

Dynamics of Root Microorganisms in Closed Hydroponic Cropping Systems

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Cover: Tomato cropping system used in the thesis experiments (upper left), tomato roots inoculated with *Pythium ultimum* (upper right), agar plate with colonies of fluorescent pseudomonads illuminated with UV-light (lower left), sporangia of *P. ultimum* seen through microscope (lower right).

(photo: Anna Karin Rosberg)

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Abstract

Greenhouse production of vegetables in closed hydroponic cropping systems is a resource-efficient technique for the production of high-quality produce with a high yield per unit area. While this type of cropping system allows savings in terms of water and nutrient use, the recirculation of water increases the risk of pathogen dispersal. Oomycetous pathogens in particular, such as *Pythium*, thrive in aquatic environments and are among the most destructive pathogens. Root pathogen outbreaks could be controlled by sustaining a stable and high level of general microbiota. It is therefore of utmost importance to understand how biotic and abiotic factors affect the indigenous root-associated microbiota.

In the present thesis, the impact of pathogen inoculation, plant age, nutrient availability and use of plant protection products on microbial communities associated with root and nutrient solution was investigated. It was demonstrated that all these factors had an effect on the indigenous microorganisms. Plant age in particular was a significant driver of microbial community structure due to its significant impact on root exudation patterns. Using 454-pyrosequencing, the effect on bacterial communities of the inoculation of *P. ultimum* was visualized. Although the effect was strongest at the fruit-bearing plant stage, clear differences were seen also in the seedling and flowering stages. Nutrient availability was revealed to have an impact on the number of colony-forming units of bacteria, fungi and fluorescent pseudomonads. Organic carbon specifically was shown to be the main driving force of microbial growth. However, not only the quantity, but also the quality and ratios between the different organic and inorganic nutrients had an effect on microbial numbers. Through DGGE analysis, a shift in the bacterial community structure was seen after the addition of a fungicide in the nutrient solution. The microorganisms present in the nutrient solution were not able to degrade the fungicide.

This thesis also demonstrates the importance of combining culture-dependent and culture-independent methods in the analysis of microbial communities.

Key words: bacteria, fungi, microbial communities, nutrient solution, organic carbon, plant age, *Pythium ultimum*, rhizosphere, root pathogens, tomato

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Dedication

To David and Gabriel

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List of Publications

This thesis is based on the work contained in the following papers, referred to in the text by Roman numerals:

- I Rosberg, A.K., Gruyer, N., Hultberg, M., Wohanka, W. & Alsanius, B.W. (2014). Monitoring rhizosphere microbial communities in healthy and *Pythium ultimum* inoculated tomato plants in soilless growing systems. *Scientia Horticulturae* 173, 106-113.
- II Rosberg, A.K., Wohanka, W., Hultberg, M. & Alsanius B.W. (2014). Impacts of *Pythium ultimum* inoculation on tomato root-associated bacterial communities, as examined by 454 pyrosequencing (submitted).
- III Rosberg, A.K., Wohanka, W., Hultberg, M. & Alsanius B.W. (2014). Organic carbon and micronutrients have an effect on microbial dynamics in nutrient solutions of diseased and non-diseased hydroponic systems (manuscript).
- IV Alsanius, B.W., Bergstrand, K-J., Burleigh, S., Gruyer, N. & Rosberg, A.K. (2013). Persistence of fenhexamid in the nutrient solution of a closed cropping system. *Agricultural Water Management* 127, 25-30.
- V Alsanius, B.W., Rosberg, A.K., Hultberg, M., Khalil, S. & Jung, V. (2014). Understanding and utilizing naturally-occurring microbes against plant pathogens in irrigation reservoirs. In: Hong, C. *et al.* Biology, Detection, and Management of Plant Pathogens in Irrigation Water. Chapter 28; 347-364. APS (in press).

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The contribution of Anna Karin Rosberg to the papers included in this thesis was as follows:

- I Planned the experiment with co-authors. Performed the experimental work and evaluated the data. Wrote the manuscript with co-authors.
- II Planned the experiment with co-authors. Performed the experimental work and evaluated the data. Wrote the manuscript with co-authors.
- III Planned the experiment with co-authors. Performed the experimental work and evaluated the data. Wrote the manuscript with co-authors.
- IV Participated in planning the experiments and performing the statistical analysis. Wrote the manuscript with co-authors.
- V Participated in creating the carbon pool model, and wrote the manuscript with the main author.

Abbreviations

ATP	adenosine triphosphate
CFU	colony forming unit
CLPP	community level physiological profiling
CMA	corn meal agar
DAPG	2,4-diacetylphloroglucinol
DFT	deep flow technique
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
EC	electrical conductivity
KB	King's B agar
MA	malt extract agar
MDA	multiple displacement amplification
NFT	nutrient film technique
PCR	polymerase chain reaction
PDA	potato dextrose agar
PLFA	phospholipid fatty acid
PM	phenotypic microarray
rRNA	ribosomal ribonucleic acid
TIC	total inorganic carbon
TOC	total organic carbon
T-RFLP	terminal restriction fragment length polymorphism
TSA	tryptic soy agar
UV	ultraviolet

1 Introduction

The production of vegetables in closed hydroponic cropping systems is a resource-efficient way of producing crops of high quality, with a high productivity in terms of yield per unit area (Stanghellini & Montero, 2012). Compared to fields, water savings in greenhouse production are in the order of 70-90 % for the same crops. This applies to closed systems in particular (Raviv & Lieth, 2008). One of the main concerns related to closed systems is the potential spread of root pathogens. With the recirculation of nutrient solution, a single infected plant could potentially be the source of infection for the entire crop (Postma *et al.*, 2008). Oomycetous pathogens in particular, such as *Pythium* and *Phytophthora*, are the most destructive diseases due to their production of motile zoospores that thrive in aquatic environments (Stanghellini & Rasmussen, 1994). Root pathogen outbreaks could be controlled by sustaining a stable and high level of general microbiota (Hoitink *et al.*, 1991). It is therefore of the utmost importance to understand how biotic and abiotic factors affect the indigenous, root-associated microbiota.

