Monitoring Occurrence and Relative Levels of Rhizosphere Microorganisms on Rockwool Tomato Crops Across the 2012/2013 Growing Season

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Abstract

The tomato is the 8th most economically important agricultural product and the 4th most economically important crop in the world. The UK delivers the 3rd highest tomato yields globally. Such intensive production is due to the adoption of soilless growing techniques in greenhouses utilising assimilation lighting and heating along with the addition of CO_2 to extend the growing season and maximise yield. Soilless growing systems reduce the abundance of soil-borne pathogens on the plant roots but may pose increase risk from water-borne pathogens such as the oomycetes. Government pressure to increase consumer safety and reduce environmental impact has led to a reduction in the use of chemical pesticides and an increase in fertigation solution recycling. This creates a situation in which disease risk may be increased and the methods by which you can control the disease are reduced. In this study the microbial populations, associated with the rhizosphere, were monitored via a small scale microarray. Crops grown on three contrasting fertigation systems pSSF (part slow sand filtration), SSF (slow sand filtration) and a run-to-waste (RTW) system were monitored every two weeks throughout the 2012-2013 growing season and two crops grown at a nursery utilising two physical treatments, heat and UV, were monitored at four points during the season. Finally, rapid in-house real-time diagnostic assays utilising loop mediated isothermal amplification (LAMP) were developed.

A number of differences were observed from the pSSF, SSF and RTW systems. The RTW system exhibited lower taxa richness and species diversity with two significant differences in diversity observed between the pSSF-RTW and SSF-RTW systems. The RTW showed most taxa declining in abundance and persistence during the second half of the season. A general decline in taxa persistence was also observed in the second half of the season at the pSSF system but with reduced severity. The SSF system, however, showed an increase in taxa persistence during the second half of the growing season and also showed the highest persistence of oomycetes in the Pythium spp. Pythium spp. tended to persist later into the growing season, however, the pSSF and SSF showed Pythium spp. persistence 20 weeks earlier than the RTW. An increased number of fungal and oomycete pathogens were observed on the pSSF and SSF systems.

There were more similarities than differences observed between the two crops grown on systems utilising physical treatments and both observed similar taxa richness and diversity. The remaining observations were apparent for all the crops monitored by microarray. A high number of possible fungal and oomycete

pathogens were observed on all crops with taxa tending to be conserved between sites. Taxa observed at only a single site were generally saprophytic fungi. The Pythium spp. showed divergent patterns of persistence and abundance compared to all other taxa monitored in this study, with first occurrence mid-way through the season and general patterns of increasing abundance in the latter stages.

Finally, LAMP assays developed for Pyrenochaeta lycopersici and Verticillium spp. targeting the ribosomal ITS region were shown to not be suitable for use. Two further assays developed targeting CDiT1 and SIX1, SIX3 and SIX4 from Pyrenochaeta lycopersici and Fusarium oxysporum f.sp lycopersici, respectively, were developed but not validated due to time constraints.

Acknowledgements

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Contents

List of Figures

List of Tables

List of Abbreviations

AFLP - Amplified fragment length polymorphism

ANOVA - Analysis of variance

BLAST - Basic local alignment search tool

CANARY- Cellular analysis and notification of antigen risks and yields

CHP - Combined heat and power

DFT - Deep flow technique

DGGS - Denaturing gradient gel electrophoresis

ds-DNA - Double stranded DNA

EC – Electrical conductivity

ELISA - Enzyme-linked immunosorbent assays

FERA – The Food and Environment Research Agency

FO - *Fusarium oxysporum*

FOL - *Fusarium oxysporum* f.sp *lycopersici*

FORL - *Fusarium oxysporum* f.sp. *radicis-lycopersici*

FS - Fertigation solution

HRP - Horseradish peroxidase

IGS - Intergenic spacer

ITS - Internal transcribed spacer

LAMP - Loop mediated amplification

LS - Lineage specific

NFT - Nutrient film technique

NGS - Next generation sequencing

PBS - Phosphate-buffered saline

PCR - Polymerase chain reaction

pSSF – Part slow sand filter

Q-PCR - Quantitative polymerase chain reaction

QTL - Quantitative trait loci

RAPD - Random amplified polymorphic DNA

rDNA - Ribosomal DNA

RFLP - Restriction fragment length polymorphism

RT-LAMP – Reverse transcriptase loop mediated amplification

RT-PCR – Reverse transcriptase polymerase chain reaction

RTW - Run -to-waste

SDW - Sterile distilled water

SIX - Secreted in xylem

SSC - Saline sodium citrate

SSF - Slow sand Filter

TBE - Tris-Borate-EDTA

TMV - Tomato mosaic virus

T-RFLP - Terminal Restriction fragment length polymorphism

Chapter 1: Introduction

1.1 Tomatoes: Taxonomy and Historical Perspectives

The tomato, *Solanum lycopersicum,* is an herbaceous perennial plant from the family Solanaceae. The Solanaceae contains over 3,000 species (Wu & Tanksley, 2010), many of which are of high economic importance. Solanaceous produce includes tobacco, harvested from members of the *Nicotiana* genus; chili and bell peppers, from cultivars of *Capsicum annuum*. Some species also harbour therapeutic uses such as *Atropa belladonna* from which atropine is extracted (Ji *et al.,* 2009). Atropine has many uses due to its antagonistic effect on the parasympathetic nervous system and can be used to treat symptoms of Parkinson's disease (Djilani & Legseir, 2005) and intoxication of nerve agents (Shih & McDonough, 1999). The most economically important genus within the Solanaceae is that of which tomato belongs, *the Solanum. Solanum* is a varied genus of annual and perennial plants containing over 1,500 species including the potato, *S. tuberosum,* the tomato's closest relative and the aubergine *S. melongena* (Peralta & Spooner, 2006).

The classification of tomatoes was in dispute until the middle of the 16th century when Linnaeus grouped seven taxa of cultivated tomatoes, previously described as separate by Tournefort (1694), and named them *Solanum lycopersicum* (Peralta & Spooner, 2006). Two hundred years prior the tomato was misidentified as the lycopersicon or "wolf-peach" by Anguillara in 1561. This classification was based on a 1,400 year old morphological description by Galen, a Greek naturalist (Peralta & Spooner, 2006). In the advent of the genetic revolution work carried out by Spooner *et* al., 1993, using chloroplast-DNA restriction site data, could confirm the tomatoes' position within the Solanaceae. This, reinforced by Olmstead *et* al., 1997, placed tomatoes within the genus *Solanum,* supporting the work done by Linnaeus over 200 years beforehand.

1.1.1 Wild Varieties of Tomato

Wild species of tomato are native to the Andean region of South America (Sims, 1980). The Andes is comprised of a wide variety of habitats to which the tomato has successfully adapted. In fact the tomato has adapted so well to its native environments it can be observed in some of the more hostile regions observed along the Andean range. Species such as *S. galapagense* can be observed growing as little as 1m above high tide and enduring high levels of salinity juxtaposed to *S. corneliomuelleri* that grows 3200m above sea level on

rocky slopes and *S. pimpinellifolium* seen in arid environments (Peralta & Spooner, 2006). It is not only the tomatoes' ability to adapt to such differing environments that has seen their commercial success. Greatly varying morphological and physiological features have also added to their success, the most notable of which are the size and colour of their fruit. The fruit of the tomato plant can be seen in an array of different colours from the red with which we are so familiar; to yellow, purple, green and orange (Peralta & Spooner 2006). These traits observed in the wild varieties of tomato have allowed for the domestic success that can be observed in the tomato industry today.

1.1.2 Early Domestication and Cultivation

The exact origin of the domestic tomato is unknown (Peralta *et al.*, 2007). There are two conflicting theories, one stating the origin as Mexico and the other Peru (Jenkins, 1948; de Candolle, 1886). Both rely heavily on historical linguistics with some support from botanical data but both fall short when analysed utilising molecular techniques, more information can be found in Bauchet & Causse, 2012.

The most likely ancestor of the modern cultivated tomato is the wild cherry tomato or *S. lycopersicum* var. *cerasiforme* which grows readily throughout the tropic and sub-tropic regions of S. America (Peralta & Spooner., 2006). The initial cultivation of tomato was for ornamental use due to fear that the fruit was toxic (Costa & Heuvenlink, 2005), a trait observed from other members of the Solanaceae who are also known as the Nightshade family. After the introduction of the tomato into Europe in the 16th century the Europeans took the tomato to China in the 17th century, still with a prevailing use as an ornamental crop (Sabine, 1820; Siemonsma & Piluek, 1993). The consumption of the tomato became widespread during 18th century as its culinary use spread across Europe from south to the north (Harvey *et al.,* 2002).

1.2 Economic Importance of the Tomato

Solanaceous crops are the third most economically important agricultural family and the tomato was the most important vegetable crop in 2011, by scientific definition (Wu & Tanksley, 2010; FAOSTAT, 2013). Moreover, the unprocessed tomato was the 8th most commercially important commodity of 2011 behind rice, milk, beef, pork, chicken, wheat and soya bean and therefore the 4th most economically important crop providing a market worth $58.2 billion

US dollars globally (FAOSTAT, 2013). Tomato production is dominated by China and mainland-China both producing over 48 million tonnes in 2011, 48.6 and 48.5 million tonnes, respectively (FAOSTAT, 2013). The sum of the following three top countries production is less than both China and mainland-China with India, Turkey and the USA producing 16.8, 11 and 12.5 million tonnes each, respectively (FAOSTAT, 2013). Although China produce far higher quantities their growing techniques are less efficient than that of countries within Europe. The Netherlands, Belgium and the UK deliver the greatest yields with 4.8, 4.6 and 4.1 million Hg/Ha, respectively (FAOSTAT, 2013).

1.2.1 The UK Tomato Market

Concentrating further on UK tomato production we can see some interesting patterns when we look at the relationships between the area of land harvested and the total yield of tomatoes (Fig. 1.1). In Fig. 1.1 we can see that over ten years, between 1990 and 2000, the general trend in area harvested is an almost 50% decrease from just below 600 hectares to 300. Conversely, the yield has grown from 2.5 Mg/Ha to 4.0 Mg/Ha giving only a slight decrease in production across the decade and a doubling in efficiency of the growing systems. This change has been observed due to the introduction of new super-nurseries such as Cornerways, Norfolk the UKs largest single greenhouse tomato nursery. The establishment of these nurseries and their advantages will be discussed in section 1.3.

The yield has remained around 4.0 Mg/Ha between 2000 and 2012, with a significant drop between 2002 and 2003, Fig. 1.1. Again, in opposition to the plateau in yield, apart from a brief rise in 2002 and 2003 the area harvested has continued to decline from 300 Ha to 200 Ha between 2000 and 2012 Fig. 1.1. This drop may have been observed due to the introduction of new nurseries with highly efficient tomato production driving down prices, to a level which is unsustainable, and out-competing smaller sites with less efficient systems. These figures have led to a general decrease in total UK tomato production which can be seen in Fig. 1.2.

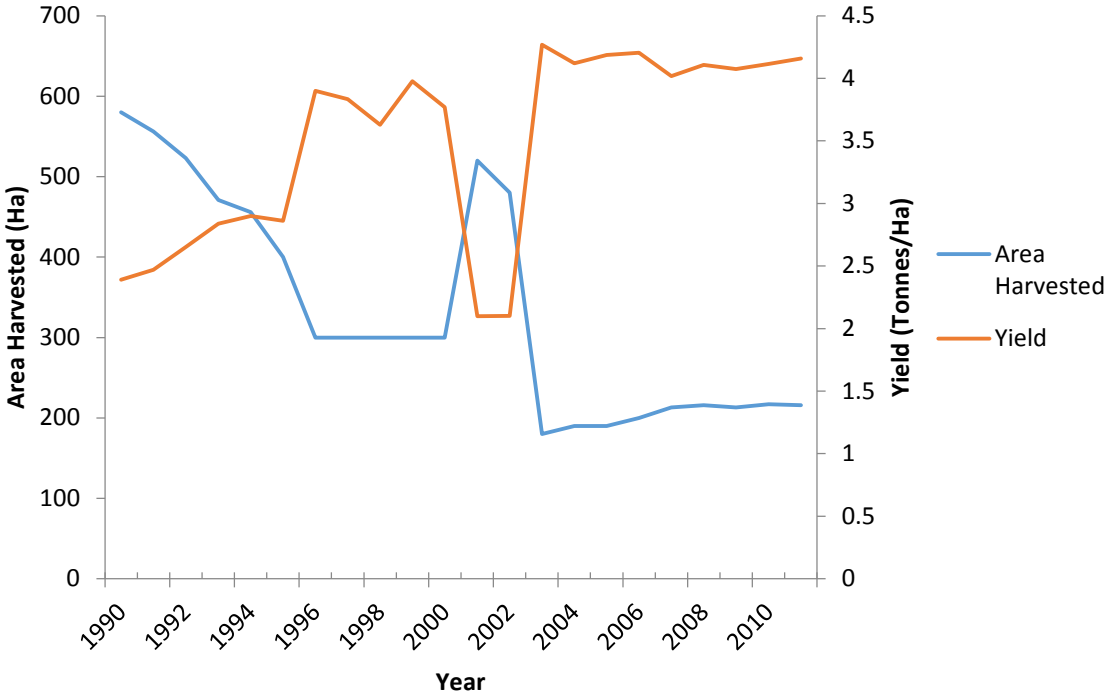


Fig. 1.1. The area harvested (Ha) and yield (Tonnes/Ha) of tomato plants within the UK between 1990 and 2012. All data was collected from FAOSTAT, 2013.

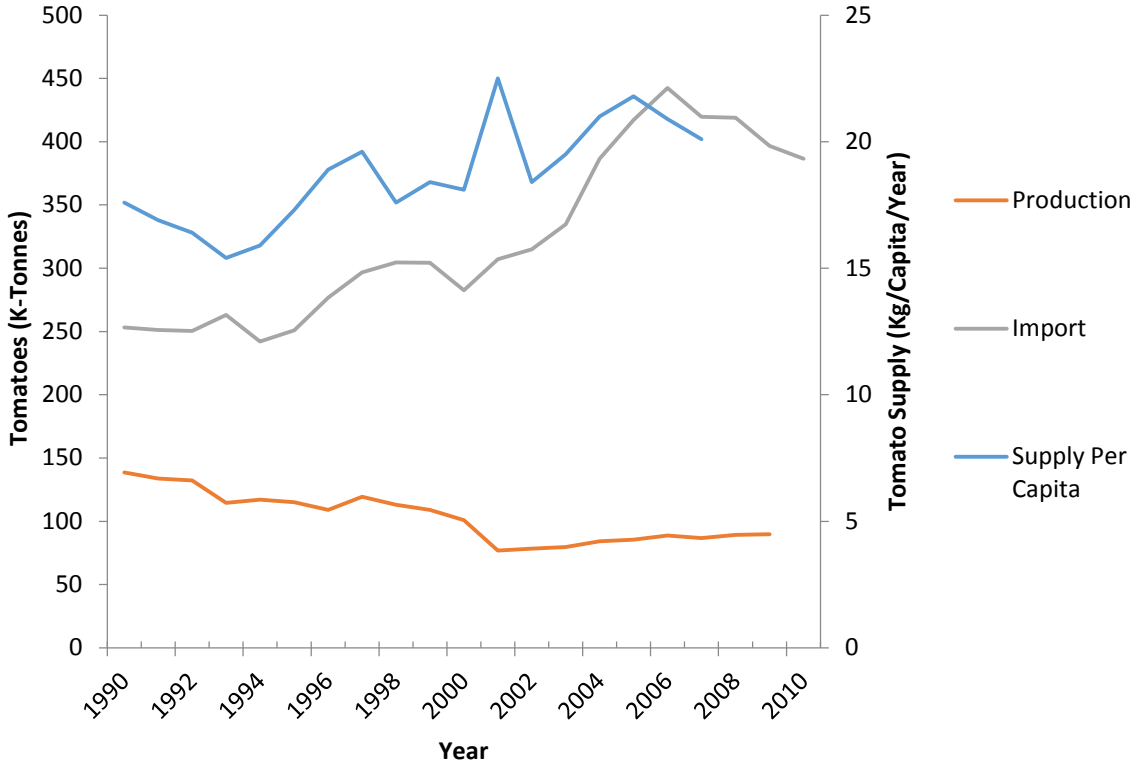


Fig. 1.2. Tonnes of tomatoes produced in and imported into the UK (K-Tonnes) along with the per capita supply of tomatoes (Kg/Capita/Year) for the UK between 1990 and 2010. All data was collected from FAOSTAT, 2013.

Although we can see a steady decline in the production of tomatoes within the UK over the last two decades, Fig. 1.2, the tomato supply per capita has risen from 17.6 Kg/Capita/Year in 1990 to 20.1 Kg/Capita/Year in 2007. This may seem only a small increase, but when applied to the population of 57.25 million in 1990 and 60.99 million in 2007 (The Office for National Statistics, 2013) an extra 218,000 tonnes of tomatoes were required to meet the needs of the British consumer. This has seen the import of tomatoes into the UK increase dramatically between 1990 and 2010, from 253,157 to 386,509 tonnes, respectively. There is a clear indication towards the growing demand for UK produced tomatoes, but a quick resolution to the problem is not so clear cut. To understand the why tomato production in the UK is in decline (see section 1.3.2) we must first understand how the modern tomato nursery is run.

1.3 The Modern Tomato Nursery in the UK

Growing tomatoes in the UK presents a number of challenges, the foremost of which is the sub-optimal climatic conditions needed for producing a profitable crop. The often harsh weather of the United Kingdom and shorter hours of daylight during the growing season would only allow an outdoor crop to be cultivated between July and October and is highly dependent on the fluctuating weather conditions (The British Tomato Growers Association, 2013). For these reasons commercial tomato growing in the UK is almost entirely limited to greenhouse production.

Production and start-up costs for greenhouse nurseries are far higher than that of a field crop. Even after the initial costs have been incurred the nurseries often have to invest in new glass for their greenhouses which is exceptionally expensive (O'Neill, 2013). To make greenhouse growing more sustainable, from a business perspective, high levels of investment have been applied to increase yields and the general efficacy of the growing process. Some major changes to the growing process include the use of soilless growing mediums, assimilation lighting and the introduction of CO_2 into the greenhouses (Raviv & Leith, 2008; O'Neill, 2013; The British Tomato Growers Association, 2013).

1.3.1 Soilless Cultivation

Soilless plant cultivation encompasses any growing technique utilising an alternate medium to soil. The soilless cultivation of plants was not commonly practiced until the 20th century due to poor understanding of the nutritional requirements of plants (Raviv & Leith, 2008). Work done by Cooper, 1975 led

the way in developing complete nutrient supplements, optimal water usage and oxygen levels.

One benefit of soilless cultivation is that the nutrients and fertilisers required for healthy plant growth are all available as soluble salts and can therefore be delivered along with the irrigation water in a process known as fertigation (Silber, 2008). From herein I shall use fertigation as the general term for the irrigation/fertigation processes. A number of soilless cultivation methods have been developed with differing methods of fertigation (Silber, 2008) see Fig 1.3.. See Table 1.1 for the advantages and disadvantages of respective soilless systems.

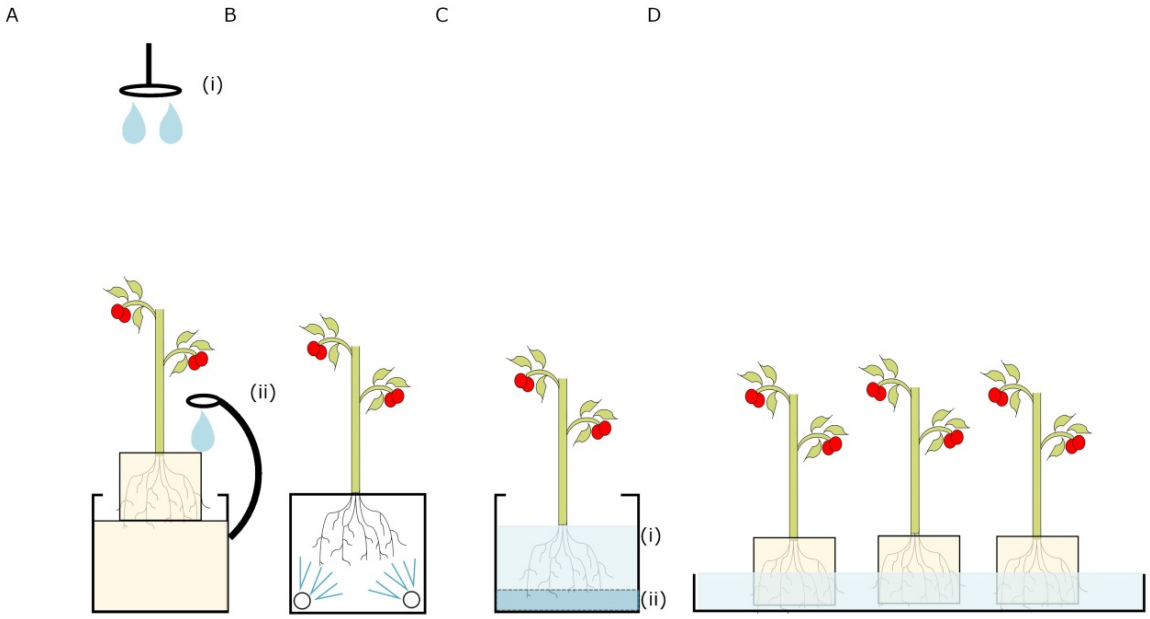


Fig. 1.3. Representational diagrams of soilless production techniques utilised in tomato greenhouses. A- i) An overhead sprinkler fertigation system that functions from above the canopy of the plant. A - ii) A drip fertigation system which applies nutrient solution directly to the soilless substrate at the crown of the plant. B) An aeroponics system which uses sprinklers within an enclosed system to give a continuous supply of nutrient solution directly to the roots. C - i) A Deep Flow Technique (DFT) system. The plant crown sits on a polystyrene float submerging the roots within 5-15cm of nutrient solution. C - ii) A Nutrient Film Technique (NFT) system. The crown and upper roots sit within a polystyrene block with only the lower roots being submerged within the "nutrient film" during watering. The DFT and NFT system troughs are set on gradients to allow the flow of water. D) An Ebb & Flood system. The plants sit in a potted substrate within a walled area. The area is completely flooded and left for 10-30 minutes to allow for proper water and nutrient uptake. The area is subsequently completely drained. (Silber, 2008)

Table 1.1- The substrates used for differing fertigation techniques and their comparative advantages and disadvantages.

Fertigation System	Substrate	Main Advantages	Main Disadvantages
Overhead sprinkler	Yes	Decreases disease risk	High cost
		Tight control of nutrient supply	Rockwool/synthetic substrate disposal is costly
		Substrate acts as a buffer to reduce fluctuations in climatic conditions	
Drip	Yes	Decreases disease risk	High cost
		Tight control of nutrient supply	Rockwool/synthetic substrate disposal is costly
		Substrate acts as a buffer to reduce fluctuations in climatic conditions	
Aeroponics	No		Poor control of Root °C
			High disease risk.
			Very high cost.
NFT	No	Moderate cost	Increased disease transmission
			Poor control of root °C
			Poor control of nutrient supply
			Downstream nutrient depletion
DFT	No	Reduced root °C fluctuations	Increased disease transmission
		Low cost	
Ebb & Flood	Yes		Increased disease transmission.
			Requires complete drainage of water
			High cost
			Substrate disposal

Another major advantage of soilless cultivation is the reduction in soil-borne pathogens that can occur within intensive soil grown crops in greenhouse environments (Raviv & Leith, 2008). Soil-borne pathogens are an increased problem due to greenhouse use generally being specific to a single crop and therefore not being open to methods such as crop rotation to allow reduction of pathogen propagules between growing seasons (Postma *et al.*, 2008).

Due to numerous advantages such as the decreased disease risk and tight control of nutrient supply, see Table 1.1, overhead and drip irrigation are the most commonly used methods for commercial production of plants (Silber, 2008). With little exception, drip irrigation is being used for nearly all fruiting vegetable production throughout the US and Europe (Silber, 2008). The precise control of fertigation is essential for tomato production with salinity and pH being closely monitored. Obtuse pH and salinity can cause physiological disorders that affect yield and quality of the crop (Peet, 2005) including blossom end rot and oedema leading to discolouration of the fruit and blistering of leaves, respectively (de Kriej *et al.,* 1992; Grimbly, 1986). Although drip irrigation clearly has advantages over its competitors it does have large overhead costs. One of the largest expenses is the disposal of the common substrate rockwool (Raviv & Leith, 2008; Sonneveld, 1988). Rockwool is an inorganic synthetic fibre favoured for its high water capacity and capillarity allowing roots to grow freely (Costa & Heuvenlink, 2005). The large economic and environmental implications of rockwool disposal has seen a drive towards the use of substrates such as coir, produced from coconut husks and easily disposed thus reducing economic and environmental impact (Raviv & Leith, 2008).

1.3.1.1 Open and Closed Fertigation Systems

Greenhouse fertigation systems have also been scrutinised for their monetary and environmental impact. There are two types of systems used, open or closed, that either run-to-waste (RTW) or recycle their fertigation solution (FS), respectively. Most open systems simply allow the waste water to drain into the ground beneath the substrate slabs (Silber, 2008). This is costly both to the environment and nursery, wasting fertiliser and polluting the local environment. Fertiliser constituents contain a high level of nitrates and excess nitrates leaked into the local environment can lead to eutrophication if the FS runs into freshwater ecosystems. Recycling of the FS in closed systems provides a more sustainable method of fertigation. Although more sustainable the closed system

may increase the risk of potential pathogen transmission. Stanghellini *et al.,* 1994, showed that oomycetes and other zoospore forming organisms could flourish in soilless culture due to their adaptation to life in liquids. This could prove devastating to tomato growers as a concentration of only 1 zoospore ml^{-1} of *Pythium aphanidermatum* can result in infection of cucumber (Postuma *et al.,* 2001). In an attempt to control the increased pathogenesis of closed systems the FS is treated before its re-use to remove detritus and prevent the spread of any potential pathogens.

The use of a slow sand filter (SSF) has the potential to completely eliminate the genera *Phytophthora* and *Pythium* and remove *Fusarium* spp., viruses and nematodes at a 90-99% success rate (Wohanka *et al.,* 1999). Heat and UV treatment can be used to target all organisms in the FS but also have the ability to target specific groups of organisms. Using heat to target specific organisms is achieved by adjusting the temperature, for example all nematodes can be removed at 55°C and all fungi at 70°C at an exposure time of 10 seconds (Runia, 1998). UV can also be adjusted to target specific organisms by adjusting the wavelengths used as certain organisms are more susceptible to different wavelengths. UVC with an optimum wavelength of 254nm, however, is the most efficacious single treatment (Runia, 1996). The antimicrobial actions of the FS sterilisation techniques appear to be highly successful in the laboratory. When used in-house, however, treatments results in only partial sterilisation of the FS (Peet & Welles, 2005). UV and heat treatments, like assimilation lighting and heating, consume large amounts of electricity and therefore profit. This has led tomato nurseries and other greenhouse based growers to work alongside energy providers to create a combined heat and power (CHP) system.

Table 1.2 The treatments and modes of action used when recycling fertigation solutions. Information was taken from Wohanka *et al.,* 1999; Runia, 1998 and Runia, 1996 for the slow sand filter, heat and UV treatments, respectively.

Treatment	Mode of action
Slow Sand Filter	Mechanical filtering of detritus
	Biofilm mediated removal of pathogens
Heat	Eliminates all bacteria, fungi, nematodes and viruses via a 10 second exposure of 80-95°
	Temperature causes lysis of cell membrane and other proteins
UV	Utilises a wavelength between 200-400nm to prevents DNA replication and elongation

1.3.2 Combined Heat and Power (CHP)

Requirement for UK growers to provide additional heat and light, to increase productivity, has made tomato production increasingly energy dependant. Greenhouse production consumes the most energy within the agricultural sector (Compernolle *et al.*, 2011). High energy requirements have led nurseries to adapting more manageable and sustainable methods of acquiring their heat and lighting. The most common method of achieving this is the CHP system, or combined heat and power.

Energy from CHP is a product of burning of gas or other combustibles such as household waste (Compernolle *et al.*, 2011; O'Neill, 2013). The CO_2 produced from combustion is filtered, removing impurities, by a catalytic converter and then passed on into the greenhouses. CO_2 levels of 750-800µmol^{-1} have seen yield increases of 30% (Peet & Welles, 2005). Heat is collected and stored by a water cooling system and then piped into the greenhouses during times off sub-optimum temperature. The bulk of energy used by growers, produced by CHP, is used to power assimilation. The combination of CO_2 introduction and assimilation lighting has led to 22-55% yield increases, dependant on growing region (Compernolle *et al.*, 2011; Marcelis *et al.*, 2002). CHP is an extremely efficient process and has led to a 30% decrease in CO_2 emissions for an equivalent nursery without a CHP (Stern, 2006; Compernolle *et al.*, 2011).

