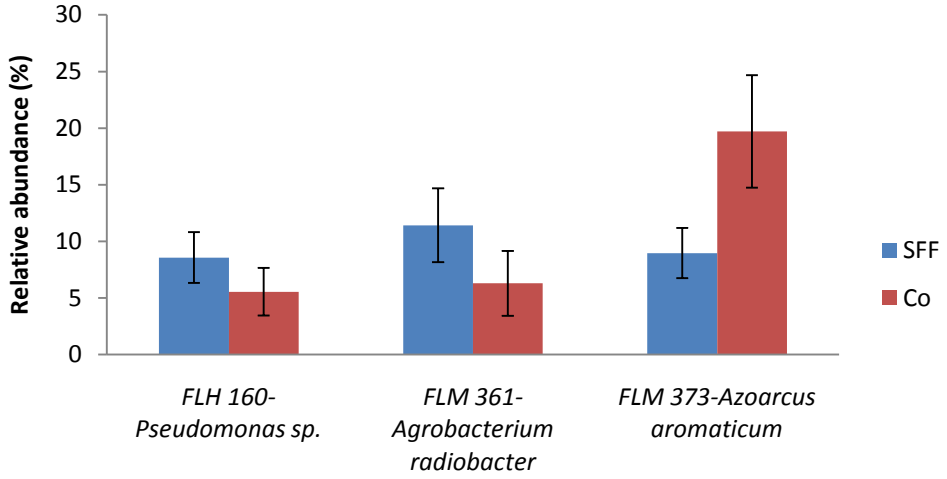

Figure 6.7: Relative abundance of enzyme and T-RF combinations that had a significant effect on PC2 groupings from PCA analysis of T-RFLP 23S rDNA NFT system datasets. Error bars represent the standard error of the mean. There were no significant differences in relative abundances of T-RFs (p=>0.05). H= *Hae*III; M= *Mse*I.

6.3.2.3 Species richness and diversity indices

For further comparison of the eukaryotic and bacterial populations between SSF treatment plants and untreated control plants, species richness and diversity estimations were provided from T-RFLP normalized datasets using the number of OTUs as a species richness estimator and the diversity indices Shannon index (H') and Simpson index (1-D), shown in Table 6.8.

In all instances eukaryotic and bacterial communities had higher species richness and were more diverse on roots in SSF treated recirculating solution than the untreated control roots. However, these differences were found not

to be significant when tested by ANOVA, except for the number of eukaryotic species as identified by the number of OTUs. This is in general agreement with ANOSIM and PCA where differences between treatment and control communities are observed but determined as having a very low level of dissimilarity.

Table 6.8: Mean diversity indices and species richness calculated from T-RFLP datasets for the two rDNA regions under examination, for both experimental NFT systems

	ITS2			23S		
	S	**1-D**	**H'**	**S**	**1-D**	**H'**
NFT system						
SSF	9.89±1.56	0.67±0.06	1.65±0.21	13.94±1.06	0.84±0.02	2.22±0.1
Co	6.08±0.62	0.6±0.05	1.27±0.12	11.92±1.01	0.81±0.02	2.02±0.1
p-values	<0.05	NS	NS	NS	NS	NS

6.3.2.4 Pathogens associated with roots supplied with SFF treated recirculated nutrient solution and an untreated control

Potential pathogens *F. oxysporum* and *A. radiobacter* were detected by T-RFLP analysis. *Fusarium oxysporum* relative abundance levels were significantly higher in control crops (Figure 6.6), whereas *A. radiobacter* was associated with SSF treatment crops but relative abundance levels were not identified to be significant between the two NFT systems (Figure 6.7). No root mat disease symptoms associated with *A. radiobacter* occurred in any of the replicate runs (Table 6.1); however, root rot symptoms associated with *F. oxysporum* were observed in dataset SSF-Co1 after 24 days. Markedly, only roots in the untreated control water showed visible root rot symptoms.

Root samples from SSF-Co1 were plated onto agar and *F. oxysporum* was isolated and confirmed by PCR amplification of ITS2 rDNA and best BLAST match (all >98% homology) results from both the SSF treatment roots and the untreated control roots. However, *F. oxysporum* was only isolated from non surface sterilized roots in SSF treatment roots indicating that the organism had not successfully infected the roots and implies that there might be some

biocontrol effects from the activity of SSF suppressing the pathogenicity of *F. oxysporum*. The pathogen could be isolated from both the non surface sterilized and surface sterilized roots in untreated control roots suggesting that *F. oxysporum* had infected the roots and was likely to be the causal agent for root rot symptoms observed (Figure 6.8).