In this thesis, the root and nutrient solution-associated microbial communities of tomato plants grown in closed hydroponic cropping systems were investigated. The microbial communities were investigated particularly for changes in community structure caused by the introduction of a pathogen, plant age, organic and inorganic nutrient availability, and the effects of the use of plant protection products.

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2 Background

2.1 Hydroponic systems

Hydroponics is a term that was coined in the 1930s to describe all methods of growing plants in liquid media for commercial purposes (Gericke, 1937). From the outset, hydroponic systems were used purely to look at plant nutrition and identify essential plant nutrients. Eventually, a growing medium was added to support and aerate plant roots; the growing medium had to be inert in order not to interfere with the nutrient composition, hence gravel and sand were commonly used. This type of growing system attracted a lot of attention early on, but due to the lack of automation and technology at the time, it took many years before it became economically sustainable (Jones, 1982). It was not until the 1970s that there was renewed interest in hydroponics. Many greenhouse growers experienced problems with soil fatigue from using the same soils for several years. The introduction of rockwool as a growing medium opened up the use of hydroponic systems in commercial settings. For the last four decades there has been considerable expansion in hydroponics. In the Netherlands, for example, the area of hydroponically-grown crops has increased from 5 ha in 1976 (Van Os, 1982) to almost 8,000 ha in 2013 (Voogt *et al.*, 2013), mainly due to its independence from soil and soil-related problems, and the possibility of precise nutrient control (Jones, 1982).

Soilless greenhouse cropping systems can be divided into open and closed systems. In open systems the drained, nutrient-enriched irrigation water is discharged into the soil, catchment or sewage treatment plant. As much as 40 % of the nitrogen supplied to the plants is estimated to be lost with the drainage water (Dorais & Dube, 2011; Hansson, 2003). This drainage contributes significantly to the eutrophication of water courses and estuarine areas in the vicinity of the greenhouse, as well as to groundwater pollution. In closed systems, the drainage is collected, mixed with fresh water, readjusted to

the accurate nutrient composition and redistributed to the plants. The use of closed systems is therefore a solution to the issues of nutrient and pesticide leakage from greenhouse production. European legislation demands a substantial reduction in nutrient release (EU, 2000), therefore growers are being encouraged to change from open to closed irrigation systems. Such changes require high investment costs, however up to 30-40 % and 50-60 % of fertilizer and water costs respectively can be saved through recirculation (Peet & Welles, 2005). The two greatest concerns associated with closed irrigation systems are the accumulation of salts in the water and the potential spread of plant root pathogens, where the presence of just one infected plant will put the entire crop at risk (Postma *et al.*, 2008). Oomycetous pathogens in particular, such as *Pythium* and *Phytophthora*, can easily spread and propagate explosively under favorable conditions, causing serious damage (Macdonald *et al.*, 1994; Stanghellini & Rasmussen, 1994).

2.2 Microbiota in hydroponic systems

Initially, it was expected that soilless systems would be the solution to evading root pathogens. It has since been shown that the diversity of root-infecting microorganisms is less than in soil. However root diseases do occur and are sometimes more serious in soilless systems (Stanghellini & Rasmussen, 1994). Before plants are introduced into soilless systems, the numbers of viable counts are low, but already within twenty hours of planting, high numbers of heterotrophic bacteria and fungi develop in the nutrient solution (Berkelmann *et al.*, 1994). The number of bacteria can be as much as 10^{10} colony-forming units (CFU) per gram root. The number of fungi is usually lower, but tends to vary over the season more than bacteria (Waechter-Kristensen *et al.*, 1994). This contamination of microorganisms in soilless systems can come from plant material, growing media, insects, workers in the greenhouse and irrigation water (Postma *et al.*, 2008). The colonization is often triggered by environmental conditions, such as steady temperature and moisture regimes, and by the fact that root colonizers are easily spread by the recirculating water. The availability of water in hydroponic systems favors all zoosporic pathogens, with the temperature of the nutrient solution being the most important environmental factor controlling the proliferation of these root diseases (Stanghellini & Rasmussen, 1994). *Pythium*, which in soil-grown cucumbers and tomatoes is not considered to be a serious threat, has become severe in hydroponic conditions. It seems that rockwool, the most common growing medium in soilless culture, is exceptionally conducive to *Pythium* diseases. This is due to its high water content relative to other types of growing media,

such as coir or pumice (Van der Gaag & Wever, 2005). It has also been suggested that the relatively low microbial diversity in hydroponic systems compared to soil (Menzies *et al.*, 2005) leads to an increased severity of opportunistic pathogens, such as *Pythium* (Paulitz, 1997). Both Craft and Nelson (1996) and Chen *et al.* (1988) have demonstrated the association of high microbial activity with suppression of *Pythium* diseases. Once a zoosporic pathogen comes into the system, it will multiply and spread by self-dispersal through the recirculating solution and by root-to-root contact (Stanghellini & Rasmussen, 1994). In periods of stress, such as during fruiting when the temperature is high and there is a low concentration of dissolved oxygen in the nutrient solution, disease epidemics can take place (Cherif *et al.*, 1997; Stanghellini & Rasmussen, 1994; Gold & Stanghellini, 1985).

2.2.1 Disinfection

In closed hydroponic systems, the risk of pathogen dispersal can be limited by the use of different disinfection techniques. The techniques are divided into active and passive methods. The active methods are, for example, UV radiation, ozone and heat treatment. One of the weaknesses of many active methods is that they also reduce the beneficial microorganisms. Zhang and Tu (2000) found that UV radiation reduced bacterial flora, but that *Pythium* was not reduced; instead, *Pythium* increased due to the lack of competition from the general microbiota. For *Pythium* to be eradicated, high doses of UV radiation were needed. The efficiency of the method was also highly dependent on the UV dose. This was also seen by Buyanovsky *et al.* (1981), who found that three hours of UV radiation per day on the nutrient solution was more efficient in reducing the microbiota than one hour per day. Three hours per day was also as efficient as having continuous UV radiation. Ozone has been shown to be efficient in eliminating pathogens such as *Fusarium oxysporum* and tomato mosaic virus (Igura *et al.*, 2004; Runia, 1994). With this method too, however, its efficiency is dependent on the dose and length of treatment. With heat treatment, the returned drainage water is heated to 95 °C for a minimum of 10 s. This temperature is generally high enough to remove most microorganisms, viruses and nematodes. However, spore-forming bacteria will survive (Os, 2009).