The heavy investment into improving productivity in UK explains why the UK has delivered some of the highest yields in the world for 2011-2012, see section 1.2.1. Granted this progress has pushed it to the forefront of tomato production but it has also had a deleterious effect on the market with a continuing decrease in the volume of tomato production seen from the UK as a whole, see Fig. 1.2 section 1.2.1. Smaller nurseries are being out-competed due to the reduced cost prices at large scale nurseries and high start-up and running costs are preventing the introduction of new growers into the market. The combination of these effects and an increasing consumer requirement for fresh tomatoes and tomato based produce is forcing the UK to be more dependent on international trade and the import of tomatoes to meet demand.

1.3.3 Modern Cultivars

Although optimum growing conditions for the tomato plant are crucially important, equally important is the selection of a suitable cultivar. The modern cultivar has been evolving since the early 20th century with the release of "Tennessee red" a fusarium wilt resistant plant in 1912 (Bauchet & Causse, 2012). Modern tomato breeding has had to tackle three main factors; biotic,

such as pathogen resistance; abiotic, temperature, water supply and salinity; and quality, colour, taste, ripening and shelf-life among others (Lindhout, 2005). Most favourable traits such as pathogen resistance have been introduced from wild relatives; others have spontaneously appeared in cultivated species or have been introduced via mutagenesis treatment (Causse *et al.,* 2007).

Successfully introducing novel genes into an elite cultivar, via selective breeding, is a laborious process. The Sw-5 gene, a monogenic tomato spotted wilt virus resistance gene, took two decades to introgress successfully (Stevens *et al.*, 1992). Moreover, many traits are controlled by quantitative trait loci (QTL) (Stevens *et al.*, 2007). QTL are regions of the genome that produce a polygenic phenotype. With the advent of the genetic revolution in the 1980s and the introduction of techniques such as RFLP (restriction fragment length polymorphism), QTL analysis, the production of high-density gene maps and the statistical computation of tailored breeding programmes has increased the success introgression and the speed at which it is achieved (Helentjaris *et al., 1985*; Tanksley & Nelson, 1996; Lander & Botstein, 1989; Stevens *et al.*, 2007).

The selective breeding of tomatoes is centred on two main techniques, often used in combination, backcrossing and pedigree selection. Backcrossing is used for introgression of monogenic traits and pedigree selection for QTL and polygenic traits (Causse *et al.,* 2007).

Progress in gene identification has led to a plethora of cultivars exhibiting numerous novel traits to fulfil both growers' and consumers' needs. A large percentage of all genes relating to fruit quality come from a single species *S. esculatum* including the *Rin* and Og^c, a ripening inhibitor and lycopene up-regulator, respectively (Vrebalov *et al.*, 2002; Ronen *et al.*, 2000). A comprehensive list of traits and their related genes can be seen in (Causse *et al.,* 2007). The introgression of disease resistance has also been successful but many cultivars rely on monogenic resistance that may not provide the durable resistance that breeders strive for.

The selection for broad-range resistance, however, has been successful as many resistance genes act in a dominant fashion. Over 12 resistance genes, derived from wild species, such as *Tm-1* and *I-2* giving resistance against tomato mosaic virus (TMV) and *Fusarium oxysporum* f.sp. *lycopersici* (FOL), respectively (Levesque *et al.*, 1990; Sarfatti *et al.*, 1989). An extensive list of resistance genes can be found in (Robertson & Labate, 2007) and further information on resistance genes can be found in section X.

Soilless tomato cultivation has also seen the introduction of grafted plants. The initial drive was to increase overall plant vigour and yield (Heijens, 2004; Peet &

Welles, 2005). Growing grafted plants have, however, also allowed the grower to diversify their scion and produce a greater range of fresh tomatoes without the need to alter root environment. New rootstocks such as Maxifort™ (De Ruiter Seeds) increase the plants tolerance to temperature, drought and salinity (Reivero *et al.*, 2003; Bhatt *et al.*, 2002; Fernandez-Garcia *et al.,* 2002). Moreover, the rootstocks provide resistance to a number of pathogens; TMV, *Fuvlia fulva,* FOL, *Fusarium oxysporum* f.sp *radicis-lycopersici* (FORL), *Oidium neolycopersici, Verticillium albo-atrum, Verticillium dahlia, Meloidogyne arenaria, Meloidogyne incognita and Meloidogyne javonica* (De Ruiter Seeds, 2012). Providing a hardy rootstock is essential to the success of greenhouse tomato production as the roots can be exposed to large fluctuations in temperature, pH, EC (electrical conductivity) and microbial population and the new vigorous and hardy rootstocks are providing some resolution to both biotic and abiotic stresses placed upon tomatoes grown in both soil and soilless systems.

1.4 The Roots and the Rhizosphere

Roots are critically important for almost all species of plant. They function as the site of water uptake, nutrient exchange and provide anchorage. Moreover, the roots are the first organ to emerge from a germinating seed and continuous root elongation is essential for a plants health and growth (Kafkafi, 2008). Upon germination, elongation of the roots occurs growing and passing through soil or substrate in response gravitropic, thigmotropic and chemotropic signals. The resulting roots create an environment where a plethora of root associated microorganisms can flourish, the rhizosphere. The rhizosphere can be defined as the interface between living roots and the soil or substrate and acts as a central commodities exchange (Lynch, 1990). The microorganisms within the rhizosphere community, when viewed from a distant perspective, are greatly influenced by plant species, rhizosphere constituents and even plant cultivar (Stephen *et al.,* 2000; Garbeva *et* al., 2004). Cultivar has shown to account for 38% of the variability observed in the abundance of plant growth promoting bacteria *Bacillus cereus* (Smith *et al.,* 1999). Research has helped to increase our understanding of the rhizosphere and has revealed a highly complex multifaceted environment.

1.4.1 The Microenvironments of the Rhizosphere

The rhizosphere is a highly heterogeneous, spatially and temporally fluctuating environment with many complex biological, chemical and physical interactions occurring between the roots, rhizosphere and its constituents (Crawford *et al.*, 2005; Cardon & Gage, 2006; Hawkes *et al.*, 2007; Bais *et al.*, 2006). Much of the spatial fluctuation within the rhizosphere is defined by the plant root and its differing developmental stages, which alters the rhizodeposition of nutrients, therefore favouring different organisms at differing regions (Cardon & Gage, 2006). Plant roots are not the only determining factor in the formation of the rhizosphere, however, conditions within the soil/substrate including the pH, EC and substrate compaction also have direct consequences on the development of plant roots and thus the rhizosphere.

At the actively growing root tip, for example, exudation is increased due to the underdeveloped vascular tissue exhibiting less control over the movement of sap (Cardon & Gage, 2006; Bretharte & Silk, 1994). In addition, to the increased exudation of sap, rhizodeposition at the root tip is also increased by the production of mucilage and sloughing of root cap cells (Whipps, 1990). This increased exudation, however, does not encompass all organic compounds and ions (Cardon & Gage, 2006). Jaeger *et al.*, 1999 and Casavant *et al.,* 2002 showed that sucrose exudation increases at the root tip, decreasing towards the crown, and tryptophan exudation decreases towards the active tip. This differential compound exudation creates microenvironments or niches in which organisms with differing metabolic capabilities can survive.

Much of the microbiota in the rhizosphere acts in a synergistic relationship with the plant (Hawkes *et al.*, 2007). Nutrients and carbon released from the plant roots is catabolised by decomposers and in turn released back into the soil (Cardon & Gage, 2006). Nutrient exchange that occurs in the rhizosphere is driven by the movement of water towards and away from the roots by transpiration and hydraulic redistribution, respectively, which occurs in a diurnal cycle and is influenced by the relative humidity (Cadwell & Richards, 1989). The diurnal cycle along with the differential nutrient uptake and exudation at specific root zones creates regions of high and low nutrient availability which also vary in distance from the root surface, dependant on carbon source (Yang & Crowley, 2000; Marilley & Aragno, 1999). Organisms that have a saprophytic lifestyle or phase are the most heavily affected by changes in the rhizosphere environment (Kerry, 2000).

Such a dramatically heterogeneous environment seems contrary to supporting a diverse microbial population but there are 60 and 12 fold more bacteria and

fungi in the rhizosphere than the surrounding environment, respectively (Griffiths, 1989). With 10-30% of carbon produced by plants being released into the rhizosphere along with inorganic ions, free O_2 and H_2O living within the rhizosphere provides an, although temporally and spatially fluctuating, abundant supply of nutrients (Lynch & Whipps, 1990; Bertin *et al*, 2003; Uren, 2000). Such fluctuations in nutrients may have driven the evolution of organisms with complex collections of metabolic pathways such as *Pseudomonas putida* a ubiquitous soil-borne bacteria (dos Santos *et al.*, 2004). *P. putida* shows high metabolic versatility utilising numerous carbon sources and oxygen independent metabolic processes (dos Santos *et al.*, 2004). Possession of such metabolic capabilities would greatly increase fitness within the microenvironments of the rhizosphere.

1.5 Microbial Communities of the Rhizosphere

The rhizosphere and its exudates can extend from 10µm to several mm from the root surface dependant on the compound and microorganism being studied (Bowen & Rouira, 1999). Within the rhizosphere a diverse assortment or microbial life can be found including fungi, bacteria, oomycetes, nematodes and archaea the latter of which will not be discussed further and is not monitored in this study. Bacteria can occupy up to 10% of the root surface with the highest densities observed at the active tip elongation zone (Kerry, 2000; Marilley & Aragno, 1999). Fungi are predominantly found in their vegetative state within the rhizosphere with hyphal densities of 12-14mm m-2 whereas in the surrounding soil 70-90% of fungi exist as spores (Kerry, 2000; Agnihothrudu, 1955). Oomycetes identified from the tomato rhizosphere, of soil crops, were mainly grouped within the *Pythium* spp. with only few *Phytophthora* spp. present (Arcate *et al.*, 2006). Little information on the location and burden of oomycetes within the rhizosphere is present in the current literature.

The microbial communities within the rhizosphere are complex and each organism is believed to have co-evolved alongside the plant roots and each other (Hawkes *et al.*, 2007) resulting in a complex community structure. Organisms have a number of effects on each other and the plant which can be competitive, additive or synergistic. Plant parasitic nematodes, which comprise <40% of nematodes in the rhizosphere, such as *Meloidogyne incognita* can increase rhizodeposition and affect the activity of other pathogens and saprophytes (Khan, 1993; Dorhout *et al.,* 1993; Dorhout *et al.,* 1988). Moreover, tomato roots infected with *M. incognita* release more water soluble carbon and metal ions altering the trophic state of *Rhizoctonia solani* into its pathogenic phase

(van Gundy *et al.*, 1977).

The roots producing a single signal can elicit varying responses from different organisms or equal responses from differing organisms resulting in a range of effects (Bais *et al.*, 2006). These responses can result in numerous root-microbe interactions including symbiotic associations and plant growth promotion but also pathogenesis and competition from plants, suggesting exudates and secretions play a large role in the outcome of rhizosphere community (Bais *et al.*, 2006).

Communication in the rhizosphere undoubtedly occurs at many levels with bacteria-bacteria quorum sensing during the formation of structures such as biofilms and electrotaxis or chemotaxis of pathogenic zoospores towards injured roots, to name a few. Such interaction gives insight into the complexity of the community structure and inter-microbe and microbe-plant interactions examples of which can be seen in Fig. 1.4

Fig. 1.4 shows us that organisms in the rhizosphere can affect each other and the plant both directly and indirectly. For example *Bacillus subtilis* has an indirect beneficial effect on the host plant by preventing tomato pathogens such as *Ralstonia solanacearum* and *Rhizoctonia solani* from colonising the rhizosphere (Asaka & Shoda, 1996; Chen *et al.,* 2013). Moreover, *B. subtilis* also directly promotes plant growth (Lugtenberg & Kamilova, 2009).The effect of the plant on the rhizosphere community was intentionally left from Fig. 1.4 as it has been previously discussed in sections 1.4 and 1.4.1.

The plant plays a huge role in determining the microbial community of the rhizosphere, but this community in turn can directly and indirectly affect plant physiology and also that of the other inhabitants of the rhizosphere in an series of “feedback loops”. Little work has been done on how rhizosphere communities interact with each other and such interactions may play an important role in the development of disease, formation of the microbial community and plant health in general. As can be seen in Fig. 1.4, which represents only a small proportion of the better studied microorganism, a plethora of microorganisms are associated with the rhizosphere. Moreover, Amann *et al*., 1995 predicted that up to 99 % of the organisms present in certain environments may not be identified by classical culture techniques, a problem which may be alleviated in future years by arising sequencing techniques such as *de novo* sequencing. For the purpose of this study, organisms known to be commonly associated with tomato roots will be focused upon.

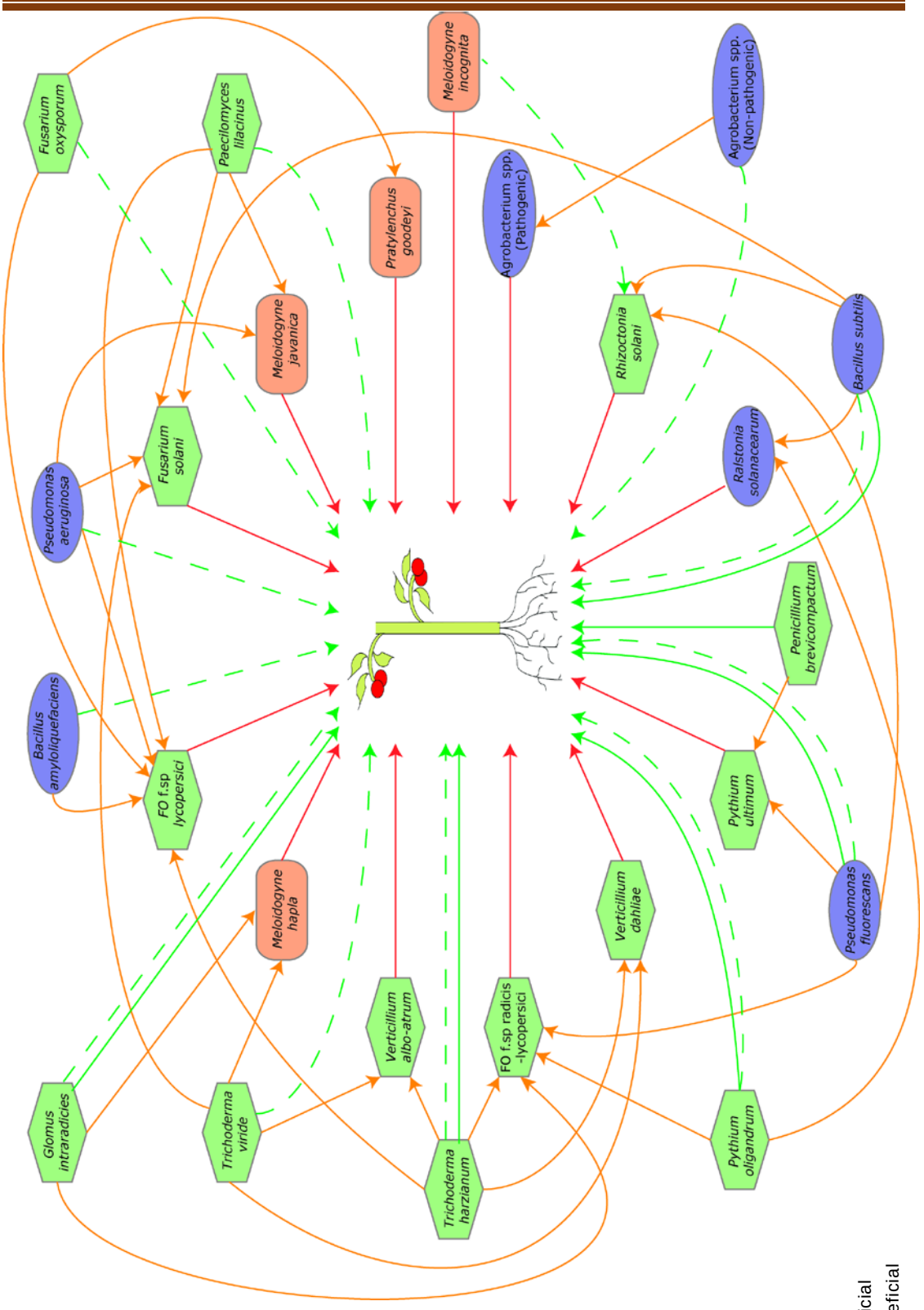


Fig. 1.4. The complex microorganisms-microorganism and microorganism-plant interactions in the tomato rhizosphere including the effect and direction of the interaction. (Alwathnani *et al.*, 2012; Amaresan *et al.*, 2012; Asaka *et al.*, 1996; Bolwerk *et al.*, 2003; Chen *et al.*, 2013; Datnoff *et al.*, 1995; le Floch *et al.*, 2005; Gravel *et al.*, 2006; Hammer *et al.*, 1997; Jabonoun-Khiareddine *et al.*, 2009; Kawaguchi *et al.*, 2008; Masadeh *et al.*, 2004; Morsy *et al.*, 2009; Mwaura *et al.*, 2010; Nzana *et al.*, 2011; Siddiqui *et al.*, 2000; Takenaka *et al.*, 2007; Vitullo *et al.*, 2012; Whipps, 2001)

1.5.1 Pathogenic Fungi of Importance

Little work has been done on the biological thresholds required for pathogens to cause disease or the effect of combinations of pathogens present on plant roots and whether they have additive, synergistic or antagonistic effects with each other (Postma *et al.*, 2008). Although soilless systems do reduce the potential risk of disease from soil-borne pathogens the nature of their fertigation systems leaves them at high risk of rapid disease dissemination, if an outbreak occurs, due to the nature of their combined fertigation systems. Moreover, growers only have the ability to effectively control a few diseases after an outbreak has occurred. Disease control is discussed further in section 1.6.

Deery, 2012 reviewed literature and identified 65 fungi and oomycetes commonly reported to cause disease on tomato. A complete list can be seen in appendix 1. This was followed by a T-RFLP (terminal restriction fragment length polymorphism) study to find the putative organisms present on tomato roots on differing growth substrates. The results gave the following pathogens to be most likely occurring on tomato roots grown on rockwool substrate, Table 1.3.

Table 1.3. Fungal pathogens commonly reported on tomato roots, their respective diseases and prevalence in soilless systems within Europe. Information adapted from; Deery, 2012, Blancard, 2012 and O'Neill, 2013b.

Organism	Disease	Prevalence in soilless systems within Europe
Alternaria solani	Early blight	-
Armillaria mellea	Armillaria root rot	-
Colletotrichum acutatum	Black dot root rot	+
Colletotrichum coccodes	Black dot root rot	++
Didymella lycopersici	Canker/ stem rot	+
Fusarium oxysporum f.sp *lycopersisi*	Fusarium wilt	-
Fusarium oxysporum f.sp *radicis-lycopersici*	Fusarium crown and root rot	++
Fusarium redolens	Fusarium wilt and root rot	-/+
Fusarium solani	Damping off/ foot and crown rot	-/+
Plectosparella cucumerina	Root and collar rot	++
Pyrenochaeta lycopersici	Corky root rot	+
Rhizoctonia solani	Damping-off	+
Thielaviopsis basicola	Black root rot	-/+
Verticillium albo-atrum	Verticillium wilt	-/+
Verticillium dahliae	Verticillium wilt	-/+
Verticillium nigrescens	Verticillium wilt	-/+

Many rhizosphere related fungal pathogens are polyphagous also causing disease on fruit such as the causal agents of anthracnose, *C. coccodes and C. acutatum*, observed as small brown depressed lesions, affecting ripe fruit pre and post-harvest (Blancard, 2012). Moreover, many pathogenic fungi have closely related saprophytic species that are difficult to discriminate using classical methods. Deery, 2012 also highlighted 75 saprophytic fungi and oomycetes, recorded to be associated with the tomato rhizosphere, see appendix 1. A number of saprophytes have been shown to act antagonistically against pathogens, see Table 1.6, directly promote plant growth, see Fig. 1.4 and also exhibit mycoparasitism such as *Pythium oligandrum* (Rey *et al.,* 2005).

Even by modern molecular techniques the specific classification of many rhizosphere related pathogenic fungi can be difficult, with only pathogenicity related regions of the genome differentiating an organism between its saprophytic and pathogenic isolates. Perhaps the most studied genera of rhizosphere associated fungal pathogens are the *Fusarium*. I shall now discuss the *Fusarium* spp. to highlight some of the molecular aspects of phytopathology and the problems faced by the modern molecular biologist when attempting diagnosis.

1.5.1.1 The *Fusarium spp.*

The genus *Fusarium* was first described over 200 years ago and has world-wide ubiquity as pathogens, endophytes and saprophytes (Summerell & Leslie, 2011; Summerell *et al*, 2010). *Fusarium* spp. are well known plant pathogens affecting bananas, wheat and tomatoes to name a few and can cause severe loss of yield (Summerell & Leslie, 2011; Dean *et al.,* 2012). Although common as pathogens it is believed that the number of species not associated with disease far outnumbers that of the pathogenic (Summerell & Leslie*.,* 2011). The *Fusarium* spp. cause diseases that include vascular wilts; stem, root and crown rots; canker, head and seed blight, affecting both juvenile and mature plants alike with species also capable of producing overlapping symptoms on some hosts (Blancard, 2012; Summerell & Leslie, 2011). Moreover, ten species of *Fusarium* cause rot of tomato fruit upon ripening and post-harvest producing mycotoxins damaging to human health (Blancard, 2012; Glenn *et al.,* 2008). One of the most damaging *Fusarium* spp. on tomato is *Fusarium solani*, capable of causing pathogenesis on most parts of the plant at differing stages of the plant's development, causing damping-off, root rot and fruit rot (Blancard, 2012).

The most common rhizosphere related species of the *Fusarium* is *Fusarium oxysporum* (*FO*) a ubiquitous soil-borne saprophyte and pathogen affecting a plethora of hosts with high specificity (Armstrong & Armstrong, 1981). The *FO* species is further divided into forma specialis with f.sp. *lycopersici* (*FOL*) and *radicis-lycopersici* (*FORL*), both pathogenic on tomato, causing Fusarium wilt and Fusarium crown and root rot, respectively. Greenhouse crops grown on soilless systems have a higher occurrence of *FORL* within Europe but the introduction of resistant cultivars has helped reduce incidence (Blancard, 2012). In 2003 over 120 different forma specialis were recognised with few plant taxa not playing host to the *FO* pathogens (Roncero *et al.*, 2003; Armstrong & Armstrong, 1981). The genus *Fusarium* provides a difficult task when attempting identification with morphological characteristics and single molecular markers not providing definitive identification (Summerell *et al.,* 2003). To add confusion, phylogenetic studies by O'Donnell *et al.*, 1998 indicated differing isolates of the same f.sp. had originated independently in evolution. It was hypothesised by Baayen *et al.*, 2000 that *FO* is a species complex as opposed to a single species.

The *FO* genome can differ vastly in size from 18.1 – 51.5 Mb and hold between 7 and 14 chromosomes (Migheli *et al.*, 1993; Migheli *et al.*, 2000). Moreover, 5% of the genome is constituted of different families of transposable elements (Daboussi *et al.*, 1992) creating a genetic environment more susceptible to change. Such genetic diversity within one species may seem outlandish but mechanics controlling *FO's* pathogenicity begins to explain why such diversity may occur. To understand the genetic diversity observed for some constituents of the *Fusarium* spp., I will use *FOL* as a model.

1.5.1.2 *Fusarium oxysporum* f.sp *lycopersici*

FOL causes fusarium wilt on tomatoes, best identified via intense discolouration of the vascular system (Blancard, 2012). *FOL* is present on every continent on earth, surviving for up to ten years in soil, disseminated through detritus and infected seed and requires natural openings or wounds for entry to roots (Blancard, 2012). Although resistance is present in most commercially used cultivars within Europe, a break in resistance could be devastating due to the ubiquitous nature of the species.

FOL can be further divided into three races defined by their ability to infect certain cultivars. Cultivar resistance is monogenic and based on the genes *I, I-2* and *I-3* that give resistance to *FOL* carrying *SIX4* (Avr1), *SIX3* (Avr2) and *SIX1* (Avr3), respectively (Armstrong & Armstrong, 1981; Lievens *et al.*, 2009; Inami *et al.*, 2012; Houterman *et al.*, 2008). The *SIX*, secreted in xylem, genes are a

group of small *in planta* secreted proteins many of which have key roles in pathogenicity (Rep *et al.*, 2005). Race 1 of FOL carries both *SIX3 and SIX4* but lost *SIX4* with the evolution of *I* a monogenic resistance gene (Houterman *et al.*, 2008). Race 2 is *SIX4*$^{-}$ and with the evolution of the *I-2* resistance gene, which gave resistance to *SIX3* carriers, race 2 isolates underwent a single nucleotide substitution, at three differing positions in separate isolates, in *SIX3* to overcome resistance and give rise to race 3 (Houterman *et al.*, 2008). All races carry *SIX1,* the target for *I-3* mediated resistance, currently utilised in most commercially available cultivars (Inami *et al.*, 2012). The loss of *I-2* mediated resistance from a single nucleotide polymorphism shows the fragility of resistances present in current cultivars and the realism of possible future breaks in resistance and their devastating effects.

Research by Ma *et al.*, 2010, revealed lineage-specific (LS) regions in the FOL genome that cover four whole chromosomes, 19Mb, encoding predominantly secreted effectors, virulence and transcription factors and proteins involved in signal transduction. Chromosome 14, in particular contained *SIX1, 3, 5, 6* and 7 two of which *1* and *3* are pathogenicity factors and are said to determine pathogenicity on tomato. Furthermore, chromosome 14, along with other LS regions can be horizontally transferred to non-pathogenic *FO* leading to a shift in lifestyle and the ability to cause disease on tomato. *FO, FS* and *F. verticillioides* have been shown to treat these pathogenicity related chromosomes as dispensable and may lose them under certain conditions (Summerell & Leslie, 2011). *FO*, however are not the only fungal pathogens to exhibit such genetic plasticity the *Verticillium* spp. also exhibit similar characteristics.

The *Verticillium* spp. cause billions of dollars in crop losses around the world each year and produce an array of symptoms on over 200 dicots and until recently was only comprised of six species of plant pathogens and saprophytes (Klosterman *et al.,* 2009; Barbara & Clews, 2004). The *Verticillium* spp., like the genus *Fusarium*, are believed to be a complex of fungal species. Research revealed at the 11th International Verticillium Symposium 2013, focusing on molecular phylogenetic groupings of the ITS region and four other genes, has proposed two new clades within the genus *Verticillium* with eight new species (Inderbitzin, 2013).Until recently there has been very little evidence towards host specificity within the *Verticillium* spp. and the molecular control of pathogenicity was poorly understood (Klosterman *et al.,* 2009; Fradin & Thomma, 2006). The molecular characterisation of genes related to and involved in pathogenicity, over the last few years, has begun to be revealed.

A plethora of 780 possible secreted effector molecules have been identified by

genome mining (Klosterman *et al.*, 2011). One investigation carried out by (Santhanam & Thomma, 2013) has identified the Sge1 transcriptional regulator, with a homolog in *FOL*, regulates the expression of six secreted effectors, of currently unknown function, that are required for pathogenicity on tomato. LS regions have also been identified in *Verticillium* spp., again enriched with putative secreted effector proteins and transposable elements that may play a role in pathogenicity (de Jonge *et al.*, 2013).

Furthermore, *FOL* and the *Verticillium* spp. have cryptic sexual cycles and it is believed that these LS regions may be the product of the extensive chromosome reshuffling predicted to drive the evolution of effectors in the ongoing arms race between host and pathogen in the absence of sexual recombination (de Jonge *et al.*, 2012). The genomic plasticity of these pathogens may pose a real problem for the modern cultivar breeder and their battle to retain resistance. Also, in the case of the *FO* and *Verticillium* spp., like many other fungi, the internal transcribed spacer (ITS) regions between the 18S and 28S ribosomal subunits, used for fungal species barcoding, does not provide the discrimination between pathogenic and saprophytic isolates.

As discussed above, rhizosphere associated fungi may exhibit increased levels of genome plasticity driven by extensive chromosome reshuffling at least in the transposon-rich pathogenicity associated LS regions. This coupled with mainly monogenic resistance being present in cultivars, that has been seamlessly broken by single nucleotide polymorphism creates a situation in which growers, breeders and pathologists are always on the back foot. In addition, the ability of *FOL* to horizontally transfer entire pathogenicity chromosomes to other members of the *FO* complex and the inferred horizontal transfer of pathogenicity genes between *FO* and the *Verticillium* spp. shows that inter and intra genera horizontal transfer may play a role in the acquisition of novel pathogenicity genes and determination of pathogenicity. Moreover, with pathogenicity related genes located within the transposon rich LS regions, the longevity of any molecular markers targeted from within this region may be uncertain.