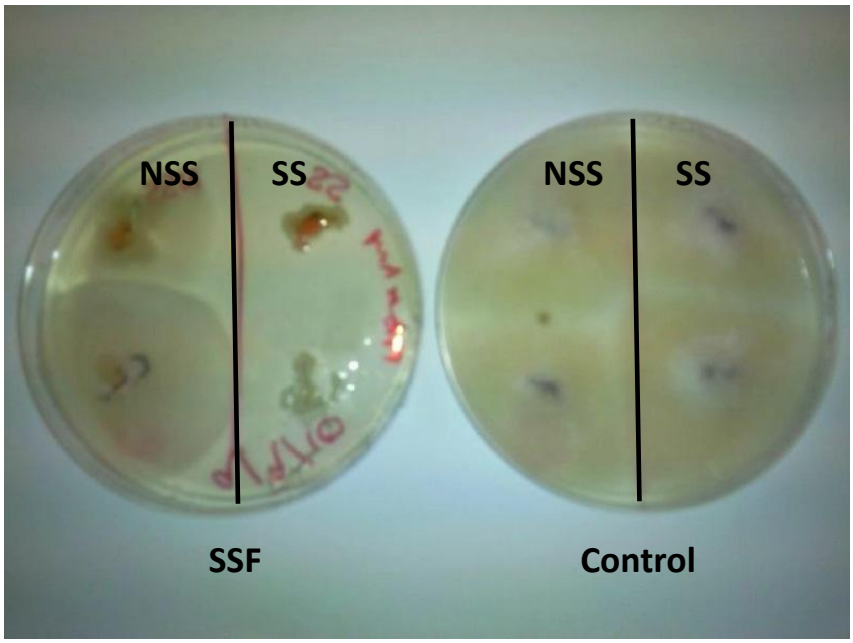

Figure 6.8: Image of PDA plates containing four root pieces from SSF-Co1 NFT run. Root samples from SSF treatment are plated on the left Petri dish and root samples from the control system and on the right Petri dish. White/pink fluffy colonies have been verified as *F. oxysporum* and are found on all four root pieces on control plate and on the non surface sterilized roots on the SSF plate. NSS: non surface sterilized; SS: surface sterilized.

Potential biocontrol agents *Coprinellus* sp. and *Pseudomonas* sp. were found to be associated with SSF treatment roots from PCA analysis (section 6.3.1.4) and have previously been reported to control *F. oxysporum* (Kamilova *et al.,* 2006; Nakasaki *et al*., 2007). These agents were also found on roots in the control treatment and were not found at significantly lower levels compared to SSF treatment when tested by ANOVA. Further studies would need to be conducted to confirm if the relative abundances of these isolates interfere with the establishment of *F. oxysporum* in the tomato rhizosphere and if their antagonistic behaviour is enhanced as a result of SSF treatment.

No disease symptoms occurred in the other replicates and this could be attributed to different environmental conditions between the replicates, which took place at different times of the year. Temperature, pH and nutrition are all known to affect pathogenesis of *F. oxysporum* and higher temperatures were recorded during the SSF-Co1 (Table 6.1) run which may have induced stress in the tomato plant and/or favour the pathogen (optimum growth at 28-35°C) (Cook and Baker, 1983). Results regarding the chemical composition of the water, the microbial community assemblages present in the recirculating solutions and in slow sand filter are currently under investigation by Giovanni Cafà (School of Biosciences, University of Nottingham). More data are required to determine the efficiency of microbial populations to reduce the pathogenicity of *F. oxysporum*, particularly over longer time periods.

6.4 DISCUSSION

Prevention of pathogenic attack from contaminated water in closed hydroponic systems has become a major challenge in the last decade (Eheret *et al*., 2001). Both active and passive methods of water disinfection used in this study were found to alter rhizosphere eukaryotic and bacterial populations when compared to untreated control plants using ANOSIM. However, the level of dissimilarity was relatively low, particularity in the active UV treatment method which found barely any separation between microbial populations when analyzing R-values generated (Clarke and Gorley, 2001).

PCA, species richness and diversity indices could not identify significant differences in microbial communities between UV treatment plants and untreated control plants. This was an unexpected result as a previous study had reported significant reductions in rhizosphere microbial populations after UV treatment (Zhang and Tu, 2000); however, other authors have reported that the reduction in microbial diversity levels in nutrient solution often disappear once solutions meet plant material (van Os *et al*., 2004b; Vallance *et al*., 2011). This could explain why no significant differences were found in the rhizosphere environment as solution had come into contact with plant material before reaching sampled plants, potentially resulting in little difference in microbial populations in solutions between treatment and control plants resulting in little or no effect on rhizosphere microbial communities.

Most importantly in this experiment, UV irradiation was found to not control levels of the root pathogens *Colletotrichum coccodes* or *Pythium dissocotum* resulting in root disease symptoms in UV treatment crops. UV treatment has previously been reported to fail to control root pathogens or reduce root rot severity (Berger *et al*., 1996; Zhang and Tu, 2000), but there is little doubt of the germicidal effect UV irradiation has on microflora colonizing water. Theoretically UV irradiation should result in total disinfection if sufficient doses reach the target organisms by manipulation of the dosage (Runia, 1994). However, total disinfection of recirculation solution is often difficult to

achieve, especially with organisms like *C. coccodes* or *Py. dissocotum* which produce dark pigmented fungal structures or thick walled oospores respectively and are thought to be relatively less susceptible to UV irradiation. This can result in these organisms being retained in solutions, multiplying and accumulating in the rhizosphere (Zhang and Tu, 2000; Vallance *et al.,* 2011). It has been suggested that the accumulation of surviving pathogenic propagules can cause minimal effects at high UV doses over a short period of time (Stanghellini *et al.,* 1984), but can build up sufficiently to cause root rot over the growing season (Buyanovsky *et al.,* 1981).