Slow filtration is an example of a passive method. Here, the nutrient solution is filtered slowly through a growing medium, *e.g.* sand, rockwool or ground volcanic stone, where it is thought that mechanical and biological factors are responsible for the elimination of pathogens. Many pathogenic bacteria and fungi are efficiently eliminated this way, and plenty of beneficial

organisms stay in the filter where they are active in suppressing pathogens (Bergstrand, 2010; Brand & Wohanka, 2001; Ehret *et al.*, 2001). Another type of passive method is the use of constructed wetlands filled with ground volcanic stone and implanted with common cattail (*Typha latifolia*), which has proven efficient in reducing *P. ultimum* in tomato production (Gruyer *et al.*, 2011).

2.2.2 Disease suppression

Suppressiveness in soil has been defined by Baker and Cook (1974) as “soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may still persist in the soil”. Two types of mechanisms of suppressiveness have been distinguished: i) general suppression and ii) specific suppression. In general suppression, the entire microbial biomass competes with the plant pathogenic microorganisms for nutrient and space resources, hence a reduction in the germination and growth of the pathogens. Specific suppression, on the other hand, is due to specific individual populations or groups of microorganisms. The main characteristic of specific suppression is that the suppressiveness is transferable (Cook & Baker, 1983). Factors thought to influence disease suppression are the activity of the total microbiota, the diversity of the microbial population, the presence of specific types of antagonists, the production of antimicrobial compounds, as well as the microbial-induced disease resistance of the plants (Clematis *et al.*, 2009; Minuto *et al.*, 2007; Weller *et al.*, 2007; Postma *et al.*, 2005; Weller *et al.*, 2002; Hultberg *et al.*, 2000; Postma *et al.*, 2000; Ongena *et al.*, 1999; Tu *et al.*, 1999; Paulitz, 1997; Stanghellini & Miller, 1997).

Several studies (Clematis *et al.*, 2009; Minuto *et al.*, 2007; Postma *et al.*, 2005; Postma *et al.*, 2000; Tu *et al.*, 1999) report on suppressiveness against root pathogens in soilless cropping systems as well. Few growing media have been described based on their disease suppressive properties. However, differences in the composition of microbial communities have been found when root samples from two different growing media used for tomato production were analyzed (Khalil, 2001). The differences found were most likely due to the diverging chemical and physical properties of the two growing media, but could also reflect differences in potential suppressiveness. It has been shown that the microbiota of re-used rockwool slabs, used originally for cucumber production, was able to suppress *Pythium aphanidermatum* in a new cucumber crop, while new, sterile rockwool slabs were not. New rockwool slabs provide a microbiological vacuum that allows a fast multiplication of

pioneer, pathogenic *Pythium* species (Postma, 2004). They are, however, inferior competitors in the presence of a fully developed microbial community (Sutton *et al.*, 2006).

There are several ways in which the suppressiveness of growing media can be improved, *e.g.* by adding antagonistic microorganisms (Castano *et al.*, 2013; Khalil *et al.*, 2011; Wohanka *et al.*, 2008; Paulitz, 1997; Nemeč *et al.*, 1996) or by nutritional amendments (St Martin & Brathwaite, 2012; van der Gaag *et al.*, 2007; Chen *et al.*, 1987). Compared to soil, in greenhouse production there is a possibility of providing the perfect environmental conditions needed to suit antagonistic agents (Paulitz & Belanger, 2001), yet the efficiency of the biocontrol agents varies depending on the growing media used (Khalil *et al.*, 2009). The efficiency of the biocontrol agent is also dependent on its mode of introduction into the cropping system. Antagonistic microorganisms have proven to be most efficient when they have been introduced into the system in the same way as the pathogen, *i.e.* if the pathogen was introduced through the nutrient solution then the antagonist should be introduced in the same way (Rattink & Postma, 1996).

A combination of adding antagonistic microorganisms and using a disinfection technique to clean the nutrient solution is also a possible approach to increasing suppressiveness. The disinfection of the nutrient solution has to be gentle in order not to eradicate the beneficial organisms along with the pathogens. Slow filtration has proven to be a suitable disinfection technique with positive effects in combination with oomycete and fungal and bacterial biocontrol agents (Deniel *et al.*, 2004; Garibaldi *et al.*, 2003; Rey *et al.*, 1999).

2.2.3 Differences in microbiota depending on the growing medium

Considering the differences in soilless growing media in terms of physical and chemical properties, it is likely that their microbial community structures are very different too. This was shown by Borrero *et al.* (2006), who found a clear difference in carbon utilization using community-level physiological profiling (CLPP) when comparing the microbiota of peat and compost. In soilless growing systems there can be inorganic growing media such as rockwool, perlite, pumice and vermiculite, and organic growing media such as coir, peat and wood fibers. Differences in terms of the microbiota inhabiting these two groups of growing media were identified by Koohakan *et al.* (2004). They found that inorganic growing media were mainly colonized by bacteria, while the organic growing media had a larger fungal population. Similar findings were established by Khalil and Alsanius (2001), where higher amounts of bacteria were found in rockwool than in peat, and the reverse was found with regard to fungi. Although it is not known exactly why there are differences in

the microbial communities in different growing media, it is thought that it could be related to the type of organic compounds available to the microorganisms (Koohakan *et al.*, 2004).

Cropping systems without growing media, such as nutrient film and deep flow techniques, also have a high microbial diversity. Despite the simplicity of these systems compared to soil-based growing systems, analyses have shown that bacterial diversity in the rhizoplane of roses was as great as when grown in soil (Chave *et al.*, 2008).