1.5.2 The Oomycota

Oomycetes are a class of organism also known as the water moulds and are closely related to brown algae. There are over 800 species of pathogenic and saprophytic oomycetes which produce both hardy oospores, persisting in water for years, and motile zoospores which exhibit both chemo and electro-taxis (Blancard, 2012). Oomycetes' ability to both lay dormant in water systems and

move towards their hosts makes them particularly well adapted to life in the soilless greenhouse. As a result, fertigation systems that either partly or fully recycle their fertigation solution are at a greater risk of oomycete disease (Postma *et al.*, 2008). There are two main genera of oomycete associated with tomato; the *Pythium* spp. and *Phytophthora* spp.; both causing major losses in the greenhouse industry for both juvenile and mature plants as a result of dampening off and stunting, respectively (Schroder *et al.*, 2013). Disease can often be identified in seedlings via classical methods but in mature plants lack of specific symptomology and reproductive structures leads to difficulties in identification (Schroder *et al.*, 2013). A number of oomycetes associated with the tomato rhizosphere were identified by Deery, 2012, Table 1.4.

Table. 1.4 Oomycete pathogens commonly reported on tomato roots, their respective diseases and prevalence in soilless systems within Europe. All information was adapted from Deery, 2012, Blancard, 2012 and O'Neill, 2013b.

Organism	Disease	Prevalence in soilless systems within Europe
Phytophthora arecae	Foot rot	+
Phytophthora cinnamomi	Root rot	+
Phytophthora cryptogea	Foot rot/damping-off	+
Phytophthora nicotianae	Root rot/stem base rot/fruit rot	+
Pythium aphanidermatum	Root rot	+/++
Pythium arrhenomanes	Damping-off/ Root rot/stem base rot	+/++
Pythium debaryanum	Root rot/stem base rot	+/++
Pythium diclinum	Damping-off	+/++
Pythium echinulatum	-	+/++
Pythium irregular	Root rot/stem base rot	+/++
Pythium megalacanthum	Damping-off/ Root rot	+/++
Pythium myriotylum	Damping-off/ Root rot/stem base rot	+/++
Pythium oligandrum	Damping-off	+/++
Pythium paroecandrum	Root rot/stem base rot	+/++
Pythium torulosum	Mild disease	+/++

There are 106 species of *Pythium* most of which are generalist necrotrophic phytopathogens with broad host ranges, although some are pathogenic on mammals, fish and fungi (Levesque & de Cock, 2004; Phillips *et* al., 2008; Miura *et al.*, 2010; Rey *et al.*, 2005). The phylogeny of the *Pythium* spp. much like the *Fusarium* and *Verticillium* genera still requires further investigation with species,

such as *Pythium irregular,* believed to be a complex of species (Schroder *et al.*, 2013).

Phytophthora spp. or "plant destroyers" are comprised of 114 species, many of which have hemibiotrophic lifestyles in contrast to the necrotrophic lifestyle of the *Py*thium spp. (Hansen *et al.*, 2012; Adams *et al.*, 2008; Jiang & Tyler, 2012). A highly diverse range of small secreted putative effectors have been identified in the *Phytophthora* by a conserved RxLR-dEER region which is believed to target proteins to the cytoplasm of the host cells (Govers & Gijzen, 2006; Bhattacharjee *et al.*, 2006). Tyler *et al.*, 2006 found over 350 putative RxLR-dEER effectors in both the *Phytophthora ramorum* and *Phytophthora sojae* genomes and like the *Fusarium* and *Verticillium* genera, the effectors were situated in LS regions of the genome which exhibit reduced homology and increased genetic fluidity. Moreover, repeat-rich regions and large numbers of transposable elements are believed to facilitate genomic plasticity for the generation of new pathogens (Grouffaud *et al.*, 2008).

The molecular basis of host range and specificity within the oomycota is not yet clear (Jiang & Tyler, 2012). Although there are many similarities, in the molecular bases of virulence and acquisition of pathogenicity gene variation, the basal processes of fungal and oomycete pathogenicity differs. Oomycete pathogenicity is vastly dependent on large and rapidly diversifying protein families such as hydrolytic enzymes, extracellular toxins and inhibitors, whereas fungal pathogenicity is more reliant on secondary metabolites and protein families are less extensively diversified (Jiang & Tyler, 2012; Tyler, 2009; Tyler & Rouxel, 2012).

1.5.3 Other Taxa of Importance

Nematodes and bacteria also play large roles in the establishment of the rhizosphere community. Nineteen studies focused on analysing rhizosphere associated bacterial populations on various host species showed that in 16 studies the Proteobacteria dominated the bacterial populations with the γ-Proteobacteria, which includes the *Pseudomonas* genera, dominating in most cases (Hawkes *et al.*, 2007). The *Pseudomonads* contain species both pathogenic and directly and indirectly beneficial to plants health. *Pseudomonas syringae* is most notorious member of the *Pseudomonads*, causing leaf spot and bacterial speck on tomato, although scarcely present in soilless systems (Blancard, 2012). Another member of the Proteobacteria widely studied and associated with disease on tomato is the *Agrobacterium* genus. Again, the *Agrobacteria* can both

promote plant growth and cause disease. The development of disease is dependent on the presence of pathogenicity plasmids *Ti* and *Ri* leading to the formation of galls and root proliferation, respectively. Both of which have been commonly observed on soilless systems within Europe (Blancard, 2012).

Plant pathogenic nematodes have been widely studied in the rhizosphere, but the majority of nematodes >60% are bacterial feeding taxa and little work has been done on their impact on the rhizosphere (Kerry, 2000). Plant pathogenic nematodes, however, have been shown to influence the quality and quantity of root exudates in turn influencing the activity of other plant pathogenic and beneficial species (Bowers *et al.*, 1996; Khan, 1993). Deery, 2012 found that *Meloidogyne incognita* and *Pratylenchus goodeyi;* root-knot and lesion nematodes, respectively; occur on greenhouse tomato plants with increased frequency towards end of the growing season.

As has become apparent in the previous section there are a wide range of organisms associated with the rhizosphere with both beneficial and pathogenic effects on the host. Yet most have high genomic homogeneity outside of pathogenicity related regions. Thus far the molecular and biochemical differences between pathogenic and non-pathogenic strains of rhizosphere related organisms are poorly understood and in some cases the function of resistance genes in certain cultivars is also uncertain. The biochemical similarities between pathogenic and non-pathogenic strains may result in the inability to specifically target pathogenic strains during attempts to control disease, inadvertently destroying the microbial populations which may protect the plant from other pathogens.

1.6 Disease Management

After the end of WWII through to the 1960s, greenhouse production relied heavily on the use of chemical pesticides to control pathogens. Over these two decades the heavy reliance on chemical crop protection and high intensity farming led to the emergence of resistance for key pathogens (van Lenteren, 2000). In the advent of resistance the use of inert soilless growing substrates became more common.

The use of new soilless substrates was able to greatly reduce the plethora of soil-borne pathogens due to reduced viability of soil-borne pathogens in an environment more aquatic in nature and the growers' ability to completely sterilise greenhouses after cropping, see section 1.3 for more information on soilless tomato production. Aside from complete sterilisation of the greenhouse, the use of pathogen free seed and good hygienic practise can help prevent the

introduction of pathogens into the greenhouse system (van Lenteren, 2000).

1.6.1 Naturally Occurring Resistance and Pesticides

Although soilless substrates provide an environment that naturally harbour less pathogenic organisms, there is still a potential for devastating outbreaks of disease increased by the use of assimilation heating providing an environment that may favours the proliferation of indigenous and non-indigenous pathogens. To overcome the potential for disease, resistance genes were found in wild relatives of tomato and introgressed into the commonly used tomato cultivars, see Table 1.5 and section 1.3.3 for more information on modern cultivar breeding. Although resistance genes for many pathogens are available, not all are used commercially or are not wholly efficient at protecting the crops and so outbreaks my still occur.

Table 1.5 A Summary of resistance genes available their target pathogens, efficacy and source. Information adapted from (Blancard, 2012; Scott & Gardener, 2006).

Pathogen	Gene	Efficiency	Source
Alternaria solani	*Ebr*	n/a	*L. esculentum*
Clavibacter michiganensis	*Cm*	n/a	*L. peruvianum*
Verticillium albo-atrum & dahliae	*Ve*	+++	*L. pimpinellifolum*
Fusarium oxysporum f.sp. *lycopersici*- Race-1	*I*	+++	*L. pimpinellifolum*
Fusarium oxysporum f.sp. *lycopersici*- Race-2	*I-2*	+++	*L. pimpinellifolum*
Fusarium oxysporum f.sp. *lycopersici*- Race-3	*I-3*	n/a	*L. pennellii*
Fusarium oxysporum f.sp. *radicis-lycopersici*	*Fr1*	+++	*L. peruvianum*
Meloidogyne incognita	*Mi*	+++	*L. peruvianum*
Pseudomonas syringae pv tomato	*Pto*	+++	*L. pimpinellifolum*
Pyrenochaeta lycopersici	*Pyl*	+	*L. peruvianum*
Ralstonia solanacerum	*Bw1, 3, 4, 5*	n/a	*L. pimpinellifolum*
Xanthomonas campestris	*Bs4*	n/a	*L. pennellii*

For a limited number of pathogens including *Alternaria solani* and *Didymella lycopersici* fungicidal treatment, after the first symptoms arise, can be achieved with fungicides such as Mancozeb or Chlorothalonil (Blancard, 2012). Application of these fungicides, however, can be costly and not always effective. For example, outbreaks of *Pythium* spp. and *Phytophthora* spp. require the drenching of all substrate in anti-oomycete fungicides such as Propamocarb-HCL preceding minimal application of fertigation solution to prevent re-emergence (Blancard, 2012). For other pathogens including *Agrobacterium tumefaciens, Clavibacter michiganensis, Colletotrichum coccodes, Fusarium oxysporum* f.sp. *lycopersici, Fusarium oxysporum* f.sp. *radicis lycopersici, Pyrenochaeta lycopersici, Ralstonia solanacearum* and *Xanthomonas campestris* there are no chemical based pesticide treatment. Some control of the disease is available by the removal or quarantining of diseased plants and increased sanitation but this is only applicable to few (Blancard, 2012). Moreover, where the use of a chemical treatment is available many may be prohibited under new government legislation to prevent the consumption of fungicidal residues by the general public.

1.6.2 Biocontrol

The limited availability of chemical control and pressure from the European Union to reduce their use has led to a boom in the research to find naturally occurring rhizosphere associated microorganisms to be used for biocontrol. Biocontrol relies on the premise that certain microorganisms can prevent pathogen colonisation of the rhizosphere or at least prevent the development of disease. Modes of action include parasitism, the production of fungicidal and bactericidal molecules and the formation of physical barriers. An array of biocontrol applications have been observed by a number of organisms, see Table 1.6, to date most have only shown success *in vitro* and in small scale lab based trials with limited success in the field (Blancard, 2012).

This variability in success may be why so few biocontrol products are commercially available. In addition, commercially available biocontrol products utilising non-pathogenic *Agrobacterium* spp. to protect against crown gall disease have shown the transfer of pathogenicity between biocontrol agent and pathogen so use has to be carefully considered. The few commercially available biocontrol products, for example BIOMEX-Plus (Omex), usually contain their microbial product in conjunction with a fertiliser.

Our inability to control disease outbreaks and dissemination increases our need

to closely monitor the rhizosphere and its microbial populations to take action on the detection of any pathogen post-haste.

Table 1.6. An overview of the biological control agents for the main pathogens of tomatoes grown on soilless substrates, their targets and the source of information.

Organism	Controls against	Source
Agrobacterium vitis	*Agrobacterium tumefaciens*	Kawaguchi *et al.*, 2008
	Agrobacterium rhizogenes	Kawaguchi *et al.*, 2008
Bacillus amyloliquefaciens	*Fusarium oxysporum* f.sp. *lycopersici*	Vitullo *et al.*, 2012
Bacillus subtilis	*Fusarium solani*	Morsy *et al.*, 2009
	Ralstonia solanacearum	Chen *et al.*, 2013
	Rhizoctonia solani	Asaka *et al.*, 1996
Fusarium oxysporum	*Fusarium oxysporum* f.sp. *lycopersici*	Whipps, 2001
	Pratylenchus goodeyi	Mwaura *et al.*, 2010
Glomus intraradices	*Fusarium oxysporum* f.sp. *radicis-lycopersici*	Datnoff *et al.*, 1995
	Meloidogyne hapla	Masadeh *et al.*, 2004
Paecilomyces lilacinus	*Fusarium oxysporum f.sp lycopersici*	Siddiqui *et al.*, 2000
	Fusarium solani	Siddiqui *et al.*, 2000
	Meloidogyne javanica	Siddiqui *et al.*, 2000
Penicillium citinum	*Fusarium oxysporum* f.sp. *radicis-lycopersici*	Alwathnani *et al.*, 2012
Penicillium brevicompactum	*Pythium ultimum*	Gravel *et al.*, 2006
Pseudomonas aeruginosa	*Fusarium oxysporum* f.sp. *lycopersici*	Siddiqui *et al.*, 2000
	Fusarium solani	Siddiqui *et al.*, 2000
	Meloidogyne javanica	Siddiqui *et al.*, 2000
Pseudomonas fluorescens	*Fusarium oxysporum* f.sp. *radicis-lycopersici*	Bolwerk *et al.*, 2003
	Pythium ultimum	Gravel *et al.*, 2006
	Rhizoctonia solani	Hammer *et al.*, 1997
Pythium oligandrum	*Botrytis cinerea*	le Floch *et al.*, 2005
	Fusarium oxysporum f.sp. *radicis-lycopersici*	le Floch *et al.*, 2005
	Pythium dissotocum	Vallance *et al.*, 2009

Trichoderma harzianum	*Ralstonia solanacearum*	Takenaka *et al*., 2007
	Fusarium oxysporum f.sp. *lycopersici*	Alwathnani *et al.*, 2012
	Fusarium oxysporum f.sp. *radicis-lycopersici*	Datnoff *et al.*, 1995
	Verticillium albo-atrum	J-Khiareddine *et al.*, 2009
	Verticillium dahlia	J-Khiareddine *et al.*, 2009
	Verticillium tricorpus	J-Khiareddine *et al.*, 2009
Trichoderma viride	*Fusarium solani*	Morsy *et al*, 2009
	Meloidogyne hapla	Masadeh *et al.*, 2004
	Verticillium albo-atrum	J-Khiareddine *et al.*, 2009
	Verticillium dahlia	J-Khiareddine *et al.*, 2009
	Verticillium tricorpus	J-Khiareddine *et al.*, 2009

1.7 Pathogen Detection and Community Analysis

The identity of much of the microbial population present in the rhizosphere is currently unknown due to our inability to culture the vast majority of microorganisms. For more information on the microbial communities of the rhizosphere see section 1.5. The microorganisms we are able to culture often have closely related pathogenic strains which cannot be discriminated via morphological features or plating on specific media. This leaves determination of pathogenicity to the observation of symptoms and in many cases once symptoms are observed it may be too late to take action. Moreover, many pathogens exhibit overlapping symptomology and some are homologous to symptoms caused by environmental stresses and therefore require vast *a priori* knowledge not always available to the commercial nursery grower. Although the classical diagnosis of disease is invaluable modern molecular techniques can help reduce the time taken to diagnose a disease and therefore improve the rapid initiation of disease management strategies.

One of the first advances in molecular detection of pathogens was the introduction of serological techniques such as enzyme-linked immunosorbent assays (ELISA) whose predecessors became available in the 1960s. Serological techniques utilise specific cell surface proteins as antigens for the antibody based

detection of pathogens. This, however, poses a problem when faced with some bacterial and fungal pathogens that lack the specific antigens often limiting its use for the detection of viruses. The basic principles of ELISA, however, were developed into a rapid in-field detection kit by FERA (The Food and Environment Research Agency) in the form of a lateral flow device whose function is analogous to that of a pregnancy test. This provided relatively cheap, yet rapid detection of pathogens although in some cases it lacked the high sensitivity and specificity required for detection of certain organisms.

1.7.1 PCR Based Detection

In the 1980s, following the advent of the genetic revolution and the development of the polymerase chain reaction (PCR), issues in the sensitivity and specificity of pathogen detection were reduced. This was mainly due to PCR's ability to amplify DNA present at low concentration and the new array of genetic information available for phytopathogens. The abundance of genetic information unlocked by PCR not only allowed the design of primers to detect specific to species, races and strains but also led to the development of numerous methods used to analyse microbial communities. Examples of which can be seen in Table 1.7. Traditional PCR techniques were confined to the laboratory due to high energy requirements and need for further analysis of products with restriction enzymes and gel electrophoresis. The development of Q-PCR (Quantitative-Polymerase Chain Reaction), its utilisation of fluorescent dyes; to detect the formation of ds-DNA; and the increased efficiency of equipment allowed the real-time quantitative viewing of diagnosis allowing its uses to be extended to in-field diagnosis. Perhaps one of the biggest advances facilitated by PCR was the development of the DNA microarray.

Table 1.7 A summary of PCR based techniques for pathogen detection and microbial community analysis including their main uses, advantages and disadvantages. Information was obtained from several sources (Fry *et al.*, 2009; Semagn *et al.*, 2006; Postma *et al.*, 2008; Schutte *et al.*, 2008).

Technique	Main use	Advantages	Disadvantages
AFLP	DNA fingerprinting	No *a priori* knowledge of genome	Variation in fragment sizing across platforms
		Increased reproducibility compared to RAPD	
		Universal protocol	
		Whole genome surveys	
		Increased sensitivity compared to RAPD and RFLP	
		Increased multiplex applications compared to RFLP and RAPD	
DGGE	DNA fingerprinting	Discrimination between fragments of equal sizes	Failure to discriminate between a small number of single nucleotide polymorphisms
		Larger scale applications in comparison to AFLP and RAPD	
RAPD	DNA fingerprinting	No *a priori* knowledge of the genome	Highly sensitive to changes within the PCR reaction mixture
			Low reproducibility
		Polymorphisms easily detected	
		Whole genome survey	
T-RFLP	DNA fingerprinting	Fingerprint based on a single gene	Cannot sequence products from gel
		High throughput ability for microbial community analysis	Putative species identification due to fragment length homology
		Relatively simple protocol	
Q-PCR	DNA ID of molecular markers	Quantitative microbial analysis	Misidentification of products with non-specific ds-DNA dyes
		Organism identification via melt analysis	Increasing specificity with TaqMan probes is highly costly
		Small scale multiplex application	
		In field use possible	
		Relatively fast	

1.7.2 Microarray Analysis of Microbial Communities

Microarrays use the same basic principle as the Southern blots used in traditional RFLP (restriction fragment length polymorphism) on a larger scale. A high density of oligonucleotides, specific to a heterologous region of an organisms' genome, are fused to the surface of a membrane, often a glass slide, at a specific position called a probe. Upon the addition of DNA from a microbial community sample regions of an organisms' genome, previously amplified by PCR, is hybridised with its respective probe. The addition of uracil-specific enzyme-linked antibodies, which bind uracil incorporated into the DNA during PCR, and a substrate cleaved by the enzyme causes a localised colour change allowing levels of hybridisation to the probes to be viewed. The intensity of the colour can then be used to infer a semi-quantitative abundance of the organism associated with the probe.

The DNA microarray was initially developed to monitor the changes in the expression of genes in *Arabidopsis thaliana* (Schena *et al.*, 1995). DNA microarrays soon expanded their use into phylogenetic studies. Microarrays used in phylogenetic studies often utilise oligonucleotide probes 15-30nt in length designed from heterologous regions of genes used in DNA barcoding such as the intergenic spacer region (IGS) and ITS (internal transcribed spacer). The IGS and ITS are situated between copies of the rDNA (ribosomal DNA) and between the small and large subunits, respectively, see Fig. 1.5.

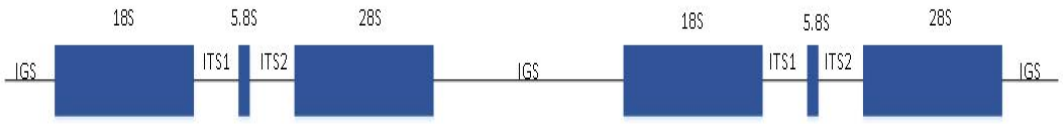


Fig. 1.5 A generic representation of the fungal rDNA (ribosomal DNA) structure including the positioning of the ITS (internal transcribed spacer) and IGS (intergenic spacer) regions.

A major advantage of phylogenetic microarrays over previous methods to study microbial communities, such as T-RFLP, is the increased specificity of detection as T-RFLP may only give putative diagnosis due homogeneity in the sizes of terminal fragments. Moreover, the possible scale of identification is also an advantage with microarrays such as the PhyloChip designed to detect over < 8,000 species (Schatz *et al* 2010). Although scale and specificity much outweighs other methods of community analysis, microarrays are not applicable to on-site diagnosis due to their relatively complex protocol. The complexity of the protocol, however, is outweighed by the scale of organism detection and

relatively short end-end process of <12hrs, dependant on protocol. Microarrays are now commonly used to monitor microbial populations across a number of fields from the marine sediments to the human gut (Briggs *et al.*, 2013; Tottey *et al.,* 2013).

Aside from microarrays, NGS (next generation sequencing) methods such as pyrosequencing have also shown to give increased phylogenetic resolution in comparison to T-RFLP (Deery, 2012) and equivalent resolution to that of microarray studies on numerous occasions (van den Bogert *et* al., 2011; Claesson *et al.*, 2009; Loy *et al.*, 2002). Although our ability to monitor complex microbial communities has advanced dramatically over the past five decades the speed in which we can specifically diagnose a broad range of phytopathogens, possibly present at low concentrations, has been limited by the speed of the general PCR protocol.

1.7.3 Emerging Diagnostic Techniques

In the last few decades much research has been focused on the rapid detection of specific microorganisms often driven by the risk of biological weapon use and prevention of potential human disease epidemics. The emerging techniques often quickly find application in the world of phytopathology for the detection of non-indigenous phytopathogens on imported goods at ports, an increasing risk with our changing climate, and for the monitoring and control of devastating disease outbreaks such as ash dieback.

One such technique; loop-mediated amplification (LAMP) developed in 2000 by (Notomi *et al.*, 2000), utilises a Bst polymerase from *Bacillus stearothermophilus* and set of four primers that form a double ended hairpin-like structure allowing isothermal DNA polymerisation. In 2002 Nagamine *et* al. developed a six primer protocol with the addition of two loop primers which drive strand displacement synthesis and half the time of diagnosis. The formation of the double ended hairpin-like structure and isothermal amplification allows rapid, continuous amplification of DNA and the use of six primers makes the amplification highly specific with a single nucleotide polymorphism able to discriminate between organisms. Inclusion of dyes that bind ds-DNA along with stable premixed reagents and battery powered portable technology have also allowed real-time diagnosis to be performed in-field with relatively little training. To date protocols have been developed to detect the presence of a pathogen in under an hour, including DNA extraction, the LAMP process taking up to 45 minutes and 15 minutes for a extraction (Tomlinson *et al.,* 2010). Such rapid extraction is possible due to the Bst polymerase being far less susceptible to contaminants

than traditional Taq polymerases used in PCR. Many uses of LAMP have already been established including detection of *Hymenoscyphus pseudoalbidus;* the causal agent of ash dieback, *Botrytis cinerea;* causal agent of grey mould on tomato and *Pepino mosaic virus* (Dickinson, 2013; Tomlinson *et al.*, 2010; Ling *et al.*, 2013).

Increased sensitivity immunoassays have also arisen in the last decade. One such technique, developed by the Lincoln laboratory at the Massachusetts Institute of Technology, for cellular analysis and notification of antigen risks and yields (CANARY) uses biosensors to detect low level antigens, <50 colony forming units, in 1-10 minutes (Petrovick *et al.*, 2007). CANARY utilises genetically modified B-cells with transmembrane antibodies which when bound to an antigen cause the B-cell to emit photons and report that a binding event has occurred (Petrovick *et al.*, 2007). CANARY is not only a more sensitive immunobiological detection system it also allows the detection of small secreted proteins (Petrovick *et al.*, 2007). This use in particular may have many applications in the world of phytopathology with many pathogens from genera including the *Fusarium, Pythium*, *Phytophthora* and *Verticillium* secreting an array of small proteins involved in pathogenesis. Many of these pathogens only differ from their non-pathogenic counterparts by the presence of these pathogenicity related genes and their proteinaceous products. CANARY has already been established for the detection of plant pathogens within the genera *Phytophthora* and *Ralstonia* along with the Potyvirus (Petrovick *et al.*, 2007).

The emerging rapid detection techniques especially LAMP; which requires little training, employs simple and rapid DNA extraction methods, can use premixed reaction mixtures which can be stored for prolonged periods of time and is available as portable battery powered kit, could be efficiently added to any greenhouse integrated pest management scheme. Such technologies, if developed and used correctly could give huge advantages to the tomato grower allowing specific detection of pathogens in under an hour potentially preventing large scale yield losses.

1.8 Aims

Previous work by Deery, 2012 utilised T-RFLP to monitor the microbial population associated with commercially grown tomatoes. This project aims to build on the results obtained in the study by utilising a small scale microarray, developed by Devine, 2013. Using microarray technologies will allow the monitoring of the microbial populations to a higher specificity than the putative

results obtained by T-RFLP and at a greater frequency across the growing season due to the relatively fast protocol.

The primary aim was to monitor the microbial populations associated with the rhizosphere of three rockwool crops across the 2012-2013 season. The crops were grown on contrasting fertigation systems pSSF (part slow sand filtration), SSF (full slow sand filtration) and run-to-waste (run-to-waste) systems. Secondly, two irrigation systems utilising physical treatments, heat and UV, for the disinfestation of their recycled fertigation solution were monitored at four points during the season. Finally, LAMP based assays for the detection of organisms pathogenic on tomato were developed.

Chapter 2: General Methods

2.1 Monitoring the Microbial Population of the Rhizosphere by Microarray Analysis

Prior to this study a small-scale microarray was developed and validated by Devine, 2013 at The University of Nottingham. The array was designed to detect 53 species and seven genera of fungi and oomycete, eight species and six genera of bacteria and two species and a genus of nematode, see Table 6.1. Probes on the array were designed utilising bioinformatics software MEGA 5.1 from regions on heterogeneity within the 18S-26/28S ITS region of eukaryotic microorganisms and the 16S-23S ITS region of prokaryotes. Validation was achieved by running DNA from reference organisms, both closely related to and distantly related to microorganisms targeted on the array. All reference microorganisms used in validation were provided by ADAS, FERA and The University of Nottingham and identified by both classical techniques and molecular barcoding of their ITS regions. Validation allowed probe specificity to be inferred through the monitoring of which organisms produced a signal from any given probe. A summary of validation results can be found in appendix 2.

Table 2.1 The probe targeted organisms on the microarray developed by Devine, 2013 and their relevant groupings.

Fungal and Oomycete Pathogens	Fungal Saprophytes	Bacteria and Nematodes
Alternaria solani	*Alternaria spp.*	*Agrobacterium rhizogenes*
Armillaria mellea	*Aspergillus flavus*	*Agrobacterium tumefaciens*
Colletotrichum acutatum	*Aspergillus sydowii*	*Bacillus amyloliquefaciens*
*Colletotrichum coccodes**	*Aspergillus terreus*	*Bacillus subtilis*
Didymella lycopersici	*Aspergillus ustus*	*Clavibacter michiganensis*
*Fusarium oxysporum**	*Cadophora spp.*	*Erwinia spp.*
*Fusarium redolens**	*Cephalosporium spp*	*Escherichia coli*
*Fusarium solani**	*Chaetomium cochliodes*	*Nitrospira spp.*
Phytophthora arecae	*Chaetomium spp.*	*Pseudomonas syringae*
Phytophthora cinnamomi	*Cladophora spp.*	*Pseudomonas spp.*
Phytophthora cryptogea	*Doratomyces microsporus*	*Ralstonia solanacearum*
Phytophthora nicotianae	*Exophiala pisciphila*	*Rhizobium spp*
Pythium aphanidermatum	*Exophiala xenobiotica*	*Xanthomonas spp.*
Pythium arrhenomanes	*Gigaspora rosea*	*Yersinia spp.*
Pythium debaryanum	*Gliocladium roseum*	*Meloidogyne incognita*[n]

Pythium diclinum	*Glomus intraradices*	*Pratylenchus goodeyi*[n]
Pythium echinulatum	*Myrothecium roridum*	*Pratylenchus spp.*[n]
Pythium irregular	*Olpidium brassicae*	
Pythium megalacanthum	*Paecilomyces lilacinus*	
Pythium myriotylum	*Penicillium brevicompactum*	
Pythium oligandrum	*Penicillium chrysogenum*	
Pythium paroecandrum	*Penicillium griseofulvum*	
Pythium torulosum	*Penicillium variabile*	
*Plectosphaerella cucumerina**	*Penicillium spp*	
*Pyrenochaeta lycopersici**	*Petriella asymmetrica*	
Rhizoctonia solani	*Phoma spp.*	
Spongospora subterranea	*Rhizopus oryzae*	
*Thielaviopsis basicola**	*Trichoderma viride*	
Verticillium albo-atrum	*Trichoderma harzianun*	
Verticillium dahliae	*Trichoderma spp.*	
Verticillium nigrescens		

Key: * = Organism targeted by two independent probes, n= Nematodes

2.1.2 Sampling

All sampling was carried out by either a member of the respective nursery team under instruction from Tim O'Neill, ADAS, or by a member of the ADAS staff. Root Sampling was carried out by the following method:-

Scissors and scalpel were sterilised before use. Approximately 2 g of young roots were cut from; the base of the rockwool cube (pre slab contact); the rockwool slab (up to four weeks after slab contact) and finally the slab corner for the remainder of the season. The slab was re-sealed with tape after sampling. Roots were stored in sterile and sealed polythene bags.