On the contrary, the use of the passive SSF method did result in control of the root pathogen *Fusarium oxysporum* in the SSF-Co1 run of our trials, with samples in untreated control water showing visible root rot symptoms while SSF treatment roots appearing visibly healthy. Markedly *F. oxysporum* was isolated from non surface sterilized SSF treatment roots and identified in these samples by T-RFLP, albeit at significantly lower levels. This implies that there might be some biocontrol effects from the activity of SSF suppressing the pathogenicity of *F. oxysporum*. Potential biocontrol agents *Coprinellus* sp. and *Pseudomonas* sp. were found to be associated with SSF treatment roots from PCA analysis and have previously been reported to control *F. oxysporum* (Kamilova *et al.,* 2006; Nakasaki *et al*., 2007). However, further studies are required to establish whether these organisms are promoted by the effect of SSF and to determine their efficiency to reduce the pathogenicity of *F. oxysporum*. In addition, trials should be set up over longer time periods to establish whether *F. oxysporum* continues to be controlled with time and over different crop growth stages.

In conclusion, this work supports the scepticism of removing total microflora from nutrient solutions via active methods resulting in the potential loss of beneficial microbes and increased availability of ecological niches (Runia *et al*., 1988; Zhang and Tu, 2000). These methods are particularly ineffective in systems where the contamination pathways are not resolved resulting in recontamination of solutions with potentially less pathogen suppressive

capabilities (Hong and Moorman, 2005). The use of passive methods such as the SSF could represent a natural and cheap biological solution to enrich and stabilize microbial communities in recycled irrigation water, resulting in the reduction and suppression of root pathogens. However, further work is required to establish appropriate maintenance of SSF, suitable operational conditions and identification of optimum levels of key microbiota to obtain consistent control of pathogens using this method. Previous work has identified SSF methods to eliminate or suppress some pathogens while fail to effectively control others (Runia, 1993; Déniel *et al*., 2006); moreover, human pathogens have been found to be supported in SSF (Calvo-Bado *et al*., 2003), which currently limits its commercial application.

7 SUMMARY AND FINAL CONCLUSIONS

Examination of the effects of growth media, plant age and disease control methods on rhizosphere microbial communities and tomato root health were achieved using two molecular methods, the fingerprinting method T-RFLP and the sequence-based method pyrosequencing, alongside traditional cultivation, microscopy and plant health assessment techniques. Furthermore, relationships between rhizosphere microbial community diversity, the presence or relative abundances of root pathogens with crop health assessments were analyzed.

7.1 MICROORGANISMS ASSOCIATED WITH TOMATO RHIZOSPHERE

The molecular analysis of ITS1 and ITS2 rDNA identified four eukaryotic kingdoms present in the tomato rhizosphere, namely Fungi, Chromista, Protozoa and Anamalia. Fungi were the most abundant and diverse Eukarya identified in the root environment predominantly belonging to Phyla Ascomycota and Basidiomycota within the subkingdom Dikarya; as well as the Phyla Zygomycota, Chytridiomycota and Glomeromycota. Chromista belonging to the Phyla Oomycota were also very abundant and relatively diverse particularly within the rhizosphere of hydroponically grown tomatoes. Protozoa from the Phyla Ciliophora and Anamalia belonging to the Phyla Nematoda were less diverse and less commonly identified; furthermore, they were only associated with media with high organic matter (Chapter 4). However, Protozoa and Anamalia were not primary targets of this study and methods of analysis were not optimized for their examination; consequently these organisms may have been more common and diverse than our results predict.

Eukaryotic taxa identified by molecular methods in this thesis have been previously described as common inhabitants of tomato rhizosphere and were backed by culturing and microscopy analysis (Appendix I; Price, 1976), indicating the validity of the results obtained. Notably, the use of

pyrosequencing increased the resolution and confidence of rDNA analysis, identifying organism within the described phyla to a genus and often species level (Bruns and Shefferson, 2004; Liu et *al*., 2008), some of which have not been previously described in the tomato rhizosphere. Moreover, this method identified that 40% of Eukarya in the rhizosphere did not match any previously published sequences when BLAST searches were conducted. This suggests that a large proportion of the eukaryotic community in the tomato rhizosphere belong to previously undescribed eukaryotic taxa.

Ecologists have long predicted that fungi and fungi-like organisms are highly diverse and poorly studied, with most species of these groups being not yet described (Hawksworth, 1991; Schmit and Mueller, 2007). Nilsson *et al*. (2009) found that only 0.9% of the estimated amount of fungal species could be identified from fully identified sequences (FIS) using BLAST searches of ITS data in public databases (International nucleotide sequence databases). They went on to predict that the sheer numbers of sequences from pyrosequencing studies are likely to dilute the presence of FIS in BLAST hits and will further complicate the identification of environmental community constituents. Clearly, from the onset of huge amounts of unidentified eukaryotic community data being deposited into public databases, from next generation sequencing, far more emphasis must be placed on improving eukaryotic taxonomy for molecular identification. It has been suggested that temporary methods for assigning clusters of unidentified Eukarya into standardized molecular species are required pending formal taxonomic assignment. Without a unified method for processing high throughput sequencing data, or open-access repositories, it will prove difficult to compare data across studies, and furthermore, increase the workload for taxonomists (Horton *et al*., 2009).