The conduciveness of different growing media to root pathogens differs. Experiments with cucumber showed that the number of diseased plants was significantly higher in rockwool than in pumice or coir. The pathogen responsible for the diseased plants was *Pythium aphanidermatum*, a common pathogen in cucumber and tomato. The water content of the growing media tested was highest in rockwool (Van der Gaag & Wever, 2005). Since high water content is favorable for the formation and spreading of zoospores (Duniway, 1979), this factor was described as the most important. With high water availability, the zoospores can move easily in the growing medium and reach more locations where they can infect roots. The height of the growing medium and also the type are important tools that can be used to control the water content in the root zone in order to decrease disease incidence (Van der Gaag & Wever, 2005).

In a comparison of rockwool, coir, nutrient film technique (NFT) and deep flow technique (DFT), *Pythium* was found to be highest in NFT. The total number of bacteria was significantly lower in DFT than in all the other growing systems. The tests were performed both on plant roots and in the nutrient solution. In the same experiment, it was also established that differences occurred in the microbial communities colonizing roots and nutrient solutions. The structure and diversity of the microbiota also differed between the cropping systems used (Koohakan *et al.*, 2004).

2.2.4 Oomycetes in closed systems with tomato

Within the Oomycota class, there are both pathogenic and non-pathogenic organisms present in hydroponic systems. The main species are *Pythium* and *Phytophthora*. Oomycetes were previously grouped with fungi owing to their filamentous growth and the presence of multinucleate hyphae. They now belong to the Chromista kingdom and the phylum Stramenopiles. The factor separating them from real fungi is the differences in the composition of cell walls, which contain cellulose instead of chitin. They have flagellate cells and a diploid, sexually reproductive phase. In most fungi this phase has been reduced, but in oomycetes it is dominant. Ecologically they are similar to fungi

with respect to mass of hyphal growth formation and decomposition of dead organic material in aquatic habitats (Madigan *et al.*, 2009).

Various species of *Pythium* are known to infect plants in hydroponic systems, with the main species being *P. aphanidermatum*, *P. dissotocum*, *P. ultimum* var. *ultimum*, *P. ultimum* var. *sporangiferum* and members of *Pythium* group F. Except for *P. ultimum*, all these species produce an abundance of zoospores. They are all able to produce oospores in infected roots and in the rhizosphere. The infection has several ways of entering the greenhouse: i) airborne dust, ii) soil, seeds, and plant fragments on tools and equipment, iii) footwear, iv) water used to prepare the nutrient solution and v) growing medium. A common way of introducing the infection is through transplants. Many growers buy small transplants instead of sowing the plants themselves, enhancing the risk of infection. Greenhouse equipment, *i.e.* pipes, tubing, nutrient solution tanks *etc.*, are common sources of infection. Even when the systems have been treated with disinfectants, it is still common to find *Pythium*. Owing to their thick cell walls, the oospores of *Pythium* are resistant to chemical disinfectants and are frequently found in biofilms in hydroponic plumbing.

Pythium infects plant roots with mycelia and zoospores. The primary infection is predominantly through zoospores produced by sporangia, which are formed by germinating oospores, or by mycelium. Subsequent infections are usually caused by zoospores formed by sporangia on infected roots. With *P. ultimum*, which has a poor zoospore production, mycelium is the primary source of inoculum. The actual infection starts with zoospore encystment on the root surface. It produces a thick cell wall, adheres to the root, germinates through a germ tube and then penetrates the root surface. In general, root tips, elongation zones and young root hairs are frequently penetrated. However, some *Pythium* species are able to infect all parts of young roots. Direct penetration with penetration pegs, fine hyphae and enzymatic action has been observed with *P. aphanidermatum*. Wounded root tissues are easy entryways for pathogens, such as damage caused by insects and larvae, as well as sites of emergence of lateral roots. Root mucilage is an important factor promoting *Pythium* populations in the root zone. *P. ultimum* is known to penetrate roots where there is increased mucilage production in junctions of cortical cells, zones of elongation and at the base of lateral roots and root hairs.

Root colonization of *Pythium* is both inter- and intracellular. The infection is commonly biotrophic at first, and then develops into a necrotrophic stage. In the biotrophic stage, it is possible to isolate the pathogen from the roots, but there are no visible symptoms. In the necrotrophic stage, the roots become discolored. The color depends on both the plant and *Pythium* species, but is

generally brownish. Symptoms on the upper parts of the plants can include stunted shoots, wilted leaves and smaller and fewer fruits. In many plants the foliage stays green until the roots are heavily infected and have started to decay. At the first signs of wilting, the wilting itself is often temporary, commonly in combination with high temperatures in the greenhouse. Eventually, however, the plants will wilt permanently (Sutton *et al.*, 2006).

In commercial tomato production, two stages of *Pythium* infection have been observed. The first stage is at the start of the crop until June, when a small population of *Pythium* is regularly detected. In July and August and at the end of the cropping season, this infection increases rapidly (Rey *et al.*, 2001). In many cases the infection occurs without symptoms, but could also lead to a reduced yield and premature plant death (Tu *et al.*, 1999).

Among the *Phytophthora* species, *P. cryptogea* and *P. nicotianae* var. *nicotianae* are well-known pathogens of tomato plants in hydroponic systems. As with *Pythium*, they cause root rots, damping-off of seedlings and rots of lower stems. Plants infected by *Phytophthora* show symptoms of drought and starvation. They are quickly weakened and thus susceptible to infection by other pathogens. When there is root infection, many of the small roots die quickly, while the older roots get necrotic lesions (Agrios, 2005). The zoospores of *Phytophthora* are produced inside sporangia, which are formed terminally on hyphae. The pathogen can infect plants not only by zoospores, but also by the sporangia which can germinate directly by means of germ tubes (Crous *et al.*, 2009).