Fertigation solution sampling was carried out by the following method:-

A sterile 20 ml syringe was used to collect 20 ml of fertigation solution from the slab base and then transferred into a sterile 20 ml universal. The universal was labelled with the appropriate information and posted first class, along with the roots, to Plant and Crop Sciences, Sutton Bonington Campus, The University of Nottingham.

2.1.3 DNA Extraction

Prior to extraction, screw-cap micro-Eppendorf tubes were filled with 8-12 2mm glass beads and autoclaved. In a class II biological safety cabinet 120 mg,

±5 mg, of roots were weighed out on sterile 110 mm Whatman® qualitative filter paper removing any excess fertigation solution. The roots were placed into the screw-cap micro-Eppendorf. Fertigation solution samples were first vortexed and then 5 ml was filtered by a sterile 25 mm, 0.20 µm Whatman® nylon membrane within a syringe-driven filter unit. The nylon membrane was then placed into the micro-Eppendorf. All samples underwent the same process following the previous steps.

Micro-Eppendorfs were placed into liquid nitrogen for five minutes and then placed into a Fastprep FP120 (Thermo-Savant) at 6.5 ms^{-1} for 45 seconds to homogenise the samples. The process was repeated three times in total. The homogenised samples were then extracted using the DNeasy Plant Mini Kit (QIAGEN) following the manufacturers' protocol. All DNA was then stored at -20°C.

2.1.4 Polymerase Chain Reaction of ITS regions

The PCR of ITS regions was used for both microarray analysis and genetic barcoding of any cultures received. Two sets of "universal" primers were used one for the amplification of the eukaryotic ITS region and one set for the Prokaryotic, see Table 2.2. All primers were produced by Sigma-Aldrich®. Primers were diluted to a stock of 100 µM and a working concentration of 10 µM (pmol/µl) with sterile distilled water (SDW) and stored at -20°C.

Table 2.2. The Primers used to amplify the ITS regions of eukaryotic and prokaryotic organisms used in this study. Eukaryotic primers were developed in Sequerra *et al.*, 1997 and prokaryotic primers in Ranjard *et al.*, 2001.

Primers	Sequence	Target
2234C^{FE}	GTTTCCGTAGGTGAACCTGC	3' of 18S
3126T^{RE}	ATATGCTTAAGTTCAGCGGGT	5' of 26/28S
S-D-Bact-1522-b-S-20^{FP}	TGCGGCTGGATCCCCTCCTT	3' of 16S
L-D-Bact-132-a-A-18^{RP}	CCGGGTTTCCCCATTCGG	5' of 23S

Key: F= Forward primer, R= Reverse primer, E=eukaryotic, P=prkaryotic

A master-mix (n+1) was made comprising of 0.5 µl of forward and reverse primer, 10.5 µl of SDW and 12.5 µl of 2X MangoMix (Bioline) and then vortexed. PCR products being added to the microarray had the further addition of 0.5 µl of Biotin 16-dUTP (Roche) per reaction into the master-mix. A 24 µl of aliquot of master-mix was then added into an autoclaved 50 µl single reaction PCR tube and 1 µl of sample DNA was added. All PCR reactions were carried out in a

BioRad® S1000™ thermal cycler programmed with the following protocols:-
Eukaryotic primers: 94°C for 2 minutes followed by 35 cycles of; 94°C for 30 seconds, 53 °C for 1 minute and 72°C for 1 minute; with a final extension of 72 °C for 5 minutes.
Prokaryotic primers: 94 °C for 3 minutes followed by 35 cycles of; 94 °C for 1 minute, 55 °C for 30 seconds and 72 °C for 1 minute; with a final extension of 72 °C for 5 minutes.

2.1.5 Gel Electrophoresis

All PCR products were visualised on a gel to confirm the successful amplification of the targeted DNA region. A 1% agarose gel was made with 1X Tris-Borate-EDTA buffer (TBE) and stained with 25 µl L^{-1} of ethidium bromide (Sigma-Aldrich). A 3 µl aliquot of PCR product was pipetted into the gel along with a 1Kb ladder and run at 100V for 60 minutes using a BioRad® PowerPac Baisic™. The gel was viewed by UV camera InGenius3 on software platform GeneSys both manufactured by Syngene, UK.

2.1.6 Hybridisation of Target DNA to the Microarray

Microarrays were manufactured by Alere™, Germany, and run following the protocol developed by Devine, 2013. All stages of the microarray protocol, apart from the final, were carried out in a Thermomixer Comfort® (Eppendorf). Microarrays were first conditioned by adding 500 µl of SDW and agitating at 500 rpm for 5 minutes at 55 °C, contents were discarded. A 500 µl aliquot of Nexterion® Hyb (Schott) was added to the array again agitating at 500rpm for 5 minutes at 55 °C and discarded afterwards. Eighty microliters of Nexterion® Hyb (Schott) were added to the array and allowed to heat to 55 °C.

The Biotin-incorporated PCR products from the prokaryotic and eukaryotic rDNA primers were now hybridised to the array by the following method. The remaining PCR products (22 µl) were heated to 95 °C for four minutes in a BioRad® S1000™ thermal cycler. A 20 µl aliquot of both Eukaryotic and Prokaryotic PCR product was added to each microarray and agitated at 500 rpm at 55 °C for 1 hour and 15 minutes. Contents of the microarray were then discarded. The array then underwent a three stage wash process with WB1, wash buffer 1, (2X SSC [saline sodium-citrate], 0.01 % Triton X100), WB2 (2X SSC) and WB3 (0.2X SSC) using 500 µl aliquots of each at 20 °C agitating at 500 rpm for 5 minutes, discarding microarray contents after each step.

A blocking solution was freshly prepared for each set of microarrays, comprising (n+2) 200 µl PBS (phosphate-buffered saline), 4 mg of semi-skimmed milk powder and 1 µl of 1 % Triton X100. The blocking solution was vortexed and 100 µl added to the microarrays for 15 minutes at 20 °C to bind any active but vacant probes. Microarray contents were then discarded. The remaining blocking solution then underwent the addition of horseradish peroxidse-linked (HRP) antibodies (Cell Signalling Technology®) antigenic against Biotin at a ratio of 1 µl of antibodies per 100 µl of blocking solution. The mixture was gently mixed to avoid antibody aggregation. A 100 µl aliquot was added to each array and incubated for 15 minutes at 20 °C and discarded afterwards.

A final wash procedure was then carried out to remove any excess conjugate and debris. Five hundred microlitre aliquots of PBST (1X PBS, 0.2% Tween 20) were added to arrays and then agitated at 500 rpm at 20 °C for 5 minutes. This step was repeated a further two times discarding array contents after each repeat. Finally, substrate was added to the array to allow optical recording of probe intensities. A 100 µl aliquot of SeramunGrün® chip (Seramun®) an o-Dianisidine substrate was added to the arrays and allowed to incubate for 16 minutes. The SeramunGrün® chip was cleaved by the horseradish peroxidase enzyme linked to the antibodies causing a localised colour change.

At 16 minutes after initial incubation, with the substrate, the arrays were placed into an ArrayTube transmission reader-03 (Alere^{TM}) and utilising software platform Iconoclust v4.1 (Alere^{TM}) images of the array were produced and subsequently analysed using the bespoke experimental template 10429_AT_Myco_2_1.0 (Alere^{TM}) to align probes with their correlating organisms. The aligned probes intensities were then normalised and corrected in respect to local background intensity by the following equation:

$$i = \frac{average\ spot\ intensity}{local\ background\ intensity}$$

2.1.7 Microarray Data Analysis

Microarray data was first filtered to remove any ambiguous data using an intensity cut-off value of 0.1 (double the value of any intensity recorded from a blank array). Data below 0.1 was removed from the data set. For the seven species for which two sets of probes were used the intensities were averaged. The combined results from each set of four microarrays performed were then used qualitatively to find a simple count of species richness, the total number of taxa observed, for each sample period. Fertigation solution data was not used quantitatively due to only a single repeat being used. The cumulative intensities observed for each taxon were then recorded to allow a semi-quantitative view of relative abundance across the growing season.

2.1.7.1 Species Diversity

Microarray intensity data was first divided into four categories. The first of which included all taxa recorded on the array. The remaining three categories were fungi, oomycete and bacteria. Only intensity data from microorganisms fitting into their respective phylogenetic groupings was used to calculate diversity indices. Where probes were designed for the identification of genera and not specific species, the data was used to represent a single species.

A species diversity score was then found for each array and each category using the Shannon-Wiener index (H') following the calculation below where a = average probe intensity of species **X** and b = total intensity of array. For the separate phylogenetic groupings b = total intensity of the group. The equation was adapted from the original where a = observed number of species and b = total number of observed species.

$$H' = -\sum \left[\left(\frac{a}{b} \right) \ln \left(\frac{a}{b} \right) \right]$$

2.1.7.2 Two-way ANOVA

The interaction between groups (fertigation system and week) could not be tested due to the assumption of homogeneity of variances not being met on any instance. As such, a model with more power than but analogous to performing two separate one-way ANOVAs was performed. The adjusted model or "main effects" two-way ANOVA was utilised to test the null hypotheses of absence of

variation between groups. All statistical analyses were performed using SPSS 20.0.0 (IBM).

2.2 LAMP Assay Development

Organisms were identified in knowledge-transfer meetings with commercial greenhouse tomato growers and then reviewed in the current literature to allow the identification of any possible novel molecular markers. Upon identification of molecular markers, query submissions were sent to GenBank (NCIB) to retrieve their respective nucleotide sequences. *In silico* techniques utilising software platform MEGA 5.1 (MegaSoftware) were then performed using the Basic Local Alignment Search Tool (BLAST) to retrieve any homologous sequences deposited into GenBank. Retrieved sequences were added to the MEGA 5.1 platform and aligned allowing the identification of homologous and heterologous regions. Regions of intra-species homology were targeted for the LAMP assay avoiding any regions of inter-species homology. Sequences were then imported into LAMP designer (Premiere Biosoft) to identify regions appropriate as use as primers. In some cases nucleotide sequences were only available in mRNA form. For these cases further steps, discussed below, were required to retrieve the genomic sequence and identify any introns.

2.2.1 Obtaining Genomic Sequences from mRNA

PCR primers were first designed targeting the 5' and 3' regions of the mRNA avoiding the poly-A-tail if present in the sequence. Primers were chosen to be approx. 15-20nt in length and 60% GC with a 3' GC clamp where possible to increase primer success. Reference DNA provided by The University of Nottingham was then used alongside the designed primers to allow the optimisation of the primers and the amplification of their products. Optimisation was achieved via gradient PCR utilising a general protocol allowing the amplification of 4kb products due to unknown product length. Eight annealing temperatures were utilised in the gradient PCR, the lowest Tm °C of the primer pair acted as the maximum annealing temperature with a minimum temperature of 10 °C below. The complete PCR cycle was as follows: 94 °C for 3 minutes followed by 35 cycles of; 94 °C for 1 minute, a gradient anneal for 1 minute and 72 °C for 1 minute; completed by a final extension of 72 °C for 5 minutes. Products were then run on gel electrophoresis following the protocol in section 2.1.5

Optimised primers were used to amplify their target sequences and run on gel electrophoresis, again following protocol 2.1.5. Amplified products were then

purified using 20 µl of the remaining reaction mixture utilising the GenElute™ PCR Clean-Up kit (Sigma-Aldrich) following the manufacturers guidelines. A 5 µl aliquot of purified product, along with 5 µl of forward primer, was sent to MWG Eurofins (Germany) for sequencing. Returned sequences were checked for integrity and if appropriate imported into MEGA 5.1 and aligned with their respective mRNA sequences to highlight any introns or regions of heterogeneity. The returned genomic DNA sequences were then used to develop LAMP assays as stated in section 2.2.

Chapter 3: Monitoring the Microbial Populations Associated with the Rhizosphere from Crops Grown on Three Contrasting Fertigation Systems Over the 2012-2013 Growing season

3.1 Introduction

The UK is one of the world's leading soilless tomato producers but the tightly regulated use of pesticides and little available treatment of disease leaves the commercial tomato grower at risk of large potential yield losses, see sections 1.6. Integrated pest management schemes have the ability to control pests such as aphids and white fly via the application of biocontrol agents (Blancard, 2012). Our poor understanding of the complex microbial community associated with the rhizosphere, however, leaves crops open to attack from pathogens utilising the roots as a point of entry. Gaining insight into the microbial populations found on tomato roots will allow us to better understand the rhizosphere as an environment and also potential risks of disease.

The implementation of soilless crop production increases the prevalence of pathogens within the oomycota. Furthermore, increasing pressure is being placed on commercial growers to reduce their environmental impact by fertigation solution recycling. Little is known about the risks involved in fertigation solution recycling and a possibility of the increased incidence of water-borne and soil-borne pathogens may occur with improper disinfestation. Further understanding of the risks associated with the recycling of fertigation solution is essential for proper crop management.

In this study the rhizosphere associated microorganisms of three crops grown on contrasting fertigation systems will be monitored. Two of which undergo fertigation solution recycling by part slow-sand filtration (pSSF) and slow-sand filtration (SSF) and a final crop was grown on a run-to-waste (RTW) system. The use of a recently developed small-scale microarray (Devine, 2013) will allow an increased frequency in sampling and increased resolution of species when compared to other techniques. Increased frequency of sampling and species resolution will hopefully permit the rhizosphere communities to be studied in much more detail allowing the identification of any potential risks associated with the recycling of fertigation solution.

3.2 Methods

Three crops grown on contrasting fertigation systems pSSF, SSF and RTW were monitored every two weeks to allow a profile of the microbial populations associated with the tomato rhizosphere to be built over the 2012-2013 growing season. Four root samples and a single fertigation solution sample were taken from each system. Root samples were taken from adjacent slabs situated towards the middle of a block. Upon the next sampling the four adjacent slabs were used so that each slab was sampled only once. Sampling followed the protocols found in section 2.1.2. All crops were grown on Maxifort™ root stocks (DeRuiter), with differing scions, in rockwool substrate. Samples underwent DNA extraction, PCR and microarray analysis following the protocols in sections 2.1.3, 2.1.4, 2.1.5 and 2.1.6, respectively. Finally, data analysis was performed following the protocols in section 2.1.7.

3.3 Results

3.3.1 Taxa Richness and Prevalence

Twenty-two of the microbial taxa targeted on the array were not detected at any of the sites in either the root or fertigation solution samples, see Table 3.1. The bacteria *Escherichia coli* and the root-knot nematode *Meloidogyne incognita* were also not present in any samples. A number of taxa, 5, 6 and 3, were only observed at specific systems on root samples for the pSSF, SSF and RTW systems, respectively, see Table 3.2.

Table 3.1 The taxa not observed on root or fertigation solution samples at any of the sites (pSSF, SSF and RTW) across the 2012-2013 growing season.

Pathogenic fungi and oomycetes	Saprophytic fungi
Armillaria mellea	*Alternaria spp.*
Didymella lycopersici	*Cephalosporium spp.*
Phytophthora cinnamomi	*Chaetomium cochliodes*
Pythium aphanidermatum	*Doratomyces microspores*
Pythium arrhenomanes	*Gigaspora rosea*
Pythium debaryanum	*Gliocladium roseum*
Pythium echinulatum	*Glomus intraradices,*
Pythium oligandrum	*Paecilomyces lilacinus*
Pythium torulosum	*Penicillium chrysogenum,*
	Trichoderma harzianum
	Trichoderma spp.

Table 3.2 The unique taxa observed from the root samples on the pSSF, SSF and RTW sites.

pSSF	SSF	RTW
Aspergillus ustus	*Aspergillus sydowii*	*Alternaria* spp.
Myrothecium roridum	*Chaetomium spp.*	*Aspergillus terrus*
Pythium megalacanthum	*Exophiala xenobiotica*	*Rhizopus oryzae*
Rhizoctonia solani	*Phytophthora arecae*	
Trichoderma viride	*Ralstonia solanacearum*	
	Rhizobium spp.	

Root samples also exhibited differences in taxa richness between the contrasting fertigation systems with the pSSF and SSF root samples exhibiting 41 and 42 different taxa, respectively, and the RTW system only 28, Fig. 3.1. The reduction in taxa richness occurred for all groups of organisms at the RTW site, see Table 3.3. Levels of nematodes, however, were relatively equal across the systems, see Fig. 3.1.

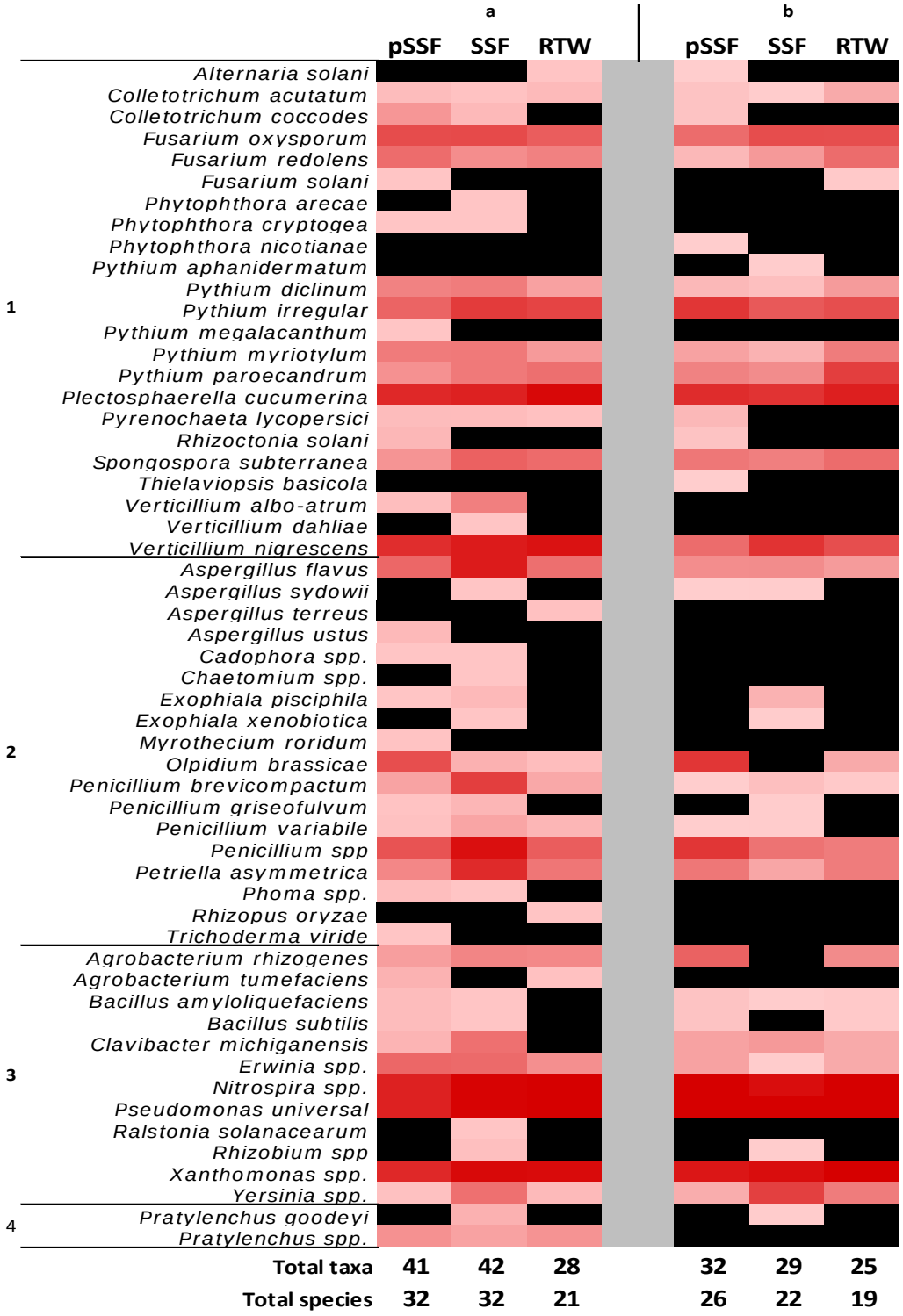
Table 3.3 The number of pathogenic fungal and oomycete, saprophytic fungal and bacterial taxa recorded on root samples from the pSSF, SSF and RTW systems.

Site	Pathogenic fungi and oomycetes	Saprophytic fungi	Bacteria
pSSF	17	13	10
SSF	16	13	11
RTW	12	8	7

Overall the fungal pathogen prevalence was high at each of the sites, see Fig 3.1. *Fusarium redolens, Pythium diclinum, Pythium myriotylum, Pythium paroecandrum* and *Spongosporra subterranea* all showed 20 > 49 % prevalence. *Fusarium oxysporum* and *Pythium irregulare* showed 50 > 70 % prevalence and *Plectospharella cucumerina* and *Verticillium nigrescens* showed 71 > 100% prevalence across the season. The RTW system exhibited the highest prevalence for *Pythium paroecandrum, Plectospharella cucumerina* and *Verticillium nigrescens;* present at 44 %, 96 % and 91 % of the season, respectively. The pSSF had the highest prevalence of *Fusarium redolens* and *Spongospora subterranea* at 46 % and 86 %, respectively. The SSF system had the highest prevalence of *Fusarium oxysporum, Pythium diclinum, Pythium irregulare* and *Pythium myriotylum* at 63 %, 38 %, 70 % and 39 %, respectively. Only two saprophytic fungal taxa were observed at over 50 % at all three sites; the fungi

Aspergillus flavus and *Penicillium* spp. The bacterial genera *Nitrospira, Pseudomonas* and *Xanthomonas* were all observed for < 80 % of the growing season on each of the systems.

Fertigation solution samples also showed varying levels of richness with 32, 29 and 25 taxa observed at the pSSF, SSF and RTW nurseries, respectively, see Fig 3.1. Taxa only observed at a single nursery were; *Alternaria solani*, *Phytophthora nicotianae* and *Thielaviopsis basicola* at the pSSF nursery; *Exophiala pisciphilia, Exophiala xenobiotica*, *Pythium aphanidermatum* and *Rhizobium* spp. at the SSF nursery and finally *Fusarium solani* at the RTW nursery. Taxa persisting for over 50 % of the season in the fertigation solution at all three sites were *Fusarium oxysporum, Pythium irregulare, Plectospharella cucumerina, Verticillium nigrescens, Nitrospira* spp., *Pseudomonas* spp. and *Xanthomonas* spp.. A general observation of an increased number of taxa with increased prevalence can be seen on the root samples in comparison to the fertigation solution samples.



Key

Percentage prevalence across the growing season (%).

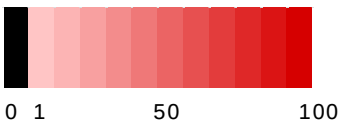


Fig. 3.1. A summary of the rhizosphere related species, including prevalence and taxa richness, occurring on the pSSF, SSF and RTW systems across the 2012-2013 growing season. Section **a** is the cumulative data from four replicate root samples. Section **b** is data from a single fertigation solution sample. Group **1**contains fungal and oomycete pathogens. Group **2** is fungal saprophytes. Group **3** is bacteria and group **4** is nematodes.

3.3.2 Persistence and Abundance of Taxa on Roots

Three Genera of bacteria, *Pseudomonas, Nitrospira* and *Xanthomonas* were present across the entire season. *Pseudomonas and Nitrospira* spp. persisted at increased levels between 3.00 and 4.00. Four taxa showed their highest abundances at the start of the season and then declined across the remainder, with mean abundances reduced in the second half of the season, see Table 3.4 and Fig 3.2.

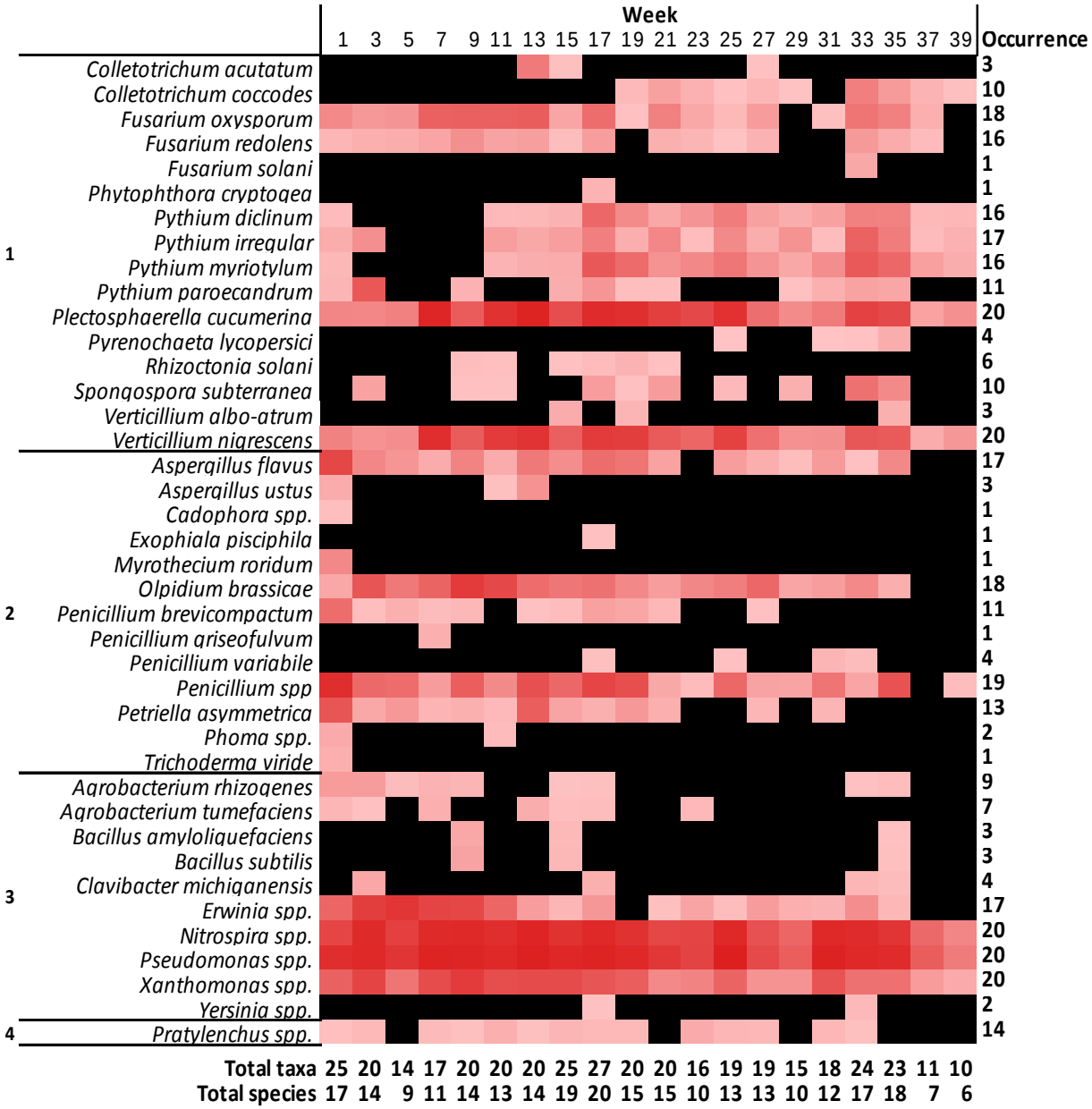
Table 3.4. The mean abundance of taxa observed on root samples, which exhibit peaks at the beginning of the season, between weeks 1-19 and 21-39 at pSSF nursery.