Prokaryotes were analyzed targeting 23S rDNA and constituents belonging to the kingdom bacteria were also found to be highly diverse and relatively abundant in the tomato rhizosphere. Bacterial communities were found to mainly consist of two major Phyla; largely Proteobacteria, followed by Firmicutes. Many of the prokaryotic taxa identified in this study have been

previously associated with the rhizosphere (Liesack and Stackebrandt, 1992; Singh *et al*., 2011; Vallance *et al*., 2011). Notably, bacterial community ecology is plagued by the same discrepancy between the numbers of described taxa and estimated number of species identified by high throughput sequencing methods (Sogin *et al*., 2006) as eukaryotic ecology. However, this field is aided by the development of analytical tools, bioinformatics pipelines, database resources and open-access repositories for high-throughput datasets of which many are not yet available or compatible with eukaryotic data (Bik *et al*., 2012).

In conclusion, the extraordinary diversity of microbial communities present in the rhizosphere, of which only a small proportion have been characterized, precludes the use of counting and naming approaches traditionally used in community ecology. The advent of next generation sequencing technologies, with the capacity to generate hundreds of thousands of limited-length sequences, facilitates accurate *en masse* biodiversity assessments of microbial communities from environmental samples . Ecologists now face the daunting task of characterizing very large numbers of environmental microorganisms in a taxonomic context in order to fully describe communities and move beyond estimations of biodiversity.

7.2 FACTORS AFFECTING RHIZOSPHERE MICROBIAL COMMUNITY ASSEMBLAGES

Various factors thought to affect the composition of rhizosphere microbial communities and deemed of concern or interest to UK tomato growers were examined; to be precise: growth media, plant age and various disease control methods. In all trials conducted during this study plant age (time) was found to be a significant factor affecting rhizosphere microbial communities, indicating there were significant shifts in the microbial populations between time points examined. In general, microbial community changes with time were found to be greater between samples taken early in the growing season than between later time points and biodiversity was generally found to

increase with time. Similar findings have been described in other studies and have been attributed to the initially sterile nature of most growing systems being rapidly colonized by microbes with the addition of plant material until a stable community is formed, leading to subtle shifts in community structures with plant development (Postma *et al*., 2000; Koohakan *et al*., 2004; Menzies *et al*., 2005; Pagliaccia *et al*., 2008).

Significant differences were also found in microbial communities associated with the rhizosphere of tomato plants grown in different media (Chapter 4). Overall, rhizosphere microbial community assemblages and diversity were relatively similar between the three crops grown in hydroponic systems with media (WF, RW and coir); whereas, crops grown in soil and NFT systems had comparatively distinctive communities associated with their roots. Soil had the highest species richness and diversity levels of all media which was expected due to higher levels of organic matter associated with soil. Furthermore, similar findings have been previously established (Postma *et al.,* 2008). NFT and RW systems were predicted to have similar microbial assemblages as soilless systems without organic components are considered poor for microbial growth. NFT crops had the lowest levels of species richness and diversity; however, RW crops were found to have relatively diverse rhizosphere communities similar to hydroponic systems with organic media. Comparable results were obtained by Menzies *et al.,* (2005) who suggested that the tightly woven nature of RW creates favourable conditions for microbial growth with plant root exudates providing organic substrates. It is thought that NFT systems were less conducive for microbial growth than other media under examination due to the lack of solid substrate acting as physical protection and space. Media also had an effect on which potential pathogens were present, with soil-borne pathogens being predominantly found in soil or organic media hydroponic systems (WF and coir) and oomycota being more commonly associated with hydroponically grown crops (Adams *et al.,* 1989; Stanghellini and Rasmussen, 1994). These findings are likely to be due to evolutionary adaptations allowing certain pathogens to

thrive in different environments. For instance, oomycota that produce zoospores are adapted well to liquid environments, whereby they can actively swim towards their hosts and can cause an epidemic in favourable conditions (Stanghellini and Rasmussen, 1994).

Conversely, the soil amendments and rootstocks examined did not significantly affect microbial community assemblages or biodiversity between treatments (Chapter 5), indicating that indigenous soil microflora and rhizosphere microbial population structure in soil grown tomato are not easily altered by the treatments used. Furthermore, the finding that microbial communities did shift with time during these trials but with community shifting being similar between treatments and rootstocks implies that other factors including time and perhaps factors not under investigation had more control over microbial community composition. Bossio *et al*. (1998) suggested that the factors influencing rhizosphere community structure can be ranked by importance with soil type and time being the most important factors for governing the composition of microbial communities, and this could explain why communities were found to change over time but not between treatments. Similar results were also obtained by Pagliaccia *et al*. (2008) who determined that host, media and time represent the most influential factors on microbial populations. Our results suggest that if the most influential factors are kept consistent then rhizosphere microbial structures are robust and difficult to perturb with changes in a factor contributing less control over microbial community composition.

7.3 EFFECT OF RHIZOPHERE MICROBIAL DIVERISTY, ROOT PATHOGENS AND PLANT HEALTH ON ROOT DISEASE

No direct link between crop health assessments and rhizosphere microbial community diversity or presence/relative abundances of root pathogens from T-RFLP and culturing methods could be established. Furthermore, the presence of potential pathogens and poor crop health assessments during the growing season did not always result in poor health or disease symptoms at

the end of cropping assessment in our trials. However, causal agents could be determined using culturing and T-RFLP in cases of symptomatic root disease as well as the identification of potential biocontrol agents (BCAs) in visibly healthy comparison crops by T-RFLP (Chapter 3; Chapter 6), indicating the reliability of the methods used.