2.3 Root exudation

Plant roots exude a vast array of different compounds into the rhizosphere. The type and amount of compounds released depend on plant species, cultivar, age and environmental conditions (Rovira, 1959). Most of the compounds are normal plant constituents produced by photosynthesis and other plant processes. The substances exuded are sugars, amino acids, organic acids, fatty acids, aromatic acids, aliphatic acids, sterols, hormones, vitamins, enzymes, phenolics and nucleotides. The suggested functions of the root exudates are that they assist in the acquisition of nutrients and water and protect against physical stress, pathogens, toxic elements and competition from other plants, as well as help establish beneficial relationships (Uren, 2001).

The mode of exudation of plant roots is thought to be through passive diffusion and active secretion. Low molecular weight compounds that are soluble in water, such as sugars, amino acids, carboxylic acids and phenolics, are passively transported along the concentration gradient between the

cytoplasm of the intact root cells and the external solution. The concentration of solutes in the cytoplasm is in the mM range, while that in the solution is usually in the μM range, creating a rather steep concentration gradient. Exudation of high amounts of specific compounds, however, is thought to be through controlled release. For example, plants such as wheat and maize exude organic acid anions through anion channels which are activated by high levels of aluminum. The organic acids chelate the aluminum and thereby protect the roots (Ma *et al.*, 2001). Anion channels, such as ATP channels for proton release and K^+ channels for potassium ions, are involved. Another mode of active exudation, primarily for high molecular weight compounds such as polysaccharides, mucilage and proteins, is exocytosis by vesicle transport (Neumann & Römheld, 2001).

The most active site of exudation is the area immediately behind the root tip. In hydroponic culture, as much as 98 % of the exuded sugars and sloughed organic matter come from the root apex (Griffin *et al.*, 1976). The plant already starts to exude carbon as a seed; as the seed imbibes, it exudes water-soluble carbon compounds and gases, which continues as the roots and shoots emerge (Whipps, 1990). It has been shown that seedlings exude the lowest amount of exudates. As the plant grows, the exudation rate increases until it sets flowers, and as it matures the rate decreases again (Aulakh *et al.*, 2001). Another correlation between exudation and growth stage was made by Garcia *et al.* (2001) who showed that actively growing root systems excrete more. The same was found by Prikryl and Vancura (1980). Plants are known to distribute assimilates from photosynthesis to actively growing parts, therefore the roots act as sinks until the plant starts to set flowers and fruits (Heuvelink & Dorais, 2005). Therefore the roots have a large amount of carbon to exude during the vegetative phase.

A rough estimate of exudation amounts has been ascertained by Uren (2001) who claimed that approximately 10 % of net fixed carbon is allocated to the root. The main part of this 10 % is thought to be root debris, and a minor part is root exudates. In terms of actual amounts, the values for rhizodeposition in nutrient solution culture trials have not exceeded 600 mg g^{-1} root dry weight. In most cases, the measured volumes were much lower (Whipps, 1990).

Intact roots exude small amounts of organic material. However, there are many factors that have an effect on root permeability, such as high and low temperatures, light intensity, anaerobic conditions, nutrient deficiencies and the presence of microorganisms (Larcher, 2003; Neumann & Römheld, 2001; Rovira, 1969).

Plants can exude both antimicrobial substances and substances that stimulate microbial growth. As an example of antimicrobial substances, it is

known that some plants can generate reactive oxygen species which places stress on the colonizing microbes and leads to a selective pressure on the rhizosphere microbiota (Apel & Hirt, 2004). Secondary plant root metabolites (Bais *et al.*, 2005) and quorum sensing inhibitors (Teplitski *et al.*, 2000) have also been found in root exudates as a response to the presence of bacteria and their signaling compounds. In contrast, many of the root exudates, such as sugars and amino acids, stimulate the chemotactic response of bacteria and encourage colonization. There are many independent evidences claiming that roots selectively stimulate specific microorganisms and thereby create unique rhizosphere communities depending on plant species (Micallef *et al.*, 2009; Broeckling *et al.*, 2008; Sharma *et al.*, 2005; Grayston *et al.*, 1998).

The exudates of tomato roots have been investigated by several researchers. Kamilova *et al.* (2006) recently discovered that the total amount of organic acids exuded by the roots is much higher than the amount of sugars, and that exudation of organic acids and sugars increases as the plants grow. They also found that citric acid, succinic acid and malic acid are the major organic acids, while fructose, glucose and xylose are the major sugars. In the same experiment it was shown that tomato roots exuded five times more sugars and organic acids when the plants were grown in rockwool compared to glass beads (Kamilova *et al.*, 2006), indicating that the growing medium is an important factor for root exudation and subsequent colonization. Aspartic acid, glutamic acid, isoleucine, leucine and lysine are the major amino acids exuded from tomato seedlings. Other amino acids were found, although in low concentrations (Simons *et al.*, 1997). Other studies claim that the major amino acids in root exudates of tomato are glycine, serine and alanine (Lesuffleur *et al.*, 2007; Vancura & Hovadik, 1965).

2.4 Temporal and spatial shifts in microbial communities

Several studies report temporal and spatial shifts in microbial communities. The causes of these shifts vary from pathogen attack to changes in plant stage and seasonal weather changes. It is a complex system with many possible influences, and one that is not easily explained.

Vallance *et al.* (2009) found that fungal populations in the rhizosphere of tomatoes grown in soilless culture were more complex towards the end of the cropping season than at the beginning. This was the case both for plants inoculated with the biocontrol agent *Pythium oligandrum* and for the control plants. Over a six-month growing period, samples were taken on three occasions and the genetic structures of the fungal populations differed significantly from one another at all three samplings. The changes in fungal

populations over the cropping season were most likely induced by changes in the plant root exudates depending on plant stage. This hypothesis is strengthened by a study by Mougél *et al.* (2006) in which the microbial community of the roots was investigated at five different plant stages (three vegetative and two reproductive) of *Medicago truncatula*. The most significant community shifts were observed at the transition from vegetative to reproductive phase. It was also shown that there were differences in how the bacterial communities changed compared to the fungal communities. During the vegetative stages the bacterial communities shifted at each plant development stage, whereas the fungal communities did not vary significantly. In the transition from vegetative to reproductive stage, both communities shifted considerably. At the last reproductive stage, the bacterial community had almost returned to what it looked like during the first vegetative stage, *i.e.* the system is able to return to a steady state after a perturbation. The fungal community also changed significantly in the last plant stage. However, it looked very different from all of the other stages. This indicates that the plant has a large rhizosphere effect (Mougél *et al.*, 2006).