Taxa	Mean abundance	
	Weeks 1-19	Weeks 21-39
Erwinia spp.	1.69	0.41
Olpidum brassicae	1.82	0.86
Petriella assymetrica	0.9	0.11
Penicillium spp.	2.03	0.89

Plectospharella cucumerina and *Verticillium nigrescens* show a peak in abundance between weeks 7 and 35, see Fig 3.2, with means of 2.55 and 2.25, lower mean abundance was observed over the remainder of the season at 1.10 and 0.97, respectively. *Fusarium oxysporum* and *Fusarium redolens* tended to persist for the whole season at mostly constant levels, see Fig 3.2, apart from weeks 7-13 where *Fusarium oxysporum* exhibits an increased mean abundance of 2.077 in comparison to 0.781 for the remainder of the year.

Pythium diclinum, Pythium irregulare and *Pythium myriotylum* are observed between weeks 1-3 of monitoring at lower abundances and then not observed again until week eleven from which they persist for the remainder of the season with increased levels between weeks 17-35. Mean abundances observed for weeks 1-15 and 17-39 were; 0.148, 0.475 and 0.212; and; 0.962, 0.869 and 0.344; for *Pythium diclinum, Pythium irregulare* and *Pythium myriotylum*, respectively. *Colletotrichum coccodes* is observed at week 19, and then persists for the remaining weeks at relatively constant abundance. A number of organisms are only present in the first few weeks of monitoring and then do not persist including *Aspergillus ustus, Cadophora* spp., *Myrothecium roridum, Phoma* spp. and *Trichoderma viride*. Finally, peak taxa richness was observed in

quarter 2 (weeks 11-19) of the growing season with mean taxa richness of 22.40 in comparison to 19.20, 17.80 and 17.20 in quarter 1, 3 and 4.



Key

Abundance of taxa

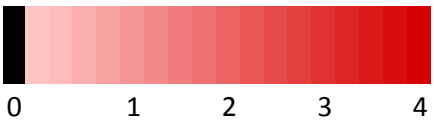


Fig. 3.2. The persistence patterns and cumulative abundance of taxa from four root sample repeats on the pSSF system over the 2012-2013 growing season. Group **1**contains fungal and oomycete pathogens. Group **2** is fungal saprophytes. Group **3** is bacteria and group **4** is nematodes.

As with the pSSF nursery, ten taxa tended to persist across the whole season at the SSF. *Nitrospira* spp. and *Pseudomonas spp.*, again, showed persistent high levels of abundance with mean abundances of 2.99 and 3.12, respectively. *Aspergillus flavus, Fusarium oxysporum, Fusarium redolens, Penicillium brevicompactum, Penicillium* spp., *Petriella assymetrica, Verticillium nigrescens* and *Xanthomonas* spp. also persisted for most of the season but with differing patterns of abundance, see Fig. 3.3.

Fusarium oxysporum and *Fusarium redolens* showed relatively constant levels of abundance with a peak for *Fusarium oxysporum* averaging at 1.09 during weeks 13-21 compared to 0.56 for the remainder of the season. Six taxa, however exhibited peak abundance between weeks 9 and 27 showing increased mean abundance in comparison to the remainder of the season, see Table 3.5 and Fig 3.3.

Table 3.5. The mean abundance of taxa observed on root samples, which exhibit a peak during weeks 9-27, from the SSF nursery.

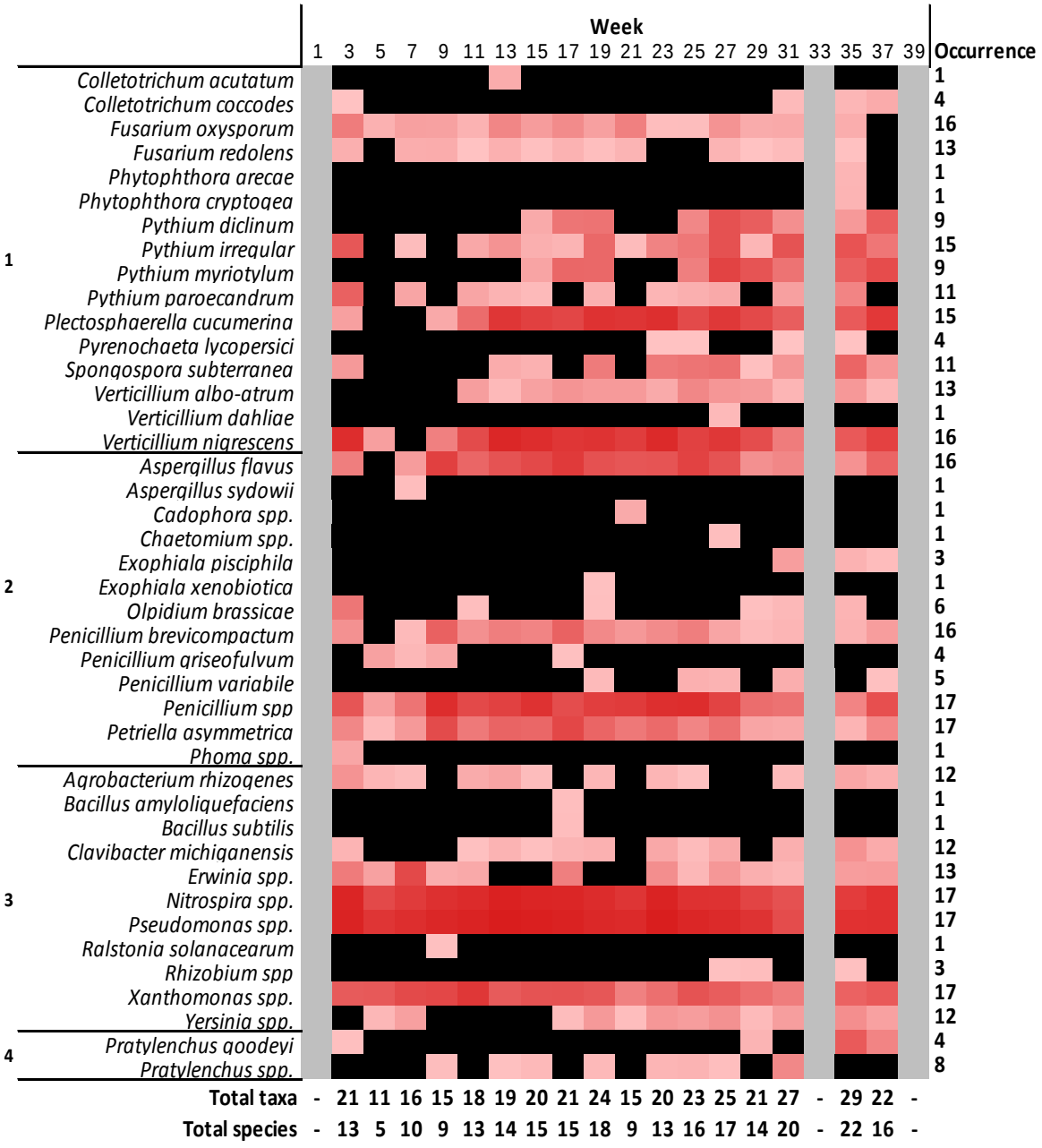
Taxa	Mean abundance	
	Weeks 9-27	Weeks 1-7 & 29-29
Aspergillus flavus	2.42	1.10
Penicillium brevicompactum	1.34	0.45
Penicillium spp.	2.81	1.71
Petriella assymetrica	1.89	0.76
Plectospharella cucumerina	2.50	1.50
Verticillium nigrescens	2.80	1.81

Nine taxa show sporadic presence at the beginning and then tend to persist from the mid-season; *Clavibacter michiganenses, Plectospharella cucumerina Pythium diclinum, Pythium irregulare, Pythium myriotylum, Pythium paroecandrum, Spongospora subterranea, Verticillium albo-atrum* and *Yersinia* spp., see Fig 3.3. Five of the taxa exhibit abundances that increase in abundance towards the end of the season, four of which show increased mean abundance between weeks 25-37, see Table 3.6. *Plectospharella cucumerina,* however, shows peak abundance from week 11 and persisting at similar levels for the remainder of the season with a mean abundance of 2.70 for weeks 11-37 and 0.55 for weeks 1-11. The remaining four taxa exhibit relatively constant abundances. Finally, peak taxa richness occurs in the final quarter of the season

for the SSF system with mean taxa richness for quarters 1, 2, 3 and 4 at 15.75, 20.40, 20.80 and 26.00, respectively.

Table 3.6 The mean abundance of taxa observed on root samples, which exhibit a peak during weeks 9-27, from the SSF nursery.

Taxa	Mean abundance	
	Weeks 25-37	Weeks 1-23
Pythium diclinum	1.64	0.43
Pythium irregulare	1.76	0.83
Pythium myriotylum	2.10	0.49
Spongospora subterranea	1.24	0.55



Key

Taxa abundance

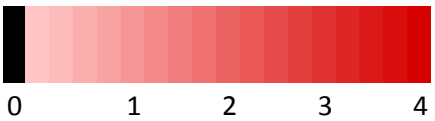


Fig. 3.3 The persistence patterns and cumulative abundance of taxa from four root sample repeats on the SSF system over the 2012-2013 growing season. Group **1**contains fungal and oomycete pathogens. Group **2** is fungal saprophytes. Group **3** is bacteria and group **4** is nematodes.

Again *Nitrospira, Pseudomonas* and *Xanthomonas* spp. were present throughout the entire season at the RTW nursery with relatively constant abundances decreasing slightly at weeks 33 and 35, see Fig. 3.4. Eight taxa were present during the first half of the season up to week 19 and then only showed sporadic presence including *Agrobacterium rhizogenes, Aspergillus flavus, Erwinia* spp., *Fusarium oxysporum, Fusarium redolens, Penicillium brevicompactum, Petriella assymetrica* and *Pythium paroecandrum*, see Fig 3.4. Six of the taxa showed similar patterns of abundance decreasing from the start to the end of the season, see Table 3.7 and Fig 3.4. The final two taxa, *Penicillium brevicompactum* and *Fusarium redolens* showed relatively consistent abundance.

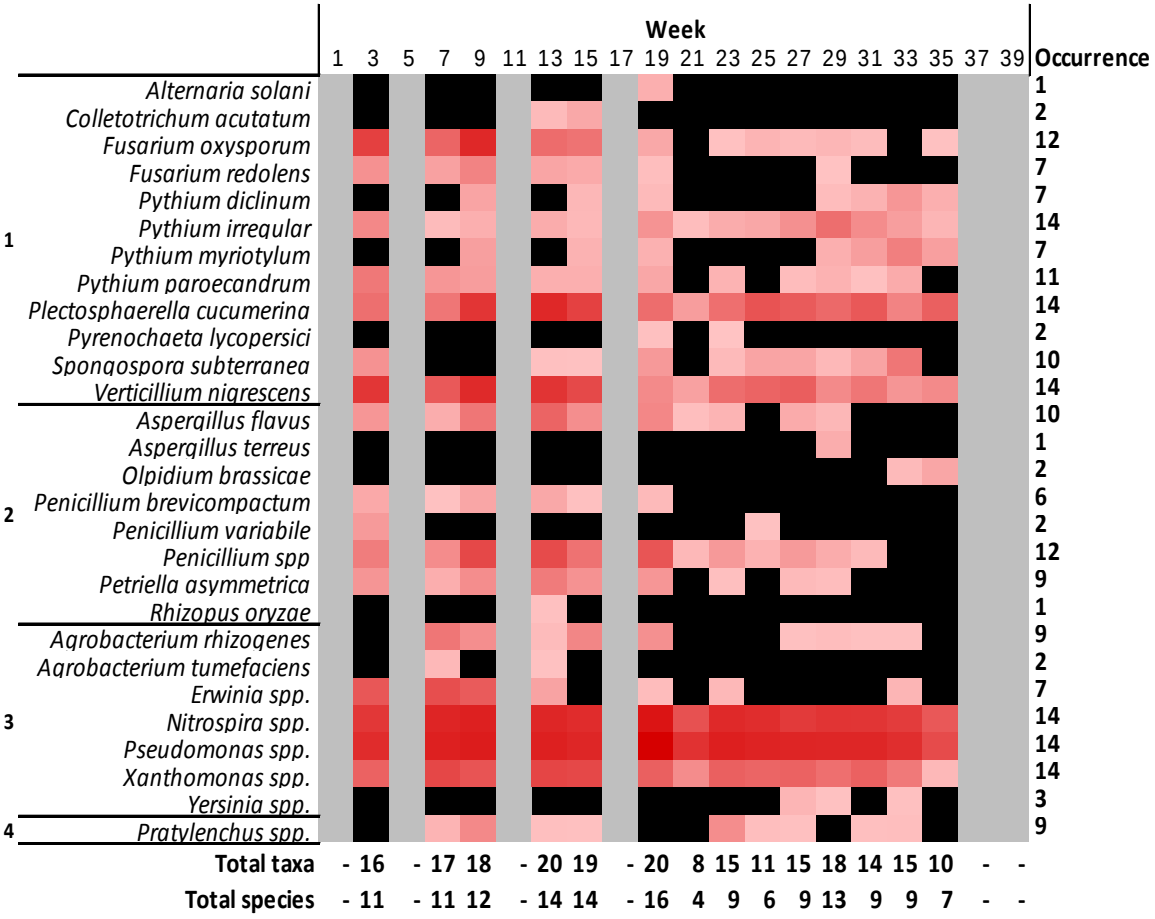
Table 3.7 The mean abundance of taxa observed on root samples, which exhibit a decline in abundance across the season, from the RTW nursery.

Taxa	Mean abundance	
	Weeks 3-19	Weeks 21-35
Agrobacterium rhizogenes	1.10	0.12
Aspergillus flavus	1.24	0.34
Erwinia spp.	1.54	0.30
Fusarium oxysporum	1.02	0.18
Petriella *assymetrica*	0.82	0.31
Pythium paroecandrum	1.99	0.22

Three taxa, that tended to persist for the whole season, exhibited peak abundance during the first 19 weeks of the season. *Penicillium* spp., *Plectospharella cucumerina* and *Verticillium nigrescens* showed mean abundances of 1.94, 2.33, and 2.49 during weeks 1-19 and 0.54, 1.82 and 1.46 during weeks 21-35, respectively. Three species *Pythium diclinum, Pythium myriotylum* and *Spongospora subterranea* showed sporadic presence until week 29 from when they persisted with increasing in mean abundance from 0.35, 0.51 and 0.49 between weeks 1-29 to 0.61, 1.06 and 1.162 during weeks 31-35, respectively. Finally, mean taxa richness was highest during quarter two of the season at 19.67 with quarters 1, 3 and 4 showing mean richness of 17.00, 13.40 and 13.00, respectively.

The RTW system exhibited differing patterns in organism abundance in comparison to the pSSF and SSF with the heat-maps giving general indications of a more constant level of abundance. Moreover, patterns of species persistence

differ with pSSF and SSF showing more organisms with sporadic, non-persisting appearance with 17 and 18 taxa, respectively, appearing at <5 points during the season and only 9 at the RTW site. Finally, mean taxa prevalence for the first and second half of the growing season shows a general pattern of a reduction taxa prevalence from the first to second half of the season for the pSSF and RTW systems exhibiting a mean prevalence of 66.67 % and 52 % for weeks 1-19 and 49.13 and 43.75 for weeks 21-39, respectively. The SSF, however, shows a general increase in prevalence over the season increasing from 43.91 % in weeks 1-19 to 54.16 % in weeks 21-39.



Key

Taxa abundance

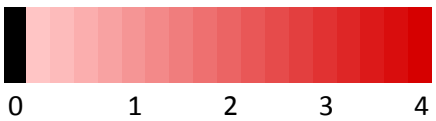


Fig. 3.4. The persistence patterns and cumulative abundance of taxa from four root sample repeats on the RTW system over the 2012-2013 growing season. Group **1**contains fungal and oomycete pathogens. Group **2** is fungal saprophytes. Group **3** is bacteria and group **4** is nematodes.

3.3.3 Species Diversity

Mean species diversity data from the root samples, in Fig. 3.5, showed that at the start of the season all three systems had a similar diversity at week 3 with diversities of 2.53, 2.60 and 2.53 for the pSSF, SSF and RTW systems, respectively. Over weeks 3-5 there was a sharp drop in diversity at the pSSF and SSF to 2.21 and 1.78 at week 5, respectively, RTW data was not available but showed decreased diversity in week 7 of 2.30. The RTW system then saw a small increase in diversity to 2.56 in week 9 but a general pattern of decreasing diversity can be seen across the season from week 9 to week 35 with a final diversity score of 1.80. The pSSF and SSF systems, however, increase in species diversity between the weeks of 5 and 17 to 2.68 and 2.58, respectively. From week 19 the SSF nursery, although showing dips in diversity at weeks 21 and 29, continues to increase in diversity to the end of the season with a final diversity of 2.75 at week 37. The pSSF system, however, continues to fall; apart from weeks 33 and 35 where diversities of 2.72 are recorded, similar to that of the SSF system; with a final diversity of 1.66 at week 39. From week 15 the SSF system tends to show increased diversity in comparison to the pSSF and RTW sites with average diversities of 2.28, 2.47 and 2.45 for weeks 1-15 and 2.65, 2.28 and 1.96 for weeks 17-39 for the SSF, pSSF and RTW systems, respectively.

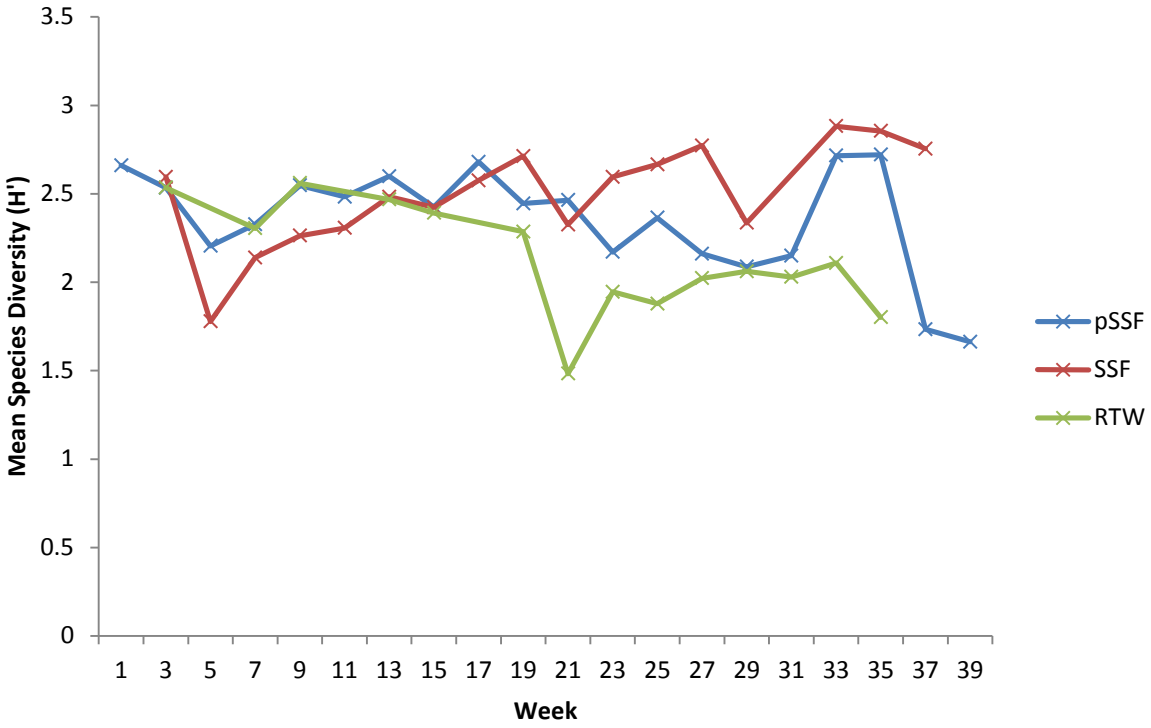


Fig.3.5 Mean species diversity observed on tomato roots at the pSSF, SSF and RTW sites across the 2012-2013 growing season.

3.3.3.1 Fungal, Oomycete and Bacterial Species Diversity

The fungal, oomycete and bacterial taxa diversities from the pSSF nursery, shown in Fig. 3.6, reveal that a general decrease in diversity is observed for the fungi and bacteria but the oomycetes show an increase in diversity over the season. This is supported by mean species diversities of 1.84, 0.43 and 1.41 between weeks 1-19 and 1.43, 0.85 and 1.12 for weeks 21-35 for the fungi, oomycetes and bacteria, respectively. The oomycetes diversity exhibits more drastic differences in diversity falling to 0.00 for weeks 5, 7 and 9 from a diversity of 0.75 at week 3 and also dropping at week 23 with a diversity of 0.34 in comparison to week 21 and 25 of 1.26 and 0.99, respectively. The bacterial diversity follows a fairly consistent trend of decline across the season from the third week to week 29 with diversities of 1.64 and 0.77, respectively, showing an increase from week 29 to 35 with a diversity of 1.32. The fungal diversity increases from weeks 3 to 13 by 0.55 but then begins to fall to week 31 with a diversity of 1.15. Although the diversities of the fungi, oomycete and bacteria differed across the season at the penultimate and final weeks all exhibited similar diversities at 1.43, 1.50 and 1.20 at week 33, respectively.

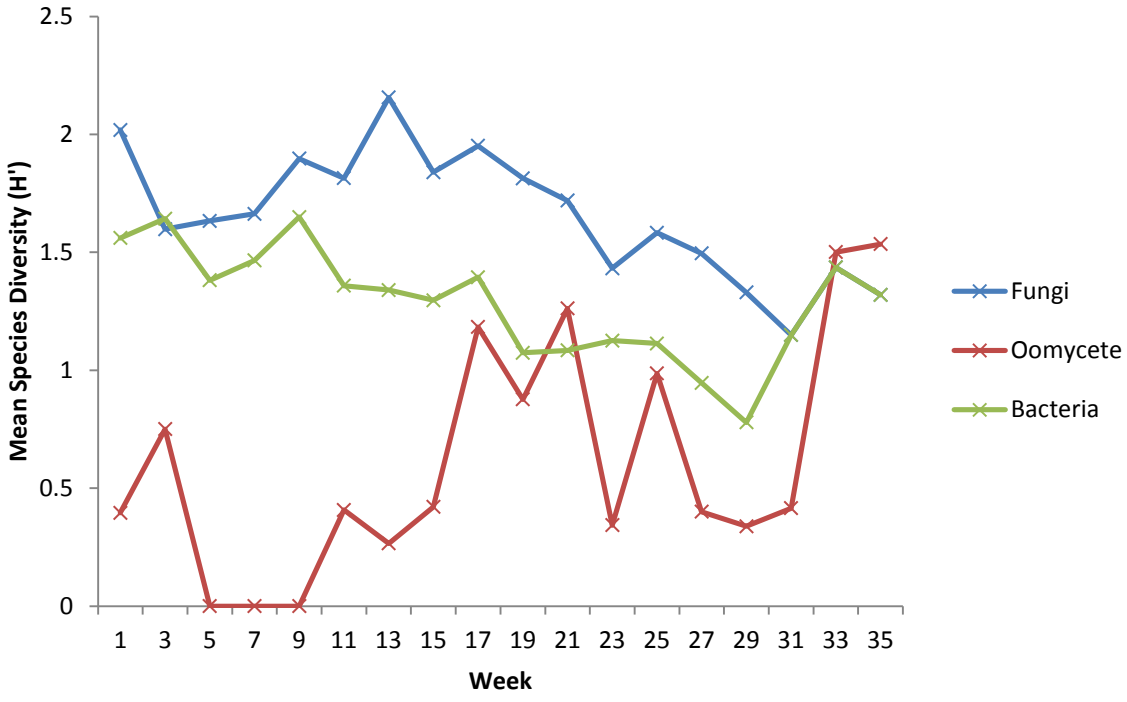


Fig. 3.6. Mean species diversity of fungi, oomycetes and bacteria observed on the roots of a tomato crop grown on the pSSF system over the 2012-2013 growing season.

The fungal and bacterial diversities at the SSF remain relatively constant across the growing season whereas the oomycete diversity increases dramatically towards the end of the season, see Fig 3.7. Only a small mean increases in diversity observed from weeks 1-19 and 21-35 of 0.21 and 0.04 for the fungi and bacteria, respectively. The oomycetes, however, almost double in diversity from with mean diversities of 0.45 to 0.98 for weeks 1-19 and 21-35, respectively. The fungal diversity at the SSF nursery increases, after an initial drop of 0.86 between weeks 3 and 5, from weeks 7-9 by 0.71 where it remains relatively stable for the remainder of the season. The oomycete diversity at the SSF, like the pSSF, also shows sudden drops in diversity of 0.94 and 0.83 at weeks 21 and 29. The bacterial diversity at the SSF site drops between weeks 3 and 21 from 1.49 to 1.01 but then increases again towards the end of the season with a final diversity of 1.72 in week 35. Again, as with the pSSF system, fungal, oomycete and bacterial diversities are divergent across the entire season until the final weeks where there final diversities are all similar at 1.84, 1.51 and 1.74, respectively.

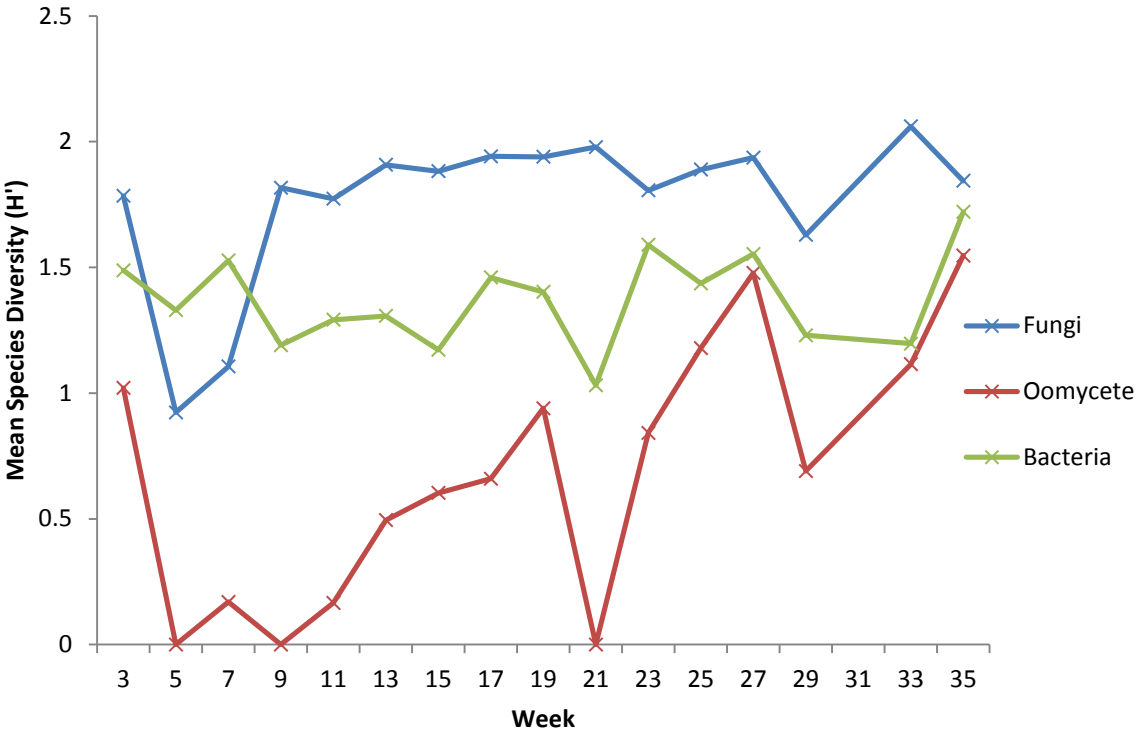


Fig. 3.7. Mean species diversity of fungi, oomycetes and bacteria observed on the roots of a tomato crop grown on the SSF system over the 2012-2013 growing season.

The nursery utilising the RTW fertigation system showed a dramatic decrease in fungal and bacterial diversities and relatively similar diversity of oomycetes across both halves of the growing season, see Fig. 3.8. Fungal and bacterial diversities dropped from 1.82 and 1.34 at week 3 to 0.92 and 0.77 at week 35, respectively. Mean diversities for the two halves of the growing season highlighted the drops in fungal and bacterial diversity and consistently sporadic oomycete diversity with mean diversities of 1.73, 0.59 and 1.36 for weeks 1-19 and 0.91, 0.58 and 1.08 for weeks 21-35 for fungi, oomycetes and bacteria, respectively. Bacterial species diversity at the RTW system declines across the season in a relatively uniform manner whereas the fungal diversity exhibits a small incline of 0.1 from week 3-15, and then a dramatic decrease of 1.24 to week 21 where it remained at low level. The oomycete diversity across the season is fairly erratic, falling to 0.14 at week 7 and 0.00 at week 21. As with the pSSF and SSF systems the final fungal, oomycete and bacterial diversities are similar at the end of the season at 0.97, 0.441 and 0.773, respectively, but the RTW exhibits oomycete diversity more divergent than that of the pSSF and SSF systems.