The lack of consistency between pathogen abundance or presence and disease are probably due to the complex chemical and physical interactions that occur in the rhizosphere, involving the microbial constituents, media and plant; all of which have an effect on the outcome of disease (Gregory, 2006). Furthermore, identification of microbial constituents, using rDNA as a molecular marker, only provided information regarding diversity of microbial populations and does not provide information regarding metabolic pathway expression. It has been previously documented that dominant microbes do not always have high levels of metabolic activity; furthermore microbes are capable of expressing multiple pathways ultimately affecting their ecological roles (Duineveld *et al.,* 2001). In addition, symptomless infections with root pathogens have been previously reported, where it was noted to affect plant growth without the expression of symptoms on host roots (Stanghellini and Kronland, 1986).

These results suggest that many factors control the rhizosphere competence and ecological role of different species and ultimately affect the outcome of disease. As no known methods are capable of efficiently assessing the fate of total microorganisms in the rhizosphere over time and space, this study could be considered as part the 'descriptive phase' in this field. The 'descriptive phase' allows microbes to be identified and changes in assemblages to be observed, but their ecological roles and the effect of changes on an ecosystem are still not known. The 'descriptive phase' has been expressed as preliminary and necessary prior to a 'testing phase', where advances in technology will facilitate the understanding of the role and function of entire ecosystems (Kent and Triplett, 2002).

Further investigations should be conducted with common and abundant pathogens identified in this study, for example: *Colletotrichum coccodes*, *Fusarium oxysporum* and *Pythium dissocotum* to further understanding of what conditions trigger pathogenesis in tomato crops. Such trials should be carried out in simpler controlled experimental environments such as hydroponic systems, which the results could be used for transfer to more complex systems such as soil. Environmental conditions should be monitored, as well as the chemical composition of the water, the microbial community assemblages in media and rhizosphere and changes in root exudation.

7.4 SUSTAINABLE ROOT DISEASE CONTROL METHODS

The development of alternative and sustainable disease control methods such as the use of composts and BCAs in soil grown crops and the safe re-use of irrigation water in soilless cultivation is a must for the tomato industry due to more strict government regulations and concerns over the sustainability of conventional chemical-intensive agriculture (Dixon and Margerison, 2009; Vallance *et al*., 2011). Composts have been commonly used for disease suppression ever since the benefits of compost were suggested in 1988 (Garibaldi, 1988). However different composts used in this thesis were found to not alter rhizosphere microbial community or affect plant health when compared to an untreated control crop. Similar results were obtained from the application of previously characterized BCAs compared to an untreated control, with evidence suggesting that BCAs could not establish themselves in the rhizosphere (Chapter 5). These results imply that rhizosphere microbial population structures in soil grown tomato are not easily altered by the treatments used. It has been proposed that for successful establishment of BCAs, microbial inoculants need to be ecologically competent, able to perturb indigenous microflora, compatible with the host plant and the environmental growth conditions to become established and to have beneficial effects (Van Os *et al*. 2004a; Tucci *et al*., 2011).

Prevention of pathogenic attack from contaminated water in closed hydroponic systems has become a major challenge in the last decade (Eheret *et al*., 2001). Both active and passive methods of water disinfection used in this study were found to alter rhizosphere eukaryotic and bacterial populations when compared to untreated control plants using ANOSIM (Chapter 6). The active method used in our study was found to not control root disease, conversely, the passive method did control root disease over the time scales measured. Our results support the scepticism of removing total microflora from nutrient solutions via active methods resulting in the potential loss of beneficial microbes and increased availability of ecological niches (Runia *et al*., 1988; Zhang and Tu, 2000). Recycled irrigation water is thought to contain a delicate microbiological composition that should be preserved in order to avoid the colonization of ecological niches by pathogens (Berkelmann *et al.,* 1994). The use of passive methods such as the SSF could represent a natural and cheap biological solution to enrich and stabilize microbial communities in recycled irrigation water, resulting in the reduction and suppression of root pathogens.

However, it is currently not possible to determine the role and function of all organisms in ecosystems and ultimately not possible to determine whether the use of compost, addition of BCAs or microflora alterations from SSF will result in disease suppression or be able to perturb indigenous microflora, presently rendering these disease control methods unreliable and limiting their application in commercial nurseries. It could be that a growers may have to use specific biocontrol methods on certain crops, production techniques and crop developmental stages to obtain reliable disease control. If this were true, biological control would have no future as a commercial product, as such products need to be produced *en masse* to be economically viable.

7.5 MOLECULAR METHODS FOR THE EXAMINATION OF MICROBIAL COMMUNITIES

The use of fingerprinting and next generation sequencing methods provided the identification of microorganisms in the tomato rhizosphere, as well as characterising patterns over time and between different treatments. In general, the comparison of the two molecular methods used in this study showed that T-RFLP results correlated well with the pyrosequencing results regarding comparable species richness levels associated with samples, community assemblage patterns and Phyla identified in the root environment.