In a study carried out by Hagn *et al.* (2008), in which cucumbers were grown in compost-amended peat, the bacterial and fungal populations were found to be different when the growing medium was infected with *P. ultimum* compared to non-infected ones. Of the bacterial populations, Actinobacteria and α -Proteobacteria were the dominant classes in the presence of *P. ultimum*, while the control was dominated by γ/δ -Proteobacteria. The γ -Proteobacteria comprises pseudomonads among other things, which are typical biocontrol organisms against *Pythium*. However, these were not present in high amounts in the pathogen-inoculated samples. The fungal community was more greatly affected by the pathogen than the bacterial community. More than 70 % of the fungal sequences in the control were linked to Homobasidiomycetes, whereas none of these were found in the *Pythium*-inoculated samples. From these data it was assumed that the presence of *P. ultimum* induced shifts in the microbial communities in the growing medium. The actual cause of the shifts was suggested to be a consequence of root damage by the pathogen, resulting in nutrient leakage, which in turn leads to secondary colonization (Hagn *et al.*, 2008).

General bacterial and fungal populations, as well as *Pythium*, have been shown to increase over the cropping season in the nutrient solution and on the roots of tomato plants grown in closed rockwool and peat systems (Tu *et al.*, 1999; Khalil & Alsanius, 2001).

2.5 Abiotic factors affecting the structure of rhizosphere microbial communities

Microbial growth occurs in response to changes in the environment. Therefore it is important to understand the relationship between the physical and chemical factors in the environment and the microorganisms' response to these. Several physical and chemical properties have been shown to correlate with differences seen in different microbial communities, e.g. pH, percentage of organic carbon, electrical conductivity and cation exchange capacity (Andrew *et al.*, 2012). Andrew *et al.* (2012) found that the level of available carbon was the limiting factor driving microbial diversity in desert soils. Neither the percentage of nitrogen present nor the C:N ratio showed any significant correlations with the rhizosphere communities. Li *et al.* (2012) saw an increase in gene copy numbers of *Bacteria*, *Archaea* and fungi in river sediment samples with high DOC concentrations compared to those with lower concentrations. Similarly, Eiler *et al.* (2003) showed that the bacterial biomass in cultures increased linearly with increasing dissolved organic carbon (DOC) concentrations. Organic carbon is not the only limiting factor in microbial growth; mineral nutrients, such as nitrogen and phosphorus are also required (Fagerbakke *et al.*, 1996). The addition of micronutrients to systems where biocontrol agents are used has been shown to increase their efficiency (Khalil & Olsson, 2013; Duffy & Defago, 1997). The effect on microbial community structure, however, is unknown. It has been shown that soil pH has as great an impact on microbial biomass as soil carbon and nitrogen concentrations (Wardle, 1992). Lauber *et al.* (2009) found that soil pH was significantly correlated with changes in soil microbial communities, and that specific bacterial classes were associated with specific pH ranges in soil samples taken from across North and South America. The pH has a clear effect on the ratio of fungi to bacteria, where the respiratory activity of fungi increases under acidic conditions while that of bacteria decreases (Blagodatskaya & Anderson, 1998).

The availability of water is a major factor controlling the survival and activity of microorganisms. Hueso *et al.* (2012) showed that soil microbial biomass and activity were severely affected by drought. The structure of the microbial communities was also affected by different water regimes, where a high moisture content results in >50 % greater fungal-to-bacterial ratio (Williams & Rice, 2007). Temperature increases corresponding to those expected from climate change (4 °C in central Europe) had no impact on microbial community structure or biomass, but increased the level of microbial metabolic activity (Schindlbacher *et al.*, 2011). In the event of more extreme temperature changes, the species of the microbial communities that are

genetically better suited to extreme temperatures will outcompete the less adaptable ones (Barcenas-Moreno *et al.*, 2009).

The use of both chemical and botanical pesticides has been shown to have a considerable impact on soil microbial community structures (Spyrou *et al.*, 2009). Despite strict regulations on pesticide registration to ensure they are only toxic to the target organism, this is rarely the case in practice (Johnsen *et al.*, 2001).

2.6 Microbial community analysis

There are several methods available for investigating microbial communities. These are commonly divided into two general types: culture dependent and culture independent. The plate count method is an example of a culture-dependent technique, in which microorganisms from natural samples can be grown on solid nutrient media. However, different microorganisms in a natural sample can have very different requirements concerning nutrient and temperature conditions. This means that only a small number of organisms can be cultured under laboratory conditions (Madigan *et al.*, 2009). By comparing plate counts with direct microscopic counts of the same samples, it has been estimated that only 0.1-1 % of all microorganisms will be revealed by the plate count method ((Hugenholtz & Pace, 1996; Torsvik *et al.*, 1990).

A commonly used culture-dependent technique for analyzing microbial communities is community-level physiological profiling (CLPP) (Houlden *et al.*, 2008; Söderberg *et al.*, 2004; Khalil & Alsanius, 2001; Waechter-Kristensen *et al.*, 1999; Zak *et al.*, 1994; Garland & Mills, 1991). The system is provided by Biolog (Hayward, CA, USA) and is based on the utilization of specific organic nutrient sources by either specific species or entire microbial populations using different sole organic compounds. The utilization of the sole nutrient sources is detected through the reduction of a tetrazolium dye, and the color change is measured spectrophotometrically. The number of sources utilized, as well as the level of utilization, can be used for comparison between different samples. Multivariate statistics are commonly used to analyze the utilization profile data (Garland, 1997). The method has been proven to differentiate spatial and temporal shifts in microbial communities consistently (Suda *et al.*, 2009; Houlden *et al.*, 2008; Correa *et al.*, 2007; Khalil & Alsanius, 2001; Garland, 1997).