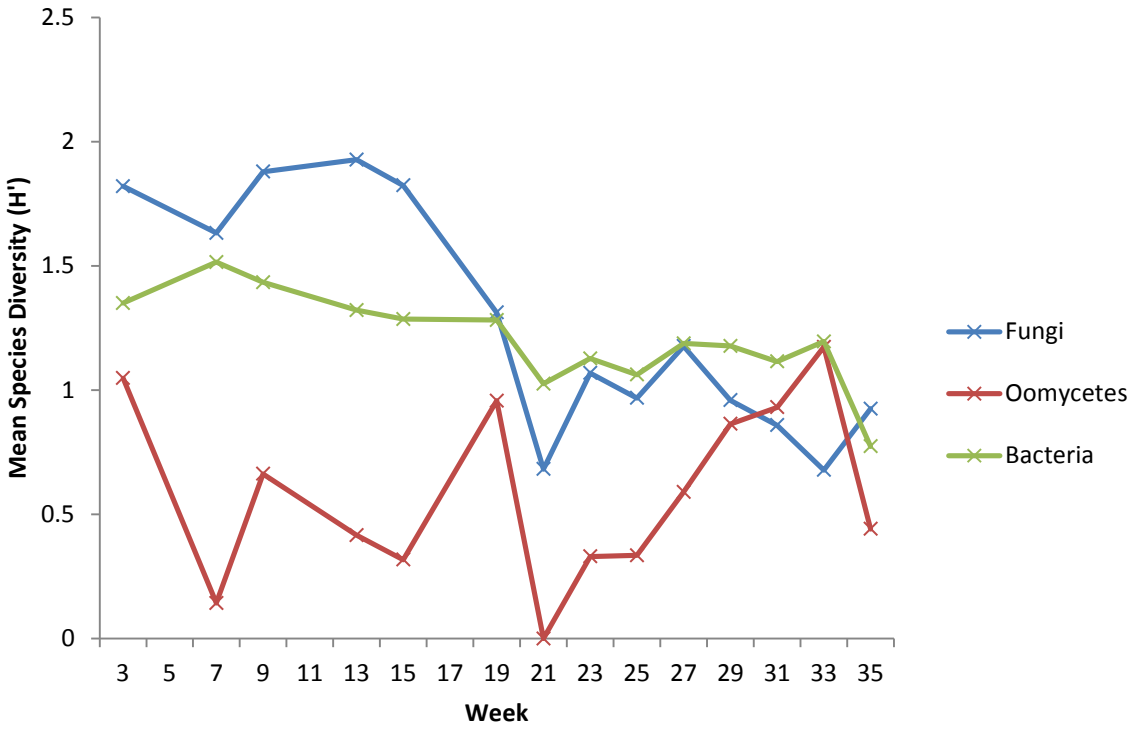


Fig. 3.8. Mean species diversity of fungi, oomycetes and bacteria observed on the roots of a tomato crop grown on the RTW system over the 2012-2013 growing season.

In contrast to the RTW and pSSF system the fungal oomycete and bacterial diversities at the site SSF undergo fewer sporadic changes, see Fig 3.6, 3.7 and 3.8. Each of the nurseries has a similar starting diversity and incurs a drop in diversity over the first week of monitoring at the respective sites. In contrast to the SSF site the fungal diversity fell dramatically at week 21 at the RTW site whereas the decline in fungal diversity at the pSSF sites is more gradual over the season. Bacterial diversity at each of the sites is fairly constant in contrast with its fungal and oomycete counterparts, excluding the fungal diversity at the SSF site, with gradual declines at each of the sites apart from the SSF where a small increase in diversity occurs. At each of the sites the oomycete diversity increases to approximately 1.00 at week 19. From this point the SSF site continues to rise to the end of the season whereas the pSSF site drops at weeks 23-31. The oomycete population at the RTW system exhibits little continuity across the season. Finally, although the fungal, oomycete and bacterial diversities at each of the sites tend to differ and follow differing general trends at each of the sites, the fungal, oomycete and bacterial diversities end the season at similar levels.

3.3.3.2 Analysis of Variance (ANOVA)

ANOVA testing of species diversity from root samples gave four significant results, see Table 3.1. The first of which was testing total species diversity from the contrasting fertigation systems which gave results $F(2,167) = 23.498, 0.088$, $p<0.001$. The F – critical value for (2, 167), $\alpha = 0.05$ was 3.05 allowing us to reject the null hypothesis and determine the size of effect exhibited between contrasting fertigation systems at 0.220, a relatively small effect. Post hoc testing allowed the identification of between group variation and its direction.

Two significant between group variations were observed between the pSSF>RTW and SSF>RTW nurseries with mean differences in diversity of 0.288 and 0.355, respectively, both with p values of <0.001. The mean difference in diversity shows a larger difference between SSF and RTW sites in comparison to the pSSF and RTW. The second significant result was observed in the difference in diversity between weeks. The ANOVA gave results of F (16,167) = 3.628, 0.88, $p<0.001$ (F-critical = 1.704) with effect size of 0.258, again a relatively small effect size. A summary of the post hoc results can be seen in Table 3.1.

Two significant variances were observed from the mean fungal and oomycete species diversity data, the first of which was from the fungal data with fertigation

system as the dependant variable. ANOVA analysis gave the results $F(2,28)$ = 9.034, 0.096, p=0.001 (F-critical= 3.340), effect size of 0.329. Post hoc analysis showed only a single significant between group variance, pSSF>RTW, at p=0.007 with a mean difference in diversity of 0.327. The second significant observation from oomycete diversity was the variance between week in the growing system, $F(16,27)$ = 2.630, 0.137, p=0.013 (F-critical= 2.035), which showed an effect size of 0.609 a moderate effect. Post hoc tests failed to discriminate the weeks between which variation occurred.

Table 3.8 A summary of the significant results from ANOVA testing used to analyse variance in microbial diversity observed, across the 2012-2013 growing season, on tomato roots from nurseries utilising three differing fertigation systems pSSF, SSF and RTW.

Taxa	Dependant Variable	P	N^2	Post Hoc		
				Direction	P	Mean difference (±)
Total	Fertigation	<0.001	0.220	pSSF>RTW	<0.001	0.288
				SSF>RTW	<0.001	0.335
	Week	<0.001	0.258	3>5	0.015	0.523
				3>21	0.047	0.427
				5<13	0.015	0.525
				5<17	0.003	0.636
				5<19	0.036	0.489
				5<33	0.044	0.486
				13>21	0.048	0.426
				17>21	0.011	0.537
Fungi	Fertigation	0.001	0.392	pSSF>RTW	0.007	0.372
				SSF>RTW	<0.001	0.499
Oom-ycete	Week	0.013	0.609	-	-	-

The mean total species diversity, see Fig. 3.9, observed at the pSSF and SSF sites are similar at 2.419 and 2.462 with standard errors of 0.036 and 0.038, respectively. The RTW system, however, exhibits a lower mean diversity of

2.106 and standard error of 0.041. The variance within the mean fungal diversities observed from the contrasting fertigation systems also show a similar pattern again with the pSSF and SSF giving similar diversities, see Fig 3.10. The pSSF and SSF nurseries show increased diversities of 1.637 and 1.739 with standard errors of 0.075 and 0.078, respectively. The RTW system again shows reduced diversity at 1.262 with standard error 0.086.

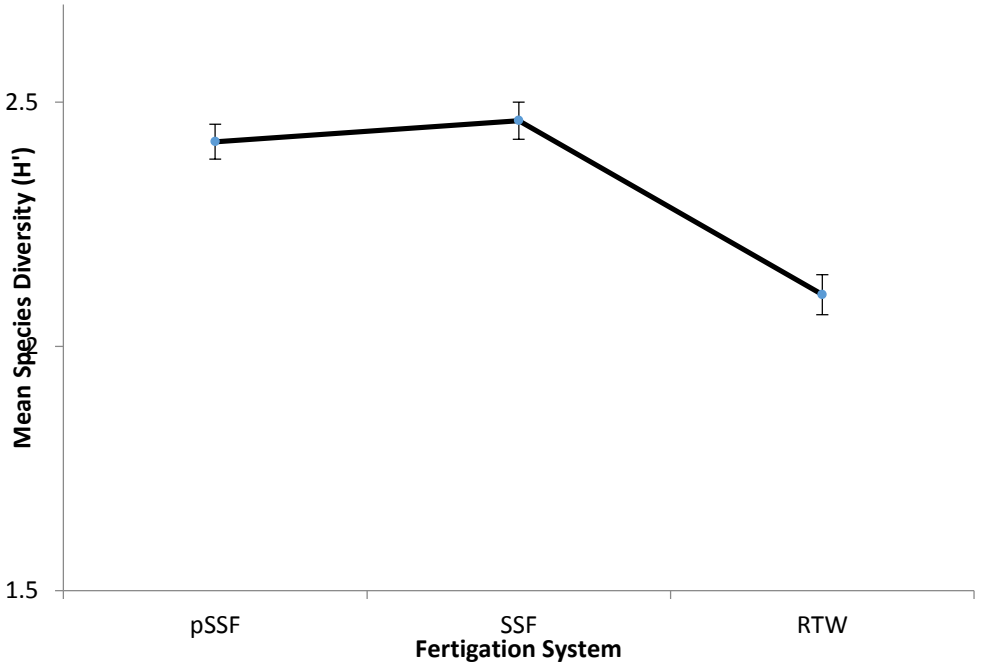


Fig. 3.9. Mean species diversity recorded on tomato roots from three contrasting fertigation systems pSSF, SSF and RTW over the 2012-2013 growing season.

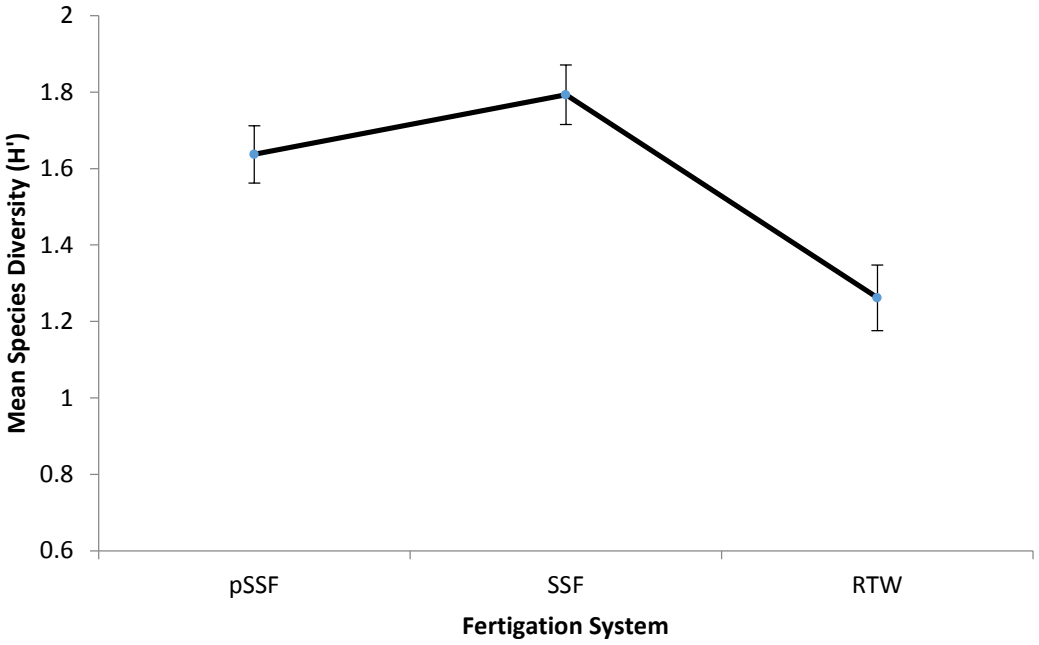


Fig. 3.10. Mean fungal species diversity recorded on tomato roots from three contrasting fertigation systems pSSF, SSF and RTW over the 2012-2013 growing season.

3.4 Discussion

The cumulative taxa richness across the growing season gave a qualitative view on the microarray data and showed reduced numbers of taxa present on the RTW system on root samples and in the fertigation samples conserved across fungi, oomycetes and bacteria (Fig. 3.1). The increased taxa richness observed on the pSSF and SSF systems may give indication towards the re-introduction of microorganisms through the recycling of fertigation solution suggesting some level of inefficacy exhibited by slow sand filtration system. A second conclusion drawn from taxa richness is from the variation in microorganisms observed only on a single system. The pSSF and SSF systems showed three pathogenic and two saprophytic taxa only present on their respective systems and the RTW system showed only 3 unique saprophytes. This shows saprophytic taxa varied more across the systems when compared to the pathogenic and that once introduced, pathogenic taxa tend to persist for longer. This is further supported by five pathogenic species of fungi and oomycetes being recorded at over 50 % of the growing season at all three sites. Only two saprophytic taxa were recorded at over 50 % on all three systems. Moreover, it may indicate the increased potential for slow sand filtration systems to introduce pathogens, due to increased variation in pathogens observed on the pSSF and SSF systems, which may lay dormant in the filtration system. That said the, the origin of the organisms cannot be determined and only a single unique pathogen, *Rhizoctonia solani,* persisted on the pSSF system.

Only two pathogenic species, *Colletotrichum coccodes* and *Verticillium albo-atrum,* that occurred solely on the pSSF and SSF sites showed <1 occurrence. Both increased in prevalence towards the end of the season, which may indicate increased risk from outbreaks of black-dot root rot and Verticillium wilt when using slow sand filtration. One would, however, expect to see increased presence of both at the SSF if this were the case but only *Verticillium albo-atrum* was more common at the SSF with 13 occurrences throughout the year compared to 3 at the pSSF system. *Colletotrichum coccodes,* however, occurred at 10 points across the season at the pSSF system and only 3 at the SSF. This indicates that other factors may be playing a role including the method of pathogen introduction to the nurseries, possible inadequate biological threshold levels occurring at the SSF site preventing the dissemination of *Colletotrichum coccodes* or other environmental conditions that may favour the occurrence of certain pathogens at their respective nurseries.

The most notable observation from Fig 3.1 is the high prevalence of pathogens across all three of the growing systems. Little incidence of disease was recorded

at either of the nurseries giving indication that the Maxifort™ root stock utilised at all three nurseries may provide some level of resistance against the pathogens recorded. This may be expected due to the root stocks vigorous and hardy nature along with its recorded resistance (De Ruiter Seeds, 2012). The ability of the pathogenic species recorded in this study to cause disease, however, cannot be determined. This is due to our inability to discriminate between pathogenic and non-pathogenic isolates of certain species in genera such as the *Fusarium*, *Verticillium, Phytophthora* and *Pythium* via the ITS molecular barcoding region targeted when developing the microarray. Further research is required to determine if the possible pathogenic species commonly recorded on tomato roots carry the necessary pathogenicity factors to cause disease.

The SSF system showed the highest prevalence for three out of four commonly recorded *Pythium* spp. This may indicate that once established in the SSF system the *Pythium* spp. show an increased tendency to persist in comparison to the pSSF and RTW systems. This could be caused by the unsuccessful removal of *Pythium* spp. propagules by slow sand filtration and therefore SSF systems may be at greater risk of *Pythium* spp. associated disease such as damping-off and root rot. The monitoring of fertigation solution directly exiting the slow sand filter, as opposed to the run-off used in this study, may give indication towards the success of slow sand filtration at removing *Pythium* spp. and other microorganisms from the used fertigation solution.

The microbial persistence and abundance data, seen in Fig. 3.2, 3.3 and 3.4 allowed a semi-quantitative view of the taxa across the growing season. As probes may react with their target DNA differentially, the abundance of microorganisms had to be viewed as relative levels of abundance of a single taxa meaning between taxa quantitative comparisons could not be made. Although this reduces the application of the abundance data it still allows any general patterns of abundance to be observed from the taxa.

The *Pythium* spp. showed a general conserved pattern of prevalence and abundance over the growing season on all three of the fertigation systems of sporadic presence on roots until week nine at the pSSF and SSF systems and week 29 on the RTW system, after which *Pythium diclinum, Pythium irregular, Pythium myriotylum* and *Pythium paroecandrum* tended to persist with increasing relative abundance as the growing season progressed. This identified a 20 week difference from the point from which *Pythium* spp. are consistantly recorded on tomato roots on the pSSF and SSF, fertigation solution recycling systems, and the RTW. Such a dramatic difference could be conceived, again,

due to the improper removal of *Pythium* spp. from fertigation solution that has already passed through the nursery. Such improper removal may lead to an accelerated increase of *Pythium* spp. on plant roots. Such an acceleration of dissemination of *Pythium* spp. propagules within pSSF and SSF systems may also vastly reduce the response time available from first visible symptoms of disease and control of disease dissemination, a situation which may be worsened for mature plants due to the non-specific symptoms produced in many *Pythium* related disease.

Furthermore, this pattern of prevalence was shown by a single fungus *Verticillium albo-atrum* at the SSF nursery which may give insight into the mode by which *Pythium* spp. escape removal by slow sand filtration. The main mode of microorganism removal by slow sand filtration is a biologically active bio-film which prevents propagule dissemination through the solution. The mechanical filtration of the slow sand filter mainly functions to remove detritus. The formation of motile zoospores by oomycetes may allow the *Pythium* and other oomycetes to physically traverse the biofilm and re-enter the fertigation solution.

A general observation from Fig. 3.2, 3.3 and 3.4 was also made. The RTW system in general exhibited far less variation in the overall abundances of its community and most peaks in abundance were visible in the first quarter. The pSSF and SSF systems, however, exhibited longer lasted peaks in abundance. For example *Plectospharella cucumreina* and *Verticillium nigrescens* showed peak abundance between weeks 7-35 on the pSSF and SSF systems, Fig. 3.2 and Fig 3.3 but both reduced in abundance after week 15 on the RTW system Fig 3.4. These extended peaks in abundance may be caused by recirculation of propagules at pSSF and SSF systems and possibly also recirculation of nutrients creating an environment that can support greater numbers of organism. This was supported by the quarter of the season in which greatest taxa richness occurred. Quarter two showed the greatest taxa richness for both RTW and pSSF and quarter four for the SSF system. This shows the microbial population continued to grow in richness throughout the season on the SSF but tended to decline on the pSSF and RTW. Decline was observed with greater speed on the RTW system.

Microorganisms present on the roots before introduction to the rockwool slab on the RTW system were more likely to persist for the first half of the season in comparison to the pSSF and SSF systems. The RTW system, however, showed the greatest reduction in persistence in the second half of the season. Both could be supported by the fewer sporadic observations of microorganisms with only 9 taxa occurring fewer than 5 times at the RTW compared to 17 and 18 on the

pSSF and SSF systems, respectively, although the origin of the organisms cannot be determined. The sporadic occurrence of microorganisms on the pSSF and SSF systems at the start of the season may reduce taxa persistence at the start of the season due to competition within a newly established environment. If the origin of the sporadically occurring microorganisms is the recycled fertigation solution, established taxa on the roots may also be re-circulated. This may cause re-distribution of organisms within the root system allowing population of any newly created environments in the rhizosphere and sustaining the populations observed on the pSSF and SSF systems. The SSF, however, was the only site that showed an increased level of average persistence during the second half of the season which indicates although increased sporadic appearances of microbial taxa occurred at both pSSF and SSF sites, the pSSF system may not allow organisms present in recycled fertigation solution to take hold due to inefficient biological thresholds in comparison to the SSF system.

Three bacteria genera showed conserved patterns of abundance across the three fertigation systems. *Nitrospira* spp., *Pseudomonas* spp. and *Xanthomonas* spp. each show high abundance throughout the growing season seeing slight reduction in abundance over the last quarter. The high abundances of γ-Proteobacteria including *Xanthomonas* spp. and *Pseudomonas* spp. can be expected as (Hawkes *et al.*, 2007) reported their dominance within the bacterial communities associated with the rhizosphere across a number of plant taxa (Hawkes *et al.*, 2007).

Species diversity data allowed the identification of two key points. The first of which is the initial drop in diversity after the first week of sampling occurring at each of the nurseries; week 1 for the pSSF and week 3 for SSF and RTW systems Fig. 3.5. The first samples were taken from juvenile plants after delivery from the propagator, before introduction to the rockwool slabs, and were grown in single rockwool cubes per plant. This drop may be observed due to the roots being introduced into a new environment with differing conditions. This change in environmental conditions may not favour all microorganisms present in the rhizosphere thus causing a drop in the diversity. This may be a crucial point in the growing season as the introduction of pathogenic organisms, at this point, may allow them to take hold in the rhizosphere due to an increased availability of nutrients from newly established roots in sterile rockwool slabs and a reduction in the microbial population. Moreover, the systems show differing levels of risk. The pSSF and SSF systems, if using recycled fertigation solution, at this point will have a greater chance of introducing pathogens due to the increased number of taxa observed and sporadic incidence of microorganisms.

The second point of interest is from week 15, at which all three systems exhibit similar diversities, to the end of the season, see Fig. 3.5. From week 17 onwards the systems begin to diverge in respect to their mean diversities observed in Fig. 3.9. These differences in diversity were supported by ANOVA testing which showed significant differences between the pSSF-RTW and SSF-RTW systems. The effect sizes observed, however, although significant were relatively small. The general climb in diversity viewed until week 15, due to its conservation between systems, may be influenced to a greater extent by the plant and from week 15 the differences may be observed due to differences incurred due to differing fertigation systems. The differences arising 15 weeks into the season may give insight into the pSSF and SSF systems. The microbial populations re-introduced to the plant roots may take time to reach a level or biological threshold where it can influence the microbial communities in the rhizosphere. Week was also shown to significantly influence species diversity in a conserved fashion across the three systems by ANOVA testing. The conservation of the variance between the sites points towards the plant and the roots playing a role in determining species diversity across the season allowing different populations to form dependant on the microenvironments and the respective nutrients provided to them by the plant during different stages of the plants development.

The fungal, oomycete and bacterial species diversities were viewed independently to identify where the differences in diversity were occurring within the microbial population, see Fig 3.6, 3.7 and 3.8. Bacterial diversity was analogous between the three fertigation systems with a general decrease in diversity across the season apart from the SSF site which saw a small increase in diversity. This reduction may be explained by the reduction in root exudates exhibited towards the latter stages of the tomato plants life. At the SSF a reduction in root exudates may be counteracted by excess nutrients passing back through into the greenhouse via the recycled fertigation solution allowing the population to remain relatively stable.

The fungal diversity which peaked at week 13 at similar levels of diversity on all three systems, however, showed to play a significant role in determining species diversity between the systems by ANOVA testing. The RTW system exhibited a much greater drop in fungal diversity than the pSSF system and the SSF remained at a consistent level for the remainder of the season. The drop in diversity at the RTW system is also highlighted by the large reduction in taxa persistence over the second half of the season. Oomycete diversity also contributed significantly to the difference seen between the three systems with

the RTW showing reduced levels of diversity when compared to the pSSF and SSF systems, however, a significant difference in oomycete diversity was not found. As discussed before these differences may be observed due to the nature of recycling fertigation solution.

The final observation from the fungal, oomycete and bacterial diversities was conserved between all three sites. The fungi and bacteria played larger roles in determining total species diversity in the initial weeks of the growing season and the oomycetes during the latter stages. This may be due to differences in lifestyle, metabolism or niche specificity. Further investigation is required to determine the origin of the observed variation between diversities to identify if environmental factors such as pH and EC are also influencing the microbial population. Furthermore, at the beginning of the season fungi, oomycetes and bacteria all exhibited differing levels of diversity, however, the differences in diversity appeared to even out in the final weeks of monitoring with similar levels of diversity exhibited by all three groups. This gives some indication that the factors limiting and influencing species diversity differ throughout the season for different taxa and between fertigation systems but with a conserved limiting factor between taxa and also between fertigation systems, possibly the plant itself.

Chapter 4: Identification of Key Differences Between the Rhizosphere Associated Microbial Populations from Tomato Crops Grown Using Recycled Fertigation Solution from Two Physical Treatments

4.1 Introduction

Over the last two decades the production of intensively grown greenhouse tomatoes on soilless systems has increased within the UK, see section 1.1.2. Soilless cultivation traditionally utilised open or "run-to-waste" systems where waste fertigation solution would simply be allowed to run-off causing considerable environmental impact. Government pressure to reduce environmental impact within the greenhouse industry has seen a move towards the use of closed systems that recycle their fertigation solution, preventing the release of nitrates and other fertiliser components escaping into the environment. Recycled fertigation solution is treated to prevent the dissemination of any pathogenic microorganisms and is achieved by the use of biological filters such as the slow sand filter and also physical treatments such as heat and UV light which cause loss of organism viability. Heat and UV can both target a wide range of organisms and have shown their success in laboratory situations but their effect on the microbial population of the rhizosphere in a commercial situation has been little studied, see section 1.3.1.1.

In this study we aim to highlight any key difference observed in the microbial population of the rhizosphere associated with the treatment of fertigation solution utilising heat and UV based physical treatments.

4.2 Methods

Two crops grown at a nursery with two differing physical treatments for fertigation solution recycling were monitored at four points, in two month increments, during the growing season. Both crops were grown on Emperador root stocks. The remaining methods follow the structure discussed in section 3.2.

4.3 Results

4.3.1 Microbial Richness, Relative Abundance and Occurrence

A number of taxa were not observed from either roots or fertigation solution samples from crops on the contrasting systems, see Table 4.1. In addition to Table 4.1 the nematode species *Meloidogyne incognita and Pratylenchus goodeyi* were also not observed. Furthermore, two species were only observed on roots from the heat treated system both of which, *Exophiala pisciphila* and *Myrothecium roridum,* were saprophytic fungi, see Fig. 4.1. Four taxa, *Alternaria solani*, *Aspergillus flavus*, *Exophiala xenobiotica* and *Trichoderma* spp., were only observed on the UV system all but *Alternaria solani* are saprophytic fungi.

Table 4.1 The taxa not observed from root or fertigation solution samples on either of the crops grown on systems utilising contrasting physical treatments for fertigation solution recycling.

Pathogenic Fungi and Oomycetes	Saprophytic fungi	Bacteria
Armillaria mellea	*Alternaria spp.*	*Bacillus amyloliquefaciens*
Colletotrichum acutatum	*Aspergillus terreus*	*Bacillus subtilis*
Colletotrichum coccodes	*Aspergillus ustus*	*Escherichia coli*
Didymella lycopersici	*Cadophora spp.*	*Pseudomonas syringae,*
Fusarium solani	*Cephalosporium spp.*	*Rhizobium spp.*
Phytophthora arecae	*Chaetomium cochliodes*	*Yersinia spp*
Phytophthora cinnamomi	*Chaetomium spp.*	
Phytophthora cryptogea	*Doratomyces microspores*	
Pythium aphanidermatum	*Gigaspora rosea*	
Pythium arrhenomanes	*Gliocladium roseum*	
Pythium debaryanum	*Glomus intraradices*	
Pythium echinulatum	*Myrothecium roridum*	
Pythium megalacanthum	*Paecilomyces lilacinus*	
Pythium oligandrum	*Penicillium chrysogenum*	
Pythium torulosum	*Penicillium griseofulvum*	
Rhizoctonia solani	*Penicillium variabile*	
Thielaviopsis basicola	*Phoma spp.*	
Verticillium nigrescens	*Rhizopus oryzae*	
	Trichoderma harzianum	

A number of organisms were also only present in the fertigation solution from a single site, the majority of which were pathogens, with six and eight unique taxa at the heat and UV systems, respectively, Fig. 4.1 .The unique taxa from fertigation solution samples at the heat treated site include the pathogens

Colletotrichum coccodes, Pythium paroecandrum and *Clavibacter michiganensis* along with the saprophytes *Exophiala pisciphila*, *Olpidium brassicae* and *Penicillium brevicompactum*. Unique taxa from the UV site include the pathogens *Agrobacterium tumefaciens, Alternaria solani, Colletotrichum acutatum, Fusarium oxysporum, Fusarium redolens, Phytophthora nicotianae, Pyrenochaeta lycopersici* and the saprophyte *Aspergillus sydowii.*

The two systems exhibited similar levels of richness with 28 and 30 taxa being recorded on the roots and 20 and 21 taxa in the fertigation solution from the heat and UV systems, respectively, Fig. 4.1. The occurrence of fungal and oomycete pathogens was increased in comparison to fungal saprophytes with and average occurrence on roots of 2.46 and 2.00 on the heat treated system and 2.43 and 1.50 at the UV site, correspondingly, out of a maximum of four occurrences. Bacterial average occurrence was 3.00 and 3.16 at the heat and UV sites with most bacterial taxa persisting on roots across the growing season. The average occurrence of fungal and oomycete pathogens in the fertigation solution was 2.00 and 1.00 at the heat treated site and 1.72 and 1.5 at the site utilising UV, respectively. Mean bacterial occurrence was 2.16 and 2.00 at the heat and UV sites, respectively.