Pyrosequencing provided detailed data on the taxonomic identity of eukaryotic community constituents often to a genus or species level, their relative abundance and community assemblage patterns, as well as information regarding the level of community coverage and sampling requirements. T-RFLP also provided detailed information of community assemblage patterns, although it gave limited detail of the taxonomic identity of Eukarya, their abundance levels and provided less coverage of the community present (chapter 4). Furthermore, clone libraries, culturing data and pyrosequencing data was necessary to verify the identity of T-RFs. On the other hand, T-RFLP was found to be a reliable method that, at a fraction of the cost of pyrosequencing, provided an overview of the eukaryotic communities present in the rhizosphere and allowed comparisons to be made regarding community constituents, their relative abundance, and microbial diversity of each environment and whether these differences were statistically significant.

Yet, the price of next generation sequencing will inevitably decrease; furthermore, the technology is constantly improving and the ease of use and accuracy of analytical tools and pipelines are likely to develop as these methods become common practice. With this in mind, next generation sequencing represents the future approach for resolving complex microbial communities in environmental samples. However, the ultimate goal for ecological studies is to move beyond descriptions of ecosystems, to an understanding of ecosystem function. Complementary approaches, examining

metagenomics, metatranscriptomics and metaproteomics of ecosystems will be essential to determine factors governing spatial distributions and factors driving ecological assemblages.

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APPENDICES

APPENDIX I

Table I.I: List of fungi and oomycota pathogens reported on tomato roots

Eukaryote	Recorded on tomato in UK	Reference
Alternaria solani	?	Ellis (1971)
Aphanomyces cladogamus	?	Domsch & Gams (1993), Farr *et al* (1995)
Armillaria mellea	Yes	Moore (1959)
Botrytis cinerea	Yes	Price (1980)
Calyptella campanula	Yes	Fletcher (1984), Clark (1983)
Colletotrichum coccodes	Yes	Blancard (1994), Jones *et al* (1991)
Didymella lycopersici	Yes	Evans (1979), Watterson (1986)
Fusarium oxysporum	Yes	O'Neill & Wedgwood (2006)
Fusarium oxysporum f. sp. lycopersici	Yes	Fletcher (1984), Jones *et al* (1991), Blancard (1994) and others
Fusarium oxysporum f. sp. radicis-lycopersici	Yes	Blancard (1994), Jones *et al* (1991)
Fusarium redolens	Yes	Moore (1959), Leslie & Summerell (2006)
Fusarium semitectum	?	Booth (1971)
Fusarium solani	Yes	Fletcher (1984), Leslie & Summerell (2006)
Fusarium spp.	Yes	Fletcher (1984)
Humicola fuscoatra	Yes	De Gruyter *et al* (1992), Menzies & Ehret (1997), Menzies *et al* (1998).
Macrophomina phaseolina	No	Smith *et al* (1988)
Plectosphaerella cucumerina	No	Smith *et al* (1988)
Phymatotrichopsis omnivora	No	Farr *et al* (1995)
Phytophthora arecae	No	Erwin & Ribeiro (1996)
Phytophthora capsici	No	Watterson (1986), Jones *et al* (1991), Smith *et al* (1988), and others.
Phytophthora cinnamomi	No	Farr *et al* (1995), Erwin & Ribeiro (1996)
Phytophthora citricola	No	Erwin & Ribeiro (1996)
Phytophthora cryptogea	Yes	Watterson (1986), O'Neill *et al* (2000), Smith *et al* (1988), and others.
Phytophthora drechsleri	No	Koike *et al* (2007)
Phytophthora erythroseptica	Yes	Evans (1979), Watterson (1986), Smith *et al* (1988)
Phytophthora fragariae var. fragariae	No	Erwin & Ribeiro (1996)
Phytophthora hibernalis	No	Erwin & Ribeiro (1996)
Phytophthora infestans	Yes (on foliage	Lievens *et al* (2004)