Culture-dependent techniques are considered inadequate to reflect the entire rhizosphere microbiota. Hence it is advised that culture-dependent and culture-independent methods are combined to reveal more complete information regarding microbial communities (Shade *et al.*, 2012; Edenborn & Sexstone,

2007; Nybroe *et al.*, 2007). Several culture-independent methods have been used to study microbial communities, both DNA based and non-DNA based. Among the non-DNA based are phospholipid fatty acid analysis (PLFA) (Söderberg *et al.*, 2004; Khalil & Alsanusi, 2001) and fatty acid methyl ester (FAME) (Ibekwe & Kennedy, 1999), where the presence of specific signature fatty acids reveals the abundance of specific groups of microorganisms (Kent & Triplett, 2002).

A number of DNA-based techniques have been applied to analyze rhizosphere microbial communities, *e.g.* terminal restriction fragment length polymorphism (T-RFLP) (Zhang *et al.*, 2011; Smalla *et al.*, 2007), denaturing gradient gel electrophoresis (DGGE) (Smalla *et al.*, 2007; Calvo-Bado *et al.*, 2006; Postma *et al.*, 2005), and 454-pyrosequencing (Bakker *et al.*, 2014; Carvalhais *et al.*, 2013; Yu *et al.*, 2012; Gottel *et al.*, 2011). In DGGE, polymerase chain reaction (PCR) products are separated on a gel containing a chemical denaturant, which allows for separation of DNA fragments of the same length on the basis of their differing GC content and distribution. The community profiles from different samples can thus be compared and the level of similarity calculated (O'Callaghan *et al.*, 2006). High-throughput sequencing, such as 454-pyrosequencing, is a culture-independent technique that allows rapid characterization of microbial communities. Compared to Sanger sequencing, 454-pyrosequencing requires only 10 % of the costs for the same number of sequences, with only a fraction of the time needed (Jones, 2010). Another advantage over Sanger sequencing is that high-throughput sequencing requires no gels or capillaries to separate products by size. The principle of 454-pyrosequencing is that genomic DNA fragments are bound individually to microscopic beads, and thereafter clonally amplified by emulsion PCR. The beads are then transferred to picotiter plates, in which each well holds a single bead. The amplified DNA is subsequently sequenced by adding the four nucleotides (A-C-T-G) one by one. When a nucleotide is incorporated into the complementary template sequence, pyrophosphate is released and transformed into adenosine tri-phosphate (ATP). ATP reacts with luciferase for the oxidation of luciferin, resulting in light being emitted from the respective wells. The sequences of the DNA fragments are determined through the light signals emitted from the wells (Thomas *et al.*, 2012; Jones, 2010; Madigan *et al.*, 2009).

3 Objectives

The main objective of the present thesis was to investigate changes occurring in the root-associated microbial communities of tomato plants in closed hydroponic cropping systems, caused by biotic (pathogen) and abiotic (nutrient concentration, plant protection products) factors. The more specific objectives were:

- to determine if community-level physiological profiling (CLPP) can be used to monitor changes in the rhizosphere microbial communities inflicted by a pathogen (Paper I)
- to characterize the bacterial communities of non-inoculated and *Pythium ultimum*-inoculated tomato plants (Paper II)
- to characterize the role of organic carbon in microbial growth in the nutrient solution (Paper III)
- to determine if fluctuations in inorganic nutrient concentration affect microbial growth in the nutrient solution (Paper III)
- to characterize changes in the microbial communities associated with reused nutrient solution caused by use of plant protection products (Paper IV).

The hypotheses included in the present thesis were as follows:

- the microbial communities of roots change in the presence of a pathogen (Papers I and II)
- certain organic nutrient sources are used more quickly in the presence of a pathogen and thus act as signature compound candidates (Paper I)
- the microbial community structure is affected by plant age (Paper I)

- there is an increase in the number of bacteria associated with the roots and nutrient solution of *P. ultimum*-inoculated plants compared with non-inoculated plants (Paper II)
- organic carbon is a driving force in microbial growth (Paper III)
- fluctuations in organic carbon and inorganic mineral nutrient composition affect microbial growth (Paper III)
- roots infected with a pathogen will release more organic carbon than healthy roots (Paper III)
- pesticides used in closed hydroponic greenhouse systems to counteract foliar diseases do not persist in the nutrient solution beyond the waiting period (Paper IV).

4 Materials and Methods

4.1 Phytotron and greenhouse experiments

4.1.1 Plant material (Papers I-III)

For the experiments conducted in Papers I-III, the tomato cultivar Tiesto was used. Seeds were germinated in Petri dishes and subsequently transferred to hydroponic containers filled with nutrient solution in a static aerated culture. The tomato seedlings were supported by a polyethylene foam mat and the nutrient solution was continuously aerated. To provide the plants with optimal conditions, they were placed in a phytotron chamber for the first six weeks of the experiments. For the trials on fruit-bearing plants in Papers I and II, however, the seedlings were placed in the greenhouse directly after germination. The nutrient solution was prepared in accordance with Sonneveld and Straver (1989) and contained KNO_3 8.01 mM, NH_4NO_3 0.6 mM, MgSO_4 1.88 mM, $\text{Ca}(\text{NO}_3)_2$ 4.2 mM, Fe-EDTA 18.73 μM , $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ 6.25 μM , $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 12.5 μM , H_3BO_3 31.25 μM , $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.94 μM , and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.63 μM . The ion strength of the nutrient solution was gradually increased during the first five weeks from EC 1.5 at planting, reaching EC 2.5 at week 5. After the initial six weeks, the plants were moved to the greenhouse for spacing and transferred to larger growing containers. Information about climate settings is provided in Table 1.