Patterns in relative abundance from root samples, section a Fig 4.1, were conserved across most organisms and growing systems showing a peak in weeks 15 and 23 at which point peak richness was also observed at 17 and 25 for the heat treated system and 20 and 22 for the UV systems, respectively. The most severe peaks in abundance at weeks 15-23 were observed for *Aspergillus flavus, Penicillium spp., Plectospharella cucumerina* and *Verticillium nigrescens* for both the heat and UV systems, see Table 4.2

Table 4.2 The taxa exhibiting peak abundance, from root samples, during weeks 15 and 23 on both the Heat and UV treated systems showing mean abundance for weeks 15 and 23 and the remainder of the season.

Taxa	Heat		UV	
	15 & 23	3 & 31	15 & 23	3 & 31
Aspergillus flavus	1.19	0.22	1.06	0.19
Penicillium spp.	1.94	0.52	1.73	0.84
Plectospharella cucumerina	2.80	1.10	2.08	0.78
Verticillium nigrescens	2.47	0.80	2.00	0.70

This, however, was generally not observed in the *Pythium* spp. which tended to show peak abundance in the final two weeks of monitoring, 23 and 31, on both growing systems for root and fertigation solution samples, see Fig.4.1 and Table 4.3. This also highlights an increase in the mean abundance of *Pythium* at the UV treated site at 0.33 for the first half of the season and 1.13 for the second half in comparison to 0.04 and 0.63.

Table 4.3 The mean abundance of commonly occurring *Pythium* spp., for weeks 7-15 and 23-31, from root samples on both the Heat and UV treated systems.

Taxa	Heat		UV	
	7-15	23-31	7-15	23-31
Pythium diclinum	0.00	0.42	0.27	0.73
Pythium irregulare	0.12	0.88	0.37	2.55
Pythium myriothylum	0.00	0.58	0.34	0.12

Finally, the bacteria generally showed consistent levels of abundance across the season from both root and fertigation solution samples. A general observation of decline in abundance (see Fig. 4.1), during the latter weeks of the season, for the commonly occurring bacteria, *Pseudomonas* spp., *Nitrospira* spp. and *Xanthomonas* spp. is highlighted by drops in mean abundance from weeks 7-15 of the season at 1.44 and 1.54 to weeks 23-31 at 1.12 and 1.07 for the heat and UV systems, respectively.

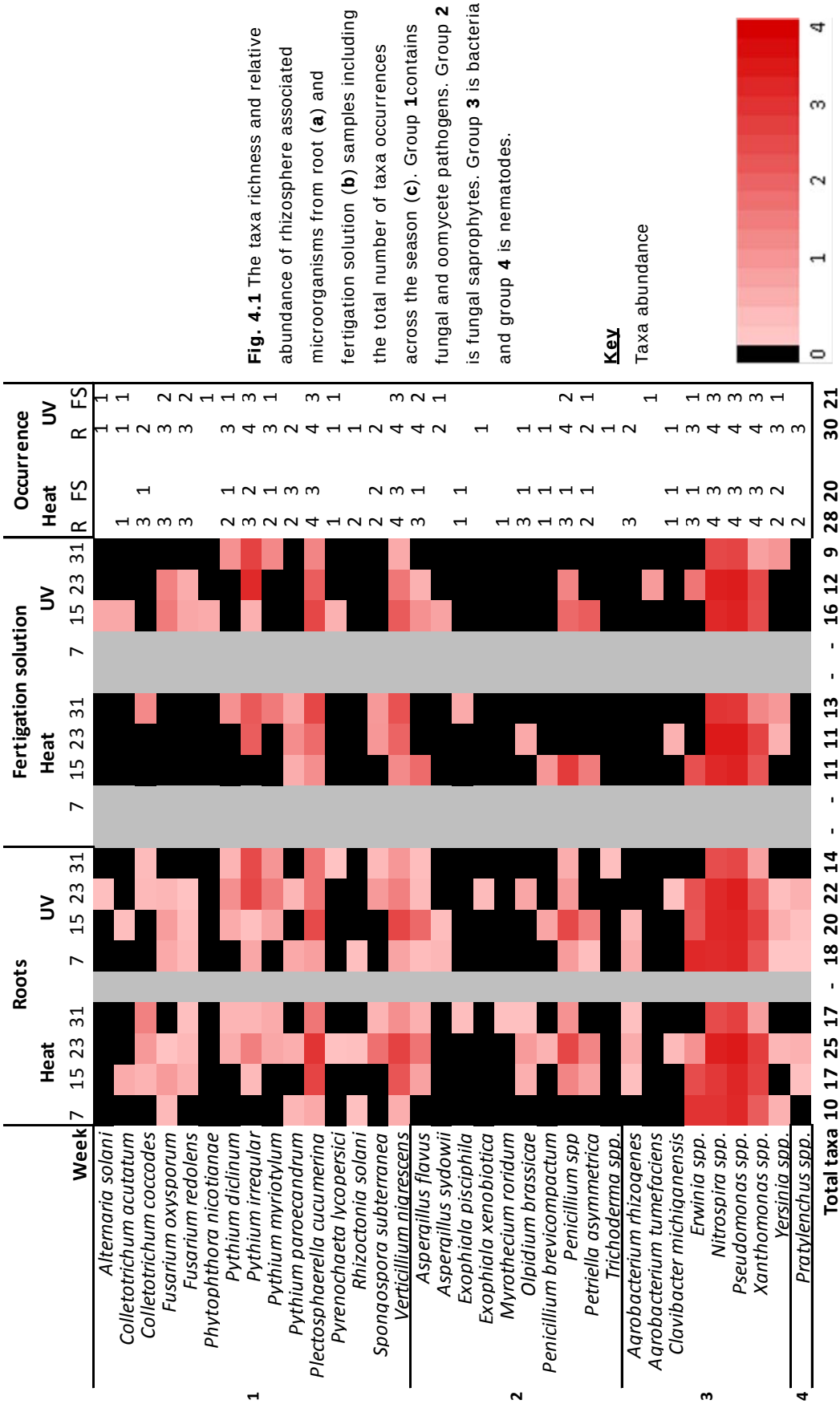


Fig. 4.1 The taxa richness and relative abundance of rhizosphere associated microorganisms from root (**a**) and fertigation solution (**b**) samples including the total number of taxa occurrences across the season (**c**). Group **1**contains fungal and oomycete pathogens. Group **2** is fungal saprophytes. Group **3** is bacteria and group **4** is nematodes.

4.3.2 Species Diversity

There was very little difference observed between the mean species diversities exhibited on root samples between the contrasting systems Fig. 4.2. The greatest difference was observed at week 7 where the heat treated system exhibited a mean diversity of 1.65 and UV 2.08. The mid two diversities, weeks 15 and 23, showed little divergence with differences of 0.10 and 0.04, respectively. The final week of study showed a slight drop at the UV site in comparison with the heat with final diversities of 2.01 and 2.22 respectively.

When the mean diversity of fungal species was viewed separately, however, increased levels of divergence were seen Fig. 4.3. The heat system showed a much lower fungal diversity at week 7 (0.17) which rose to 1.49 in week 15 and then showed a gradual increase across the final two observations at week 23 and 31 with diversities of 1.58 and 1.62, respectively. The UV system showed increased diversity at week 7 with a diversity of 1.18. This also increased to week 15 (1.82) and then declined to week 31 exhibiting a considerably lower diversity than the heat system with 0.94 compared to 1.61.

Mean oomycete diversity also showed differential patterning at the contrasting systems Fig 4.4. Oomycete diversity on the roots of the crop grown on the UV system showed an increased level of diversity across the entire season with a mean diversity of 1.36 in comparison with the heat system which exhibited a mean diversity of 0.35 over the season. Different patterns in oomycete diversity were also observed with diversity increasing from week 7-15 by 0.15 and then declining by 0.67 to week 31 at the UV site. The heat treated system, however, showed no change in diversity between weeks 7 to 15 remaining at 0.00 and then increasing to week 31 with a final diversity of 0.69. Bacterial species diversity was similar at both sites with average diversities of 1.40 and 1.35 for the heat and UV site, respectively. A general pattern decline can be seen in bacterial diversity dropping by 0.38 and 0.46 from week 7-31 for the heat and UV sites, respectively, see Fig 4.5. The main variation arising between bacterial diversity was observed at week 23 where the heat system exhibited a diversity of 1.64 in comparison to the UV system with 1.36.

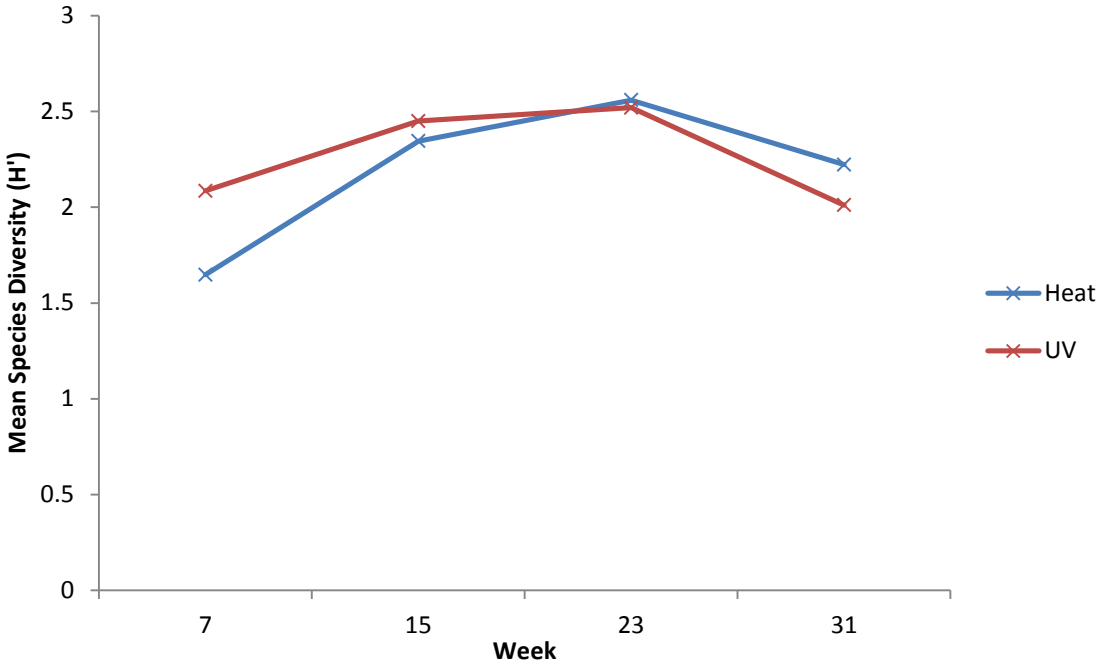


Fig. 4.2. Mean species diversity of root samples taken from two contrasting physical treatments across the 2012-2013 growing season.

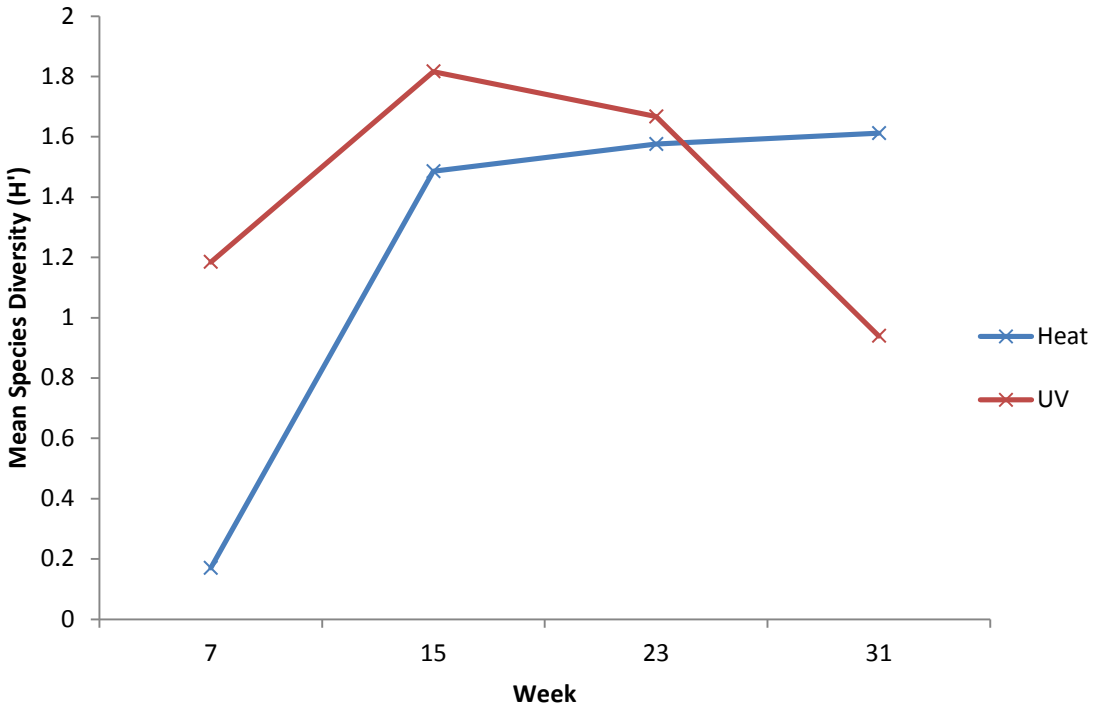


Fig. 4.3. Mean fungal species diversity of root samples taken from two contrasting physical treatments across the 2012-2013 growing season.

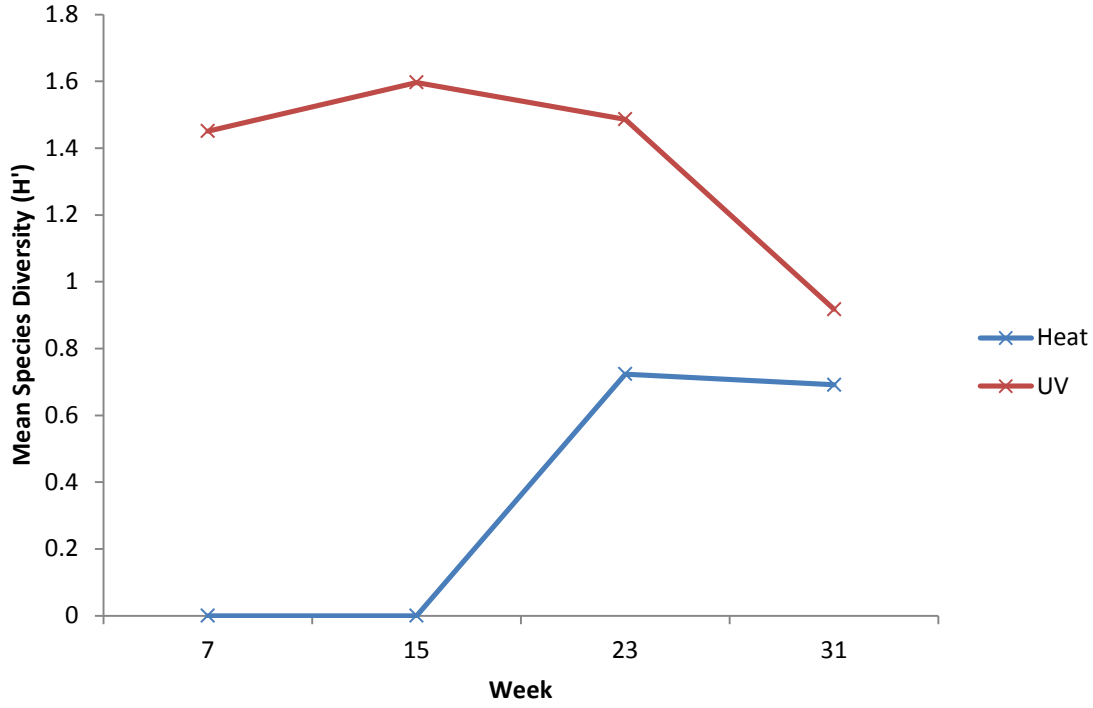


Fig. 4.4. Mean oomycete species diversity of root samples taken from two contrasting physical treatments across the 2012-2013 growing season.

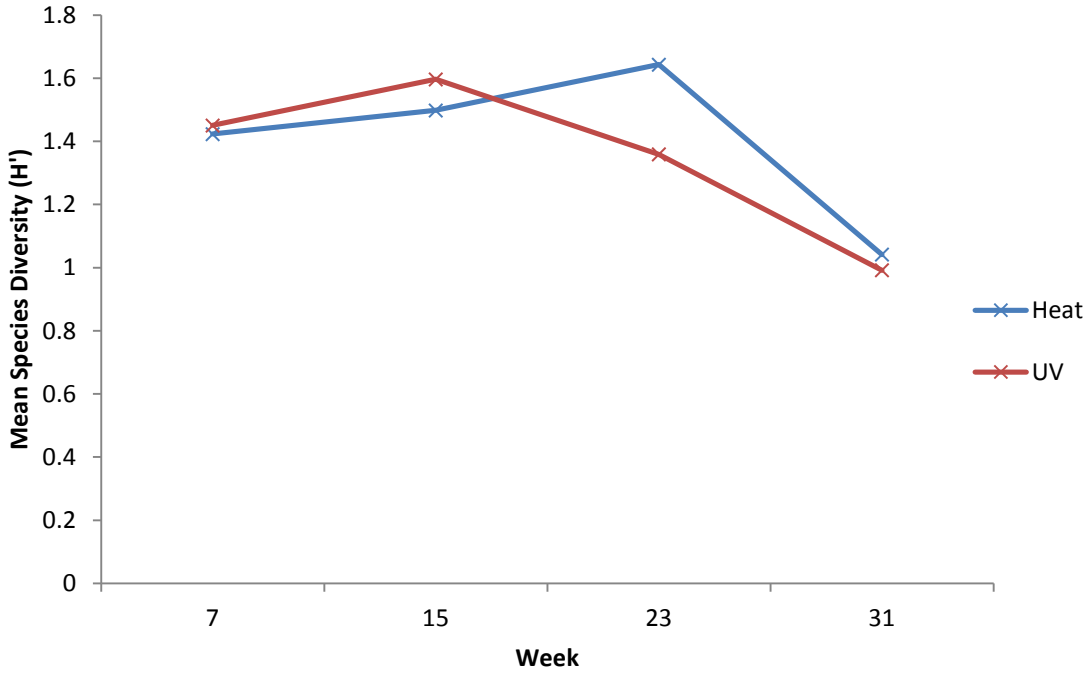


Fig. 4.5. Mean bacterial species diversity of root samples taken from two contrasting physical treatments across the 2012-2013 growing season.

4.3.2.1 Analysis of variance

Analysis of variance identified only one significant difference between the two physical treatments which occurred for the oomycete diversity giving result $F(1,27) = 54.175, 0.136, p<0.001$ (F critical= 4.21) and shows a moderate size of effect at 0.667. Week was shown to cause significant variation in diversity for all four groupings. The largest effect was observed for the bacterial diversity with $N^2= 0.766$ with results $F(3,27) = 29.481, 0.014, p<0.001$. Post hoc tests allowed the identification of three significant differences in diversity between week 31 and 7, 15 and 23 with $p<0.001$ for all differences, see Table 4.4. The fungal and oomycete population was affected less severely by week with effect sizes of 0.429 [$F(3,27) = 6.762, 0.259, p=0.002$] and 0.305 [$F(3,27) = 3.941, 0.136, p= 0.019$], respectively. The total diversity was also moderately affected by week in the growing season with an effect size of 0.613 [$F(3,27) = 14.244, 0.051, p<0.001$]. *F* critical for all week based ANOVAs was 2.96.

Table 4.4 A summary of the significant results from ANOVA testing used to analyse variance in microbial diversity observed, across the 2012-2013 growing season, on tomato roots from nurseries utilising two contrasting physical treatments, heat and UV for the disinfestation of recycled fertigation solution.

Taxa	Sample	Dependant Variable	P	N^2	Post Hoc		
					Direction	P	Mean difference (±)
Total	Root	Week	<0.001	0.613	7<15	0.001	0.498
					7<31	<0.001	0.684
					23>31	0.002	0.457
Fungal	Root	Week	0.002	0.429	7<15	0.003	1.009
					7<23	0.004	0.974
Oomycete	Root	Treatment	<0.001	0.667	-	-	-
		Week	0.019	0.305	7<15	0.034	0.538
					23>31	0.045	0.514
Bacterial	Root	Week	<0.001	0.766	7>31	<0.001	0.453
					15>31	<0.001	0.480
					23>31	<0.001	0.438

4.4 Discussion

Little difference was observed between the microbial populations associated with the rhizosphere from crops grown utilising recycled fertigation solution undergoing two contrasting physical treatments, heat and UV, for disinfestation. Taxa richness, from both root and fertigation solution samples, of fungal and oomycete pathogens, fungal saprophytes and bacteria from both sites were similar and both showed increased occurrence of pathogenic fungi and oomycetes in comparison to saprophytic taxa. Saprophytic taxa recorded on roots, however, were more likely to be unique to a treatment system whereas most pathogenic taxa recorded were present on roots at both systems.

Fertigation solution samples, conversely, showed more unique pathogens predominantly on the UV system. This, however, is not supported by root data and may be due to the detection of non-viable organisms which could be expected at increased levels in the UV system due the nature of the physical treatment as heat treatment may break down DNA from non-viable organisms. Additionally, bacterial occurrence was increased in comparison to fungi and oomycetes and as discussed previously in section 3.3 this can be expected due to the high abundance of of γ- Proteobacteria (*Xanthomonas* spp. and *Pseudomonas* spp.) within the rhizosphere.

Most of the recorded organisms, apart from the *Pythium* spp. also showed conserved patterns of abundance with peaks during the mid-points during the season. Peaks in the mid-season may be caused by continuing plant growth and increased exudates from the roots allowing growth of the microbial population in the rhizosphere. The *Pythium* spp., however, showed increased abundance in the final two points of monitoring and may be due to sub-optimal environmental conditions during the first half of the growing season or differences in lifestyle and nutrient acquisition.

Species diversity was also consistent between plants grown on the contrasting systems. When fungal, bacterial and oomycete diversities were viewed independently, the fungal and oomycete diversities showed to contribute more towards the variation observed in the total diversity. Oomycete diversity was shown to exhibit a significant difference between the two sites with a treatment shown to have a moderate effect on species diversity through ANOVA testing. This may infer a difference in the efficacy of physical treatments in the disinfestation of oomycetes with an increased diversity of oomycetes being observed in the UV system. In section 3.3.3, however, *Pythium* spp. tended to make up most of the oomycete population and showed sporadic appearances on roots throughout the earlier months in the growing season. Such sporadic

appearances may skew the data due to the limited number of observations during the growing season as it is unknown if the organism persisted for more than just the single week of observation.

Week was shown to significantly affect the entire microbial population of the root samples monitored in this study when diversities were viewed as a total population and phylogenetically grouped populations. These, as discussed earlier, may be due to morphological and physiological changes occurring at the plant roots but also environmental conditions such as; EC, pH and relative humidity; require further investigation to determine their effect on the rhizosphere microbial community.

To conclude, more similarities than differences were observed between the rhizosphere related microorganisms recorded on tomato crops grown on two systems utilising heat and UV for the disinfestation of recycled fertigation solution. To analyse the possible risks posed by these systems further analysis is required with monitoring at an increased frequency to determine at which site pathogens are more likely to persist.

Chapter 5: Development of an In-House Disease Diagnostic Kit Utilising Loop-Mediated Isothermal Amplification (LAMP)

5.1 Introduction

Greenhouse tomatoes grown on soilless substrates are open to attack from a plethora of pathogens including *Colletotrichum coccodes* and *Fusarium oxysporum* f.sp. *lycopersici* causing black-dot root rot and Fusarium wilt, respectively, a comprehensive list can be seen in section 1.5.1. Additionally, once symptoms have been observed a limited number of control methods are available for only a few diseases, see section 1.6 for more details. Rapid identification of the causal agent is key as quick removal of infected plants is often the only option. Classical identification, in some cases, can be a laborious and time consuming and new molecular methods that utilise a single established barcoding region such as the ITS region cannot always discriminate between closely related pathogenic and non-pathogenic species. To overcome this, novel molecular markers need to be identified and targeted for rapid on-site diagnosis.

Established methods such as Q-PCR are used for in-house real-time viewing of diagnosis but are energy inefficient, bulky, require thorough extraction of DNA and complicated training is required. An emerging technique; loop-mediated isothermal amplification allows highly specific and rapid detection of organisms in an end to end process that can be achieved in under an hour including DNA extraction, see section 1.7.3.

In this study key organisms are identified, for in-house rapid detection, which would benefit commercial tomato growers. LAMP assays are then developed and validated for their detection.

5.2 Methods

Meetings with commercial tomato growers were undertaken to identify any key organisms for which to develop LAMP assays for. Possible molecular markers were then identified through literature research. Where appropriate, if sequences were only available from mRNA, PCR primers were designed to identify any introns present in the genomic DNA following the procedures in section 2.2.1. Genomic DNA sequences were then used to design the LAMP assays following the protocol in section 2.2. The designed LAMP primers were then self-validated and validated with closely related taxa and taxa commonly occurring on tomato.

5.3 Results

Knowledge transfer meetings allowed the identification of six pathogens to be targeted by LAMP assays including; *Colletotrichum coccodes, Verticillium albo-atrum, Fusarium oxysporum, Thielaviopsis basicola, Pyrenochaeta lycopersici* and *Botrytis cinerea*

5.3.1 LAMP Primers

Prior to this study LAMP primers were designed to target the ITS region of *Pyrenochaeta lycopersici* and species within the genus *Verticillium*. Two sets of primers were identified from heterologous regions that would allow their specific detection, see Table 6.1.

Table 5.1 The LAMP primers developed prior to this study for the detection of *Pyrenochaeta lycopersici* and the *Verticillium* genus using the ribosomal ITS as a target.

Pathogen	Marker	Primers
Pyrenochaeta lycopersici	ITS	F3 - GCGTCATTTGTACCCTCAA
		B3 – CTTAAGTTCAGCGGGTATCC
		FIP(F1c+F2) – TGCGCTCTATAACCAGATTGCCTGGTGTTGGGTGTTTGTC
		BIP(B1c+B2) – TTCTTGCCACGGATGTCGGCCTGATCCGAGGTCAAGA
		LoopF - CCAATTGCTTTGAGGCGAG
		LoopB -CGTCCATCAAGCCTACACTT
Verticillium spp.	ITS	F3 – CAGTATCCTGGGAGGCAT
		B3 – ATTCCTACCTGATCCGAGG
		FIP(F1c+F2) – TCGGTCCGCCACTGATTTTAAGGTCGTTTCAACCATCTCGA
		BIP(B1c+B2) - TTTCGGTCGCATCGGAGTCTACGAGACGGGCTTGTAG
		LoopF – CAGAAGTAGATCCCCAACACC
		LoopB - GCACCAGCCTCTAAACCC

5.3.1.1 *Pyrenochaeta lycopersici*

A possible molecular marker for *Pyrenochaeta lycopersici* targeted LAMP assay was identified in literature based research. A 35kDa protein CDiT1 was identified in a paper by Clergeot *et al.* 2012. A GenBank query was submitted which returned an mRNA sequence of *CDiT1* accession HE615138.1. A BLAST search performed the *CDiT1* mRNA sequence returned no results. PCR primers designed from the mRNA sequence, see Table 5.2, showed optimal amplification with an anneal temperature of 61.1 °C and 1 µl of each primer per reaction, see Table 5.3. Purified PCR products from genomic DNA allowed the identification of an intron at position 259 of the mRNA sequence 81 nt in length, see Fig 6.1. The retrieved genomic sequence allowed the design of a LAMP assay to target the *CDiT1* gene, see Table 5.4.

Table 5.2 PCR primers designed from the *Pyrenochaeta lycopersici CDiT1* mRNA.

Primer	Sequence	Position	GC %	Tm °C
PlyCDiT1F	CGTCCTGGCTTTCGCTAG	30-47	61.1	63.7
PlyCDiT1R	GGAGTAATCAAGACCGGGTTC	543-563*	52.3	63.5

*Position of the reverse complementary sequence

Table 5.3 *Pyrenochaeta lycopersici* PCR primer optimisation results.

Anneal temperature	Amplification at 0.5 µl	Amplification at 1 µl
63.0	+	+
62.3	+	++
61.1	++	+++
59.3	±	+
57.0	±	±
55.3	±	±
54.0	±	±
53.6	±	±

Key: - no amplification, ± very low level amplification, + low level amplification, ++ good amplification, +++ high levels of amplification

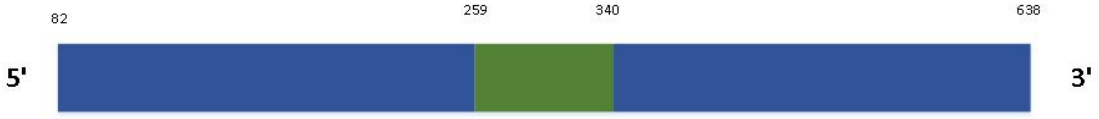


Fig. 5.1. Positioning of the intron identified from the genomic sequence of the *CDiT1* gene from *Pyrenochaeta lycopersici* showing the introns relative positioning within the mRNA sequence.