	and fruit)	
Phytophthora megasperma var. megasperma	Yes	CSL checklist of fungal pathogens
Phytophthora mexicana	No	Erwin & Ribeiro (1996)
Phytophthora nicotianae var. nicotianae	Yes	Dixon (1981)
Phytophthora nicotianae var. parasitica	Yes	Fletcher (1984), Jones *et al* (1991), Blancard (1994) and others
Phytophthora palmivora	No	Farr *et al* (1995)
Phytophthora phaseoli	No	Erwin & Ribeiro (1996)
Phytophthora richardiae	No?	Hull (1991)
Phytophthora verrucosa	Yes	Baker (1972), Erwin & Ribeiro (1996)
Pyrenochaeta lycopersici	Yes	Fletcher (1984), Jones *et al* (1991), Blancard (1994) and others
Pyrenochaeta terrestris	No	Farr *et al* (1995), Westcott (2001)
Pythium arrhenomanes	?	Jones *et al* (1991), Farr *et al* (1995)
Pythium butleri	Yes	CSL checklist of plant pathogens
Pythium debaryanum	Yes	Jones *et al* (1991), Farr *et al* (1995)
Pythium diclinum	?	Rafin & Tirilly (1995)
Pythium echinulatum	?	Rafin & Tirilly (1995)
*Pythium d*iclinum	Yes	CSL checklist of plant pathogens
Pythium irregulare	Yes	Domsch & Gams (1993)
Pythium megalacanthum	?	Farr *et al* (1995)
Pythium myriotylum	?	Jones *et al* (1991), Farr *et al* (1995)
Pythium oligandrum	Yes	Farr *et al* (1995), Price (1980)
Pythium paroecandrum	Yes	British Mycological Society database
Pythium periplocum	?	Rafin & Tirilly (1995)
Pythium salpingophorum	?	Farr *et al* (1995)
Pythium torulosum	?	Domsch & Gams (1993)
Pythium ultimum	Yes	Jones *et al* (1991), Farr *et al* (1995), Rafin & Tirilly (1995)
Pythium vexans	?	
Pythium 'group F'	?	Rafin & Tirilly (1995)
Pythium 'group G'	?	Rafin & Tirilly (1995)
Rhizoctonia solani	Yes	Fletcher (1984), Jones *et al* (1991), Blancard (1994) and others
Sclerotium rolfsii	No	Watterson (1986), Jones *et al* (1991)
Spongospora subterranea	Yes	Fletcher (1984), Blancard (1994), Farr *et al* (1995)
Thielaviopsis basicola	Yes	Jones *et al* (1991), O'Neill *et al* (2000) and others
Verticillium albo-atrum	Yes	O'Neill (2005, 2006) and others
Verticillium dahliae	Yes	Fletcher (1984), Jones *et al* (1991), Blancard (1994) and others
Verticillium nigrescens	Yes	Isaac (1953)
Verticillium nubilum	Yes	Isaac (1953)
Verticillium tricorpus	Yes	Isaac (1953), Moore (1959), Jones *et al* (1991)

Table I.II: List of fungi and oomycota saprotrophs reported associated with tomato roots or growing medium

Eukaryote	Present in UK	Reference
Acremonium atricum	Yes	Price (1980)
Acremonium sp.	Yes	British Mycological Society database
Agaricus arvensis	Yes	British Mycological Society database
Alternaria humicola	Yes	Price (1980)
Alternaria sp.	Yes	Ebben & Williams (1956)
Aspergillus flavus	Yes	Domsch & Gams (1993)
Aspergillus sydowii	Yes	Domsch & Gams (1993)
Aspergillus ?terreus	Yes	Baker (1972)
Aspergillus ustus	Yes	Domsch & Gams (1993)
Aspergillus sp.	Yes	Price (1980)
Aureobasidium pullulans	Yes	Price (1980)
Blastomyces sp.	Yes	Ebben (1959)
Calyptella capula	Yes	British Mycological Society database
Cephalosporium acremonium	Yes	Price (1980)
Cephalosporium spp.	Yes	Ebben & Williams (1956)
Chaetomium cochliodes	Yes	Ebben & Williams (1956)
Chaetomium elatum	Yes	Domsch & Gams (1993)
Chaetomium olivaceum	Yes	Price (1980)
Chaetomium spp.	Yes	Ebben & Williams (1956)
Chromalosporium ochraceum	Yes	Price (1980)
Conidiobolus coronatus	Yes	Price (1980)
Coprinopsis gonophylla	Yes	British Mycological Society database
Cryptococcus albidus	Yes	Price (1980)
Cunninghamella echinulata	?	Domsch & Gams (1993)
Cylindrocarpon didymium	Yes	Price (1980)
Doratomyces microsporus	Yes	Domsch & Gams (1993), Price (1980)
Epicoccum purpurascens	Yes	Price (1980)
Fusarium oxysporum	Yes	Dababat & Sikora (2007)
Fusarium torulosum	?	Leslie & Summerell (2006)
Gelasinospora reticulata	Yes	British Mycological Society database
Gilmaniella humicola	Yes	Domsch & Gams (1993), British Mycological Society database, Price (1980)
Gliocladium roseum	Yes	Domsch & Gams (1972)
Idriella lunata	?	Domsch & Gams (1993)
Lepiota efibulis	Yes	British Mycological Society database

Lycoperdon sp.		Baker (1972)
Mortierella polycephala	?	Domsch & Gams (1993)
Mortierella zychae	Yes	British Mycological Society database
Mortierella sp.	Yes	Price (1980)
Mucor sp.	Yes	Price (1980)
Mycotypha microspora	Yes	Price (1980)
Myrothecium roridum	Yes	Domsch & Gams (1993), Ebben (1959)
Nectria gliocladioides	Yes	Price (1980)
Neurospora crassa	Yes	Price (1980)
Oedocephalum sp.	Yes	Price (1980)
Olpidium brassicae	Yes	Moore (1959)
Olpidium sp.	Yes	Baker (1972), Blancard (1994)
Paecilomyces lilacinus	Yes	Domsch & Gams (1993)
Penicillium brevicompactum	Yes	Price (1980)
Penicillium chrysogenum	Yes	Domsch & Gams (1993)
Penicillium griseofulvum	Yes	Domsch & Gams (1993)
Penicillium janthinellum	?	Domsch & Gams (1993)
Penicillium jensenii	Yes	Domsch & Gams (1993)
Penicillium lividum	Yes	Domsch & Gams (1993)
Penicillium nigricans	Yes	Price (1980)
Penicillium purpurogenum	Yes	Domsch & Gams (1993)
Penicillium stoloniferum	?	Domsch & Gams (1993)
Penicillium thomii	Yes	Domsch & Gams (1993)
Penicillium variabile	Yes	Domsch & Gams (1993)
Penicillium verrucosum	Yes	Domsch & Gams (1993)
Penicillium verrucosum var. corymbiferum	Yes	Domsch & Gams (1993)
Penicillium verrucosum var. cyclopium	Yes	Domsch & Gams (1993)
Penicillium verrucosum var. melanochlorum	Yes	Domsch & Gams (1993)
Petriella asymmetrica	Yes	Ebben & Williams (1956)
Peziza ostracoderma	Yes	British Mycological Society database
Pyronema amphalodes	Yes	Baker, 1972
Rhizopus nigricans	Yes	Price (1980)
Rhizopus oryzae	Yes	Domsch & Gams (1993), Price (1980)
Rhodotorula glutinis	Yes	Price (1980)
Sporobolomyces roseus	Yes	Price (1980)
Torulopsis famata	Yes	Price (1980)
Tricocladium adspersum	Yes	British Mycological Society database
Trichoderma koningii	Yes	Price (1980)
Trichoderma viride	Yes	Domsch & Gams (1993), Baker (1972)