Table 1

	Temperature	Light	Light source	Photoperiod
Phytotron	19 °C day; 18 °C night	400 $\mu\text{mol m}^{-2} \text{s}^{-1}$	Fluorescent lamps	16 h
Greenhouse	19 °C day; 18 °C night	200 W m^{-2}	High-pressure sodium	12 h*

*Provided when natural light conditions were low



Figure 1. Experimental set-up in the phytotron and in the greenhouse (Photo: Anna Karin Rosberg).

4.1.2 Oomycete inoculum preparation and inoculation (Papers I-III)

For plant inoculation, a kanamycin-resistant *Pythium ultimum* isolate was used (Hultberg, 1999). The isolate was kept on potato dextrose agar (PDA) supplemented with kanamycin ($200 \mu\text{g mL}^{-1}$) during laboratory storage. To avoid loss of pathogenicity, the isolate was repeatedly re-isolated from diseased tomato seedlings. For inoculation of the tomato plants, mycelium was used in the form of entire mycelium mats and as a mycelium fragment suspension for inoculation of the seedlings in the Petri dishes. The mycelium mats were produced, as described by Hultberg *et al.* (2011), by taking mycelium of *P. ultimum* growing on PDA and transferring it to water agar plates and leaving it to grow for two days. Small plugs were then taken from the periphery of the colonies and placed individually in Petri dishes containing 15 mL of cleared V8 broth (Table 2), and incubated at $25 \text{ }^{\circ}\text{C}$ in darkness for three days. The mycelium mats were then washed twice in a sterile mineral solution and once in sterile deionized water before being used to inoculate the plants. For the mycelium fragment suspension, mycelium mats were mixed with sterile deionized water using a high-frequency blender (Polytron, Kinematica GmbH, Switzerland) to produce a suspension with a mycelial fragment concentration of $2.7 \cdot 10^7 \text{ L}^{-1}$.

Half of the tomato plants were inoculated with *P. ultimum*, and the other half were used as healthy control plants. In Papers I and II the seedlings were inoculated five days after sowing, the flowering plants were inoculated two and six weeks after the sowing date, and the fruit-bearing plants were inoculated 14 and 16 weeks after the sowing date. In Paper III all plants used in the experiment were inoculated three and six weeks after sowing. The seedlings were inoculated by giving each Petri dish five mL of the mycelium fragment suspension, while the plants growing in nutrient solution were given one mycelial mat each in their respective growing containers. The fruit-bearing

plants in Papers I and II were inoculated with three mycelial mats each at both inoculation events to ensure heavy infection considering the large plant size.

Table 2. Overview of solutions used for inoculum preparation and recovery from roots

V8 broth	Mineral solution	Wash solution
11.5 g oatmeal	0.145 g $\text{Ca}(\text{NO}_3)_2$	1 g peptone from meat
125 mL V8	0.012 g MgSO_4	(enzymatic digest: Fluka Chemika, Buchs, Switzerland)
500 mL deionized water	0.099 g KH_2PO_4	2 g Na-hexametaphosphate
0.94 g CaCO_2	0.016 g FeCl_3	(Fluka Chemika, Buchs, Switzerland)
	1 L deionized water	1 L deionized water



Figure 2. Left to right: mycelium mat of *P. ultimum* grown in a Petri dish; tomato roots inoculated with *P. ultimum*; non-inoculated tomato roots (Photos: Anna Karin Rosberg)

4.2 Sampling from a commercial greenhouse

In Paper IV samples of nutrient solution were collected from a commercial greenhouse in the south of Sweden with a tomato production area of 0.4 ha. The greenhouse was equipped with a closed hydroponic system in which the plants were grown in plastic containers filled with pumice. Fertigation was supplied to the plants through drip irrigation, and drained nutrient solution was collected in an open gutter system. The drainage was filtered through a slow filter consisting of mineral wool and supplemented with fresh water, as well as adjusted for pH and EC, before it was resupplied to the crop. At the point of sampling, the nutrient solution had been recycled since the start of the cropping season without any discharges.

4.3 Analyses

4.3.1 Plant analyses (Papers I-III)

Destructive plant analyses were performed each week for Paper III, and on three occasions for Papers I and II, to study the possible physiological consequences of inoculation with a root pathogen. The analyses included fresh and dry weight of roots, leaves, stems and fruits. For the dry weight measurements, the plant parts were placed in aluminum foil envelopes and placed in a drying cabinet at 105 °C for five days. Leaf area was measured using a LI-COR Li 3100 Area Meter (LI-COR Instruments Inc., USA).

4.3.2 Nutrient solution analyses (Papers II, III and IV)

Nutrient content

The analysis of nutrient concentration (mg L^{-1}) in the nutrient solution was performed by the accredited laboratory LMI AB (Helsingborg, Sweden). The method used was inductively coupled plasma optical emission spectrometry (ICP-OES). Nutrient solution samples were taken from both non-inoculated and *P. ultimum*-inoculated plants (Paper III).

Organic carbon

In Papers II and III, the level of total organic carbon (TOC, mg L^{-1}) and dissolved organic carbon (DOC) was measured in the nutrient solution of each individual plant using the HACH LANGE system (TOC cuvette test LCK 385, HACH LANGE GmbH, Düsseldorf, Germany). A sample of the nutrient solution was added to a cuvette and then placed in a shaker (TOC-X5) for 5 min where all of the total inorganic carbon (TIC) was expelled. After this the cuvette was placed in a heating block (LT 200, HACH LANGE GmbH, Düsseldorf, Germany) set to 100 °C for 120 min. During this time the TOC is oxidized to CO_2 which passes through a membrane and into an indicator cuvette where it causes a color change. The color reaction was read using a photometer (Xion 500, HACH LANGE GmbH, Düsseldorf, Germany).

Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS)

For quantification of fenhexamid in the nutrient solution samples taken from the commercial greenhouse, ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was undertaken by the accredited laboratory Eurofins Food and Agro Testing (Kristianstad, Sweden).

4.3.3 Harvesting of plants and extraction of microbiota (Papers I-III)

For Papers I and II, plants were harvested on three occasions – as seedlings, flowering plants and fruit-bearing plants – while in Paper