Table 5.4 The *Pyrenochaeta lycopersici CDiT1* targeted LAMP primers

Pathogen	Marker	Primers	Position
Pyrenochaeta lycopersici	CDiT1	F3- AGGCATCGTAAGTATCTCC	198
		B3- CCCTGGTAAAGAACATCCG	459
		FIP(F1c+F2) – CATGTGCGGCCAAG AGGAACAGCCAGCTAACCAATG	(339/263)
		BIP(B1c+B2) – GCGGCTACTGGAC TGTGACGAAATCCGTGGTCGAGAAA	(360/418)
		LoopF – CTTCGTGAACCACTTAGCCT	313
		LoopB – CGTTCTCGAAGGCACCAA	379

5.3.1.2 *Fusarium oxysporum* f.sp. *lycopersici*

Molecular markers were identified for the race specific identification of *Fusarium oxysporum* f.sp. *lycopersici* through reviewing literature from Houterman *et al.*, 2008 and Inami *et al.*, 2012. Utilising *SIX1, SIX4* and polymorphisms in *SIX3* may allow the specific identification of races, see Table 5.5.

Table 5.5. Race specific identification of *Fusarium oxysporum* f.sp. *lycopersici* through targeting the molecular markers *SIX1, SIX3* and *SIX4.*

	Race1	Race 2	Race 3
SIX1	+	+	+
SIX3*	+	+	
SIX4	+		

**SIX3* is present in race 3 but contains polymorphisms which may be target

Submission of GenBank queries returned genomic sequences for all of the molecular markers identified in the literature review, see Table 5.6. *In sillico* analysis of the sequences allowed the identification of homologous regions between isolates for *SIX1. SIX1* sequences from accessions AJ608703.3 and GQ268948.1 gave an 855nt region of 100% homology, the entire length of GQ268948.1, when aligned. Thus the latter accession was used to design *SIX1*

specific primers. *SIX3* reference sequences, see Table 5.6 for accessions, gave an alignment with three point mutations; G121A, G134A and G137C; see Fig. 5.2.

Only a single genomic sequence was available for *SIX4.* Suitable primer sets were identified for all molecular markers targeted, see tabe 5.7. The *SIX3* primers were able to target the single nucleotide polymorphisms, highlighted in Fig 5.2, at the 5' of B1c region of the BIP primer in race 3 isolates 14844 and IP03 accessions GQ268956.1 and GQ268955.1, respectively. The final race 3 isolates (FOL-MM10) polymorphism could not be targeted by any primer sets reviewed.

Table 5.6 The sequences retrieved through GenBank query submissions of the molecular markers identified for race specific identification of *Fusarium oxysporum* f.sp. *lycopersici.*

Gene	Accession	Gene	Origin	Length (nt)
SIX1	AJ608703.3	*SIX1/FOT5/SHH1*	Genomic DNA	14531
	GQ268948.1	*SIX1*	Genomic DNA	855
SIX3	GQ268950.1	*SIX3*: Isolate BFOL-51 (race 1)	Genomic DNA	492
	GQ268955.1	*SIX3*: Isolate 14844 (race 3)	Genomic DNA	492
	GQ268956.1	*SIX3*: Isolate IP03 (race 3)	Genomic DNA	492
	GQ268957.1	*SIX3*: Isolate FOL-MM10 (race 3)	Genomic DNA	492
SIX4	AM234064.2	*SIX4*: Exons1-2 including intron	Genomic DNA	2085

Fig 5.2. The positioning of the single nucleotide polymorphisms observed between three race 3 isolates and a race 1 isolate of *Fusarium oxysporum* f.sp lycopersici.

Table 5.7. The LAMP primers designed for race specific identification of *Fusarium oxysporum* f.sp. *lycopersici* .

Race	Target	Primers	Position
1, 2 & 3	SIX1	F3 - CTCAATCCTTGGGTTTGGG	36
		B3 - CCTGGAGCATGTCTTGTG	340
		FIP(F1c+F2) – AGGGCAAATCACTCTTGTCTGGAACTT GACCTACACGGAATATC	(175/100)
		BIP(B1c+F2) –CCTGGAAACGAGACGGACGAGGTTGACA ATGTGACCAC	(199/275)
		LoopF - GATGCTGCCACCTTATCCA	140
		LoopB - ATGGTGTCCAGACCGAGA	218
1 & 2	SIX3	F3- TTCCTTCTGCTTATAGCCATG	7
		B3- AATGCGGTGATTCAGTCC	255
		FIP(F1c+F2)- CTTGGAGCTGACCGACAGATGTGGGTTT GCTCTATTGCTG	(100/37)
		BIP(B1c+F2) - CGCCGCACGTCTTCTACTTGTCTCTGTGTA ACATTCTAGCA	(133/210)
		LoopF- AATCGGCATCTTCCACAGG	79
		LoopB- TTACTACGAGCTTCAGCACC	155
1	SIX4	F3 - GAATCTCAAGGCACTCGTT	3
		B3- ACGAGATTGGAGCCTAAGT	231
		FIP(F1c+F2) –GTTCCGATGATGTCACCCTCCGTTATTGC TTCGGTGGCT	(102/22)
		BIP(B1c+F2) –CTCAGACAGTCAGCCGCTTAAGACATCTG AAGCCATTGACT	(102/177)
		LoopF- CCCTTTGGAAGAGCAGATGT	62
		LoopB- AGATACTCCGGATAGTTCAGG T	135

5.3.2 LAMP assay Validation

The *Verticillium* spp. primers targeting the ITS region showed no amplification for *Verticillium dahliae* var. *longisporium, Verticillium nigrescens* or *Verticillium lecanii.* DNA from *Verticillium albo-atrum* was unavailable at the time of testing. The assay targeting the ITS of *Pyrenochaeta lycopersici*, however, did show successful amplification, see Table 5.8, at times between 23 minutes and 45 seconds and 28 minutes and 15 seconds. Four of the melt temperatures

exhibited were between 90.29 °C and 90.74 °C. Two melt temperatures, however, were lower at 88.80 °C and 88.72 °C. When validated against closely related species the primers also showed amplification for a number of species, see Table 5.8, with similar and preferential amplification times with *Cladosporium cladosporide* amplification occurring at 19 minutes and 30 seconds and *Alternaria brassicola* and *Alternaria alternata* both at 29 minutes and 30 seconds. The melting temperatures of the closely related taxa were similar with all samples exhibiting temperatures between 88.91 °C and 89.24 °C.

Table 5.8 LAMP assay validation results

Primer target	Organism	Amplification (minutes)	Melt (°C)
ITS	*Pyrenochaeta lycopersici*	28.15	90.29
		23.45	90.64
		25.30	90.40
		27.30	88.80
		26.30	90.74
		25.15	88.72
	Alternaria tenuissima	22.45	-
	Alternaria brassicola	29.30	88.98
	Alternaria alternata	29.30	89.13
	Cladosporium cladosporide	19.30	89.24
	Trichoderma harzianum	29.30	88.91
ITS	*Verticillium dahliae* var. *longisporium*	-	-
	Verticillium nigrescens	-	-
	Verticillium lecanii	-	-

5.4 Discussion

The LAMP assays designed prior to the study, targeting the ITS region, may both be inappropriate for use. The *Verticillium* spp. assay failed to show amplification for any species tested and further validation against *Verticillium albo-atrum* is required. The failure to amplify products may be due to a number of reasons, the first of which may be the quality of the sequence used to design the primers. If the sequences used were of poor quality then any misread bases

may lead to improper priming of the reaction and failure to produce the double-ended hairpin-like structure essential to amplification. Secondly, the primers may exhibit regions of homology causing the formation of primer dimers and preventing binding to the template DNA. Finally the GC content may be inadequate, preventing primer-template binding at 63 °C. Further investigation is required to determine if a lower reaction temperature may allow amplification.

The *Pyrenochaeta lycopersici* ITS targeted assay may also be unsuitable for specific detection of the pathogen. Amplification was also observed for a number of closely related species which also exhibited melting temperatures that overlapped with that exhibited by *Pyrenochaeta lycopersici* samples. *Pyrenochaeta lycopersici* may therefore not be distinguished by melt temperature. Only two melting temperatures observed by *Pyrenochaeta lycopersici* (88.80 °C and 88.72 °C) were observed within the range of the other organisms used during validation and may be due to contamination. Performing a greater number of repeats may permit the discrediting of the results observed and allow specific *Pyrenochaeta lycopersici* detection utilising melt temperature analysis. If the two lower melt temperatures recorded are dismissed the primers, however, require further validation which could not be completed during this study due to time constraints. Unfortunately molecular markers for all the organisms highlighted during knowledge transfer could not be identified and furthermore the primers designed in this study were also not validated both due to time constraints.

The *Pyrenochaeta lycopersici* secreted effector *CDiT1* has been shown to play a role in pathogenicity in tomato (Clergeot *et al.,* 2012). Sequencing of the genomic *CDiT1* allowed the design of a LAMP assay and BLAST analysis of the mRNA sequence and genomic DNA sequence, including intron, only returned the original sequence. This gives indication that it may provide a good molecular marker for the specific detection of *Pyrenochaeta lycopersici*, however, putative orthologs have been identified in closely related species within the Pleosporales (Clergeot *et al.,* 2012).

Finally, the assay designed to detect specific races of *Fusarium oxysporum* f.sp. *lycopersici* may also have large applications for pathogen detection in tomato greenhouses due to the nature of resistance present in differing commercial cultivars. The ability to discriminate not only between pathogenic and non-pathogenic *Fusarium oxysporum* but also the races would be a huge advantage due to the ubiquitous nature of the species. The *SIX1* assay may provide identification of all three races and *SIX4* of only race 1. The *SIX3* based assay relies on three single nucleotide polymorphisms occurring in differing

isolates. Two of which are target by their positioning at the 5' of the B1c component of the BIP primer and thus hypothetically resulting in the improper formation of the double-ended hairpin-like structure, preventing amplification, and allowing differentiation between races 1 and 2 and race 3. The third isolate identified with a single nucleotide polymorphism at G121A, however, could not be targeted. This polymorphism may possibly be detected via product melt analysis due to the change in GC content of the product.

Summary, Final Conclusions and Future Research

Chapter five was focused on the development of rapid in-house LAMP assays for the detection of pathogens from which commercial tomato producers would benefit. Assays targeting the ribosomal ITS region of *Pyrenochaeta lycopersici* and *Verticillium* spp. may be inappropriate for their specific detection. The *Verticillium* assay failed to detect a number of *Verticillium* species but further testing is required. The *Pyrenochaeta lycopersici* assay showed amplification during self-validation but also with a number of closely related species which exhibited similar amplification times and melting temperatures to that of *Pyrenochaeta lycopersici* and so could not be differentiated via melt analysis. Further assays were developed for *Pyrenochaeta lycopersici* targeting the *CDiT1* gene and for race specific detection of *Fusarium oxysporum* f.sp. *lycopersici* via targeting of the *SIX1, SIX3* and *SIX4* genes. Assay testing and validation was not performed due to time constraints.

Further research is required to design and develop assays based on the remainder of the pathogens highlighted by commercial tomato growers. Upon assay development and validation, however, LAMP may provide rapid real-time pathogen detection which could be included in the integrated pest management schemes implemented by the modern tomato grower. This is due to a number of factors including; ease of use, requiring little training; the ability to store ready-to-use reaction mixtures for prolonged periods of time; portability through the use of battery powered technologies such as the Genie II (Optigene) and the rapid and specific detection of pathogens in an end-to-end process which can be achieved in under an hour.

Chapter three and four utilised a small scale microarray to monitor the rhizosphere associated microbial populations of tomato crops grown on soilless systems. Chapter three was focused on monitoring crops grown on three contrasting fertigation systems, pSSF, SSF and RTW, and a number of key observations were drawn which I shall now summarise.

A reduced number of taxa were observed on the RTW system and a significant difference in species diversity was found with lower species diversity being observed on the RTW system in comparison to the pSSF and SSF system. Species diversity showed a conserved drop at all three sites after the introduction of juvenile plants. Additionally, week during the growing season was shown to significantly affect the total, fungal and oomycete species diversities. Fertigation system, however, only significantly affected the total and fungal species diversity. Taxa showed less variation in abundance across the growing

season on the RTW system and many taxa, present in the first half of the season, did not persist during the second. The nursery utilising pSSF showed a reduction in prevalence during the second half of the season, however, the reduction was not as severe. The SSF was the only system to show increased levels of taxa persistence during the second half of the season. There were a high number of possibly pathogenic taxa observed on all three systems but the pSSF and SSF sites showed increased variation in pathogenic taxa. Finally, the SSF site showed the greatest prevalence of oomycetes from which the *Pythium* spp. dominated. The *Pythium* spp. exhibited differing patterns of persistence and abundance in comparison with the other taxa monitored showing persistence from the middle to end of the season with increasing abundance. At the pSSF and SSF sites *Pythium* spp. tended to persist 20 weeks earlier in the growing season in comparison to the RTW site.

Chapter 4 was focused on identifying any key differences between crops grown on systems utilising recycled fertigation solution disinfested by two physical treatments; heat and UV. Little difference was observed in the microbial populations between the heat and UV sites both exhibiting similar taxa richness and species diversity. One significant difference was observed between the crops, with plants grown on the UV system exhibiting significantly higher oomycete diversity, possible causes of this were discussed in section 4.4. Many of the points highlighted for chapter 3 were conserved and also observed on the two crops monitored in this chapter including; a high number of possible pathogens occurring across the season, saprophytes accounting for more of the variation observed between systems, differing patterns in *Pythium* spp. persistence and abundance and significant variation in species diversity being observed between the weeks in the growing season.

Microarray analysis has allowed us to view the microbial populations associated with the rhizosphere in great detail across the growing season and provides a greater level of specificity in comparison with results possibly obtained from other molecular techniques such as T-RFLP, which only provides putative identifications. The data collected in this study does, however, still have to be used with some level of caution and any inferred causality is not definitive for reasons which I shall now discuss.

Biases can be introduced into the data at multiple points during the general protocol for microarray analysis. The first of which may occur during DNA extraction. DNA extraction is commonly known to introduce biases in community DNA analysis and the levels of DNA returned is dependent on the style of kit used, the manufacturer and the origin of the sample with differing groups of

organisms favoured by differing techniques. The second point of bias introduction is during PCR amplification of the community DNA. “Universal” primers targeting conserved regions, firstly, may have mismatched bases with certain taxa causing template DNA to be amplified at reduced levels or not at all. Secondly, separate PCR amplification reactions of the same community DNA sample may cause the amplification of differing templates to be favoured. The final introduction of bias is during DNA hybridisation to the microarray. Differing probes on the array exhibit varying levels of efficacy in binding to their designated targets.

These biases, to a degree, have been addressed through the experimental design utilising four repeats of microarray analysis to build a single microbial profile to reduce the levels of any potential biases incurred during procedures. Differing probe binding efficacy has also been addressed by not directly comparing any specific two probes. The final determinants, of the use of caution when interpreting the results, are defined by the molecular markers used to target taxa on the array. The ribosomal ITS region used in this study has been shown to not allow the specific detection of certain species within the genera *Fusarium, Verticillium, Phytophthora* and *Pythium* and some probes used on the array in this study do show some level of cross hybridisation, see appendix 2. Cross hybridisation, however, was shown to decrease during the analysis of community samples. Furthermore, the ability of the pathogenic taxa, monitored by the microarray, to cause disease cannot be determined and further research is required through the targeting of specific pathogenicity factors. Large scale studies, such as microarray analysis, based on specific pathogenicity factors, however, would be difficult due to the number of different genetic targets and thus a smaller scale approach utilising Q-PCR or LAMP is probably more favourable. The final note on the data collected in this study is the inability to determine the viability of the organisms detected due to the region targeted during microarray development. A possible solution would be to develop an array based on mRNA transcripts. Targeting mRNA would give increased resolution on an organism’s viability due to its reduced half-life in comparison to DNA.

Although microarray analysis does incur biases the process itself is relatively new and the technologies for manufacturing the arrays and the software for array analysis are being developed to help improve the reliability of the data collected. For instance each probe, on the array used in this study, is repeated twice on the array and an average intensity is calculated which helps reduce the impact of any cross hybridisation that occurred for each respective probe. Moreover, the quality of the printing of oligonucleotide probes onto the array is

increasing allowing increased levels of reproducibility.

The microarray used in this study has provided a good tool for the exploratory analysis of a microbial community that has been little studied. The results, however, are notoriously difficult to draw conclusions from due to the dynamic nature of microbial populations, especially the rhizosphere. Within the rhizosphere a number of environmental conditions may influence organisms preferentially which, in turn, may influence, in a beneficial or antagonistic manner, different members of the rhizosphere community or the plant itself. Such a complex and dynamic environment may lead to artefacts in results which can lead to the improper identification or dismissal of causality.

The results obtained, however, do allow us to draw some general conclusions from which further work can be built upon. The number of possible pathogenic species occurring on the plant roots at all sites (pSSF, SSF, RTW, heat and UV) indicates that more work, initially, is required to provide definitive diagnoses that they harbour the ability to cause disease. If the ability to cause disease is found then further research is required to determine what, if any, interactions occur between pathogens in the rhizosphere and how this affects plant health and the rhizosphere community as a whole. Most research, in recent years, has been focused on non-pathogenic organisms and their interactions within the rhizosphere due to the drive to find new biological control agents to control plant disease. Secondly, the largest numbers of taxa unique to the fertigation systems were saprophytic fungi which tended not to persist. Furthermore, saprophytic fungi that were present at all the systems (pSSF, SSF and RTW) tended not to persist over 50 % of the season and the possibly pathogenic species were generally present on the roots upon delivery of the plants from the propagator. This gives some indication that pathogenic and closely related species colonise the rhizosphere at higher levels on soilless systems. The inoculation of rockwool cubes, used during propagation, with an array of saprophytic fungi and beneficial bacteria, before the rootstocks are introduced, may give a competitive advantage and allow them to colonise the rhizosphere preferentially. Monitoring the microbial population and plant health may determine if the early introduction on saprophytic microorganisms could supress pathogen colonisation of the rhizosphere and benefit plant health.

At the pSSF and SSF sites *Pythium* spp. were shown to persist earlier in the season and the SSF site showed the highest prevalence of *Pythium* spp. This may infer that slow sand filtration may lack efficacy in the disinfestation of *Pythium* spp. allowing them to re-enter the greenhouse. A significant difference, however, in the oomycete species diversity was not observed at the pSSF and

SSF systems which may be due to the relatively small number of oomycete species being monitored on the array. The origin and viability of the observed *Pythium* spp. cannot be determined via microarray and further research is required to determine if *Pythium* spp. can pass through the slow sand filtration process. The development of RT-PCR or RT-LAMP (reverse transcriptase) assays, targeting mRNA, could be used to monitor recycled fertigation solution passing straight from the slow sand filter, opposed to the run-off from slabs use in this study, to give a better resolution on organism origin and viability.

Finally, in an additional investigation, which could not be discussed in this thesis due to word restrictions, the microarray's suitability for disease diagnosis and prediction was also assessed. Due to the lack of disease at the main nurseries studied, in chapter three and four, diseased samples were sent from nurseries across the UK who utilised differing substrates, soil and soilless, and rootstock to those used in routine monitoring. Symptoms were recorded by a member of ADAS staff and then conventional diagnosis of disease was performed. Three replicate microarrays were then for both diseased and control root samples. The microarray complimented conventional diagnostic results but also returned a greater number of potential pathogens. Potential pathogens, however, occurred on both diseased and control plants for the majority of the samples. It was difficult to assess whether the causal agent of disease had disseminated throughout the system and had reached the control plants but were yet to express any disease symptoms or if other environmental factors, such as the microbial population of the rhizosphere, had led to the disease taking hold on certain plants due to the fact that populations were only monitored after the detection of disease.

Due to the differing substrates, cultivars and differing nucleic acid extraction techniques that had been utilised during diseased crop monitoring the data could not be used alongside the routine monitoring data and therefore led to very small sample sizes. The small sample sizes prevented any conclusions being drawn from the data and further investigation is required to asses if the microarray could be used to monitor change in the microbial populations and predict disease development. The microarray's inability to identify an organism's viability, its host specificity and specific species, in some cases, makes specific disease diagnosis sub-optimal when compared to other diagnostic methods available such as Q-PCR and LAMP which allow highly specific and speedy detection of pathogens.

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Appendices

Appendix 1. The fungal and oomycete pathogens and saprophytes reported to be commonly observed on the tomato rhizosphere as reported by Deery, 2012.

Fungal and oomycete pathogens	Fungal and oomycete saprophytes
Alternaria solani	*Acremonium atricum*
Aphanomyces cladogamus	*Acremonium sp.*
Armillaria mellea	*Agaricus arvensis*
Botrytis cinerea	*Alternaria humicola*
Calyptella campanula	*Alternaria sp.*
Colletotrichum coccodes	*Aspergillus flavus*
Didymella lycopersici	*Aspergillus sydowii*
Fusarium oxysporum	*Aspergillus ?terreus*
Fusarium oxysporum f. sp. lycopersici	*Aspergillus ustus*
Fusarium oxysporum f. sp. radicis-lycopersici	*Aspergillus sp.*
Fusarium redolens	*Aureobasidium pullulans*
Fusarium semitectum	*Blastomyces sp.*
Fusarium solani	*Calyptella capula*
Fusarium spp.	*Cephalosporium acremonium*
Humicola fuscoatra	*Cephalosporium spp.*
Macrophomina phaseolina	*Chaetomium cochliodes*
Plectosphaerella cucumerina	*Chaetomium elatum*
Phymatotrichopsis omnivora	*Chaetomium olivaceum*
Phytophthora arecae	*Chaetomium spp.*
Phytophthora capsici	*Chromalosporium ochraceum*
Phytophthora cinnamomi	*Conidiobolus coronatus*
Phytophthora citricola	*Coprinopsis gonophylla*
Phytophthora cryptogea	*Cryptococcus albidus*
Phytophthora drechsleri	*Cunninghamella echinulata*
Phytophthora erythroseptica	*Cylindrocarpon didymium*
Phytophthora fragariae var. fragariae	*Doratomyces microsporus*
Phytophthora hibernalis	*Epicoccum purpurascens*
Phytophthora infestans	*Fusarium oxysporum*
Phytophthora megasperma var. megasperma	*Fusarium torulosum*
Phytophthora mexicana	*Gelasinospora reticulata*
Phytophthora nicotianae var. nicotianae	*Gilmaniella humicola*
Phytophthora nicotianae var. parasitica	*Gliocladium roseum*
Phytophthora palmivora	*Idriella lunata*
Phytophthora phaseoli	*Lepiota efibulis*
Phytophthora richardiae	*Lycoperdon sp.*
Phytophthora verrucosa	*Mortierella polycephala*

Pyrenochaeta lycopersici	*Mortierella zychae*
Pyrenochaeta terrestris	*Mortierella sp.*
Pythium arrhenomanes	*Mucor sp.*
Pythium butleri	*Mycotypha microspora*
Pythium debaryanum	*Myrothecium roridum*
Pythium diclinum	*Nectria gliocladioides*
Pythium echinulatum	*Neurospora crassa*
Pythium diclinum	*Oedocephalum sp.*
Pythium irregulare	*Olpidium brassicae*
Pythium megalacanthum	*Olpidium sp.*
Pythium myriotylum	*Paecilomyces lilacinus*
Pythium oligandrum	*Penicillium brevicompactum*
Pythium paroecandrum	*Penicillium chrysogenum*
Pythium periplocum	*Penicillium griseofulvum*
Pythium salpingophorum	*Penicillium janthinellum*
Pythium torulosum	*Penicillium jensenii*
Pythium ultimum	*Penicillium lividum*
Pythium vexans	*Penicillium nigricans*
Pythium 'group F'	*Penicillium purpurogenum*
Pythium 'group G'	*Penicillium stoloniferum*
Rhizoctonia solani	*Penicillium thomii*
Sclerotium rolfsii	*Penicillium variabile*
Spongospora subterranea	*Penicillium verrucosum*
Thielaviopsis basicola	*Penicillium verrucosum var. corymbiferum*
Verticillium albo-atrum	*Penicillium verrucosum var. cyclopium*
Verticillium dahliae	*Penicillium verrucosum var. melanochlorum*
Verticillium nigrescens	*Petriella asymmetrica*
Verticillium nubilum	*Peziza ostracoderma*
Verticillium tricorpus	*Pyronema amphalodes*
	Rhizopus nigricans
	Rhizopus oryzae
	Rhodotorula glutinis
	Sporobolomyces roseus
	Torulopsis famata
	Tricocladium adspersum
	Trichoderma koningii
	Trichoderma viride
	Trichurus spiralis
	Volutella ciliata

Appendix 2. A summary of the microarray validation results as reported by Devine, 2013.

Microorganism species/genus	Type[a]	Self-validated	Cross-hybridisation[b]	
Pathogens				
Alternaria solani	F	+		(+)
Armillaria mellea	F	NT		
Colletotrichum acutatum	F	+		(+)
Colletotrichum coccodes	F	+	(++)	(++)
Didymella lycopersici	F	NT		(+)
Fusarium oxysporum	F	+	(+)	(+)
Fusarium redolens	F	NT	(++)	(++)
Fusarium solani	F	+		(+)
Phytophthora arecae	O	NT	(+)	
Phytophthora cinnamomi	O	NT		
Phytophthora cryptogea	O	+	(++)	(+)
Phytophthora nicotianae	O	NT	(++)	
Plectosphaerella cucumerina	F	+		(+)
Pyrenochaeta lycopersici	F	+		(++)
Pythium aphanidermatum	O	NT		
Pythium arrhenomones	O	NT		
Pythium debaryum	O	NT		
Pythium diclinum	O	NT		
Pythium echinulatum	O	NT		
Pythium irregulare	O	+	(++)	(+)
Pythium megalacanthum	O	NT		
Pythium myriotylum	O	NT		(+)
Pythium oligandrum	O	NT		
Pythium paroencandrum	O	NT	(++)	(+)
Pythium torulosum	O	NT		
Rhizoctonia solani	F	+	+	
Spongospora subterranea	F	NT		(+)
Thielaviopsis basicola	F	+		(+)
Verticillium albo-atrum	F	NT	(++)	
Verticillium dahliae	F	+	(+)	
Verticillium nigrescens	F	+		(++)

Saprophytes					
Alternaria spp.	F	+	+		
Aspergillus flavus	F	+		(+)	(++)
Aspergillus sydowii	F	+		(+)	(++)
Aspergillus terreus	F	+		(++)	
Aspergillus ustus	F	NT		(+)	
Cadophora spp.	F	+			(+)
Cephalosporium spp.	F	NT			(±)
Chaetomium cochliodes	F	+	+		
Chaetomium spp.	F	+			(++)
Cladosporium spp.	-	NT			(++)
Doratomyces microsporus	F	NT			
Exophiala pisciphila	F	+	+		
Exophiala xenobiotica	F	NT			
Gigaspora rosea	F	NT			
Gliocladium roseum	F	+			(+)
Glomus intraradices	F	NT			(+)
Myrothecium roridum	F	NT			
Olpidium brassicae	F	NT			(+)
Paecilomyces lilacinus	F	NT			
Penicillium brevicompactum	F	NT		(+)	(+)
Pencillium chrysogenum	F	+	+		
Penicillium griseofulvum	F	NT		(++)	(+)
Pencillium variabile	F	NT			
Penicillium spp.	F	+			(++)
Petriella asymmetrica	F	NT			(++)
Phoma sp.	F	+	+		
Rhizopus oryzae	F	NT			
Trichoderma viride	F	+		(++)	(+)
Trichoderma harzianum	F	+	+		
Trichoderma spp.	F	+	+		
Bacteria					
Agrobacterium rhizogenes		+			(+)
Agrobacterium tumefaciens		+			
Bacillus amyloliquefaciens		+			
Bacillus subtilis		+			(+)

Clavibacter sp.	+		(+)
Erwinia spp.	+		
Escherichia coli	+	+	
Nitrospira spp. (nitrite oxidising bacterium)	NT		(+)
Pseudomonas syringae	NT		
Pseudomonas universal	+		(++)
Ralstonia solanacearum	+	+	
Rhizobium sp.	+		
Xanthomonas spp.	+		(+)
Yersinia spp.	NT		
Nematodes			
Meloidogyne incognita	+		
Pratylenchus goodeyi	NT		
Pratylenchus spp.	NT		(+)

A; F – fungus: O – oomycete

B; signal intensity; (±) ambiguous; (+) strong; (++) very strong.

NT – not tested