Trichurus spiralis	?	Domsch & Gams (1993)
Volutella ciliata	Yes	Domsch & Gams (1993), Ebben (1959)

Table I.III: List of bacterial pathogens reported on tomato roots

Bacterium	Recorded on tomato in UK	Reference
Agrobacterium radiobacter	Yes	Weller & O'Neill (2006)
Agrobacterium rhizogenes	Yes	Weller *et al*., 2000
Agrobacterium tumefaciens	Yes	Blancard (1994)
Clavibacter michiganensis	Yes	Blancard (1994), Jones *et al* (1991), O'Neill *et al* (2000)
Pseudomonas syringae pv. *Tomato*	Yes	Watterson (1986), Blancard (1994), Jones *et al* (1991), Schneider & Grogan (1977)
Ralstonia solanacearum	Yes	Blancard (1994), Jones *et al* (1991), O'Neill *et al* (2000)

APPENDIX II

Table II.I: Calculations for plant and root health sickness scores at the end of cropping in 10 tomato crops – 2009

Assessment	RW		Soil		NFT		Coir		WF	
	1	2	3	4	5	6	7	8	9	10
Incidence (of 9)										
Number plants dead	0	0	4	4	0	0	4	1	0	1
Incidence of stem vascular browning	9	5	6	5	3	7	5	8	9	8
Severity (0-3)										
Major roots decayed/brown	1	0	2	3	0	0	2	0	2	0
Minor roots brown or black	1	0	3	3	2	2	2	0	1	1
Corky roots present	0	0	2	2	0	0	0	0	0	0
Plant and root health (based on the 9 monitored plants)										
Plant sickness score (2 x no. dead + no. with vascular brown) (0 – 27)	9	5	14	13	3	7	14	10	9	10
Root rot score (2 x major root decay + no. minor root decay+ no. corky) (0 – 12)	3	0	9	11	2	2	6	0	5	1

Table II.II: Calculations for plant and root health sickness scores at the end of cropping in 10 tomato crops – 2010

Assessment	RW		Soil		NFT		Coir		WF	
	1	2	3	4	5	6	7	8	9	10
Incidence (of 9)										
Number plants dead	0	1	0	0	0	1	1	0	0	0
Incidence of stem vascular browning	1	1	2	3	0	6	0	0	1	0
Severity (0-3)										
Major roots decayed/brown	2	2	2	1	0	0	1	1	1	1
Minor roots brown or black	1	1	2	1	2	2	0	0	0	0
Corky roots present	0	0	1	1	0	0	0	0	0	0
Plant and root health (based on the 9 monitored plants)										
Plant sickness score (2 x no. dead + no. with vascular brown) (0 – 27)	1	3	2	3	0	8	2	0	1	0
Root rot score (2 x major root decay + no. minor root decay+ no. corky) (0 – 12)	5	5	7	4	2	2	2	2	2	2

Table II.III: Summary of visual health of plants sampled for routine root monitoring – 2010

Growing medium and dataset code	Sample time	Number of plants (of 3) affected by:						
		Leaf yellow	Leaf wilt	Leaf necrosis	Stem disease	Leaf disease	Brown roots	Root rots/ spots
RW								
1	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	2	0
2	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	0	0
3	Early	1	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	2	0
4	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	0	0
Soil								
1	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	3	0	0	0	0	0
2	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	0	0
3	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	0	0
4	Early	0	0	0	0	0	0	0
	Mid	1	0	1	0	0	0	0
	Late	3	0	3	0	0	0	0
NFT								
1	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	3	0	0	0	0	3	0
2	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	3	0
3	Early	0	0	0	0	0	0	0
	Mid	0	0	3	0	0	3	0
	Late	Missing (data not supplied)						
4	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	2	0
Coir								
1	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	0	0
2	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	3	0	0	0	3	0
3	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	3	0	0	0	3	0
4	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	0	0
WF								
1	Early	0	0	0	0	0	0	0

	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	0	0
2	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	1	0
3	Early	0	1	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	0	0
4	Early	0	0	0	0	0	0	0
	Mid	0	0	0	0	0	0	0
	Late	0	0	0	0	0	0	0

1-2: crop samples in 2009, 3-4: crop samples in 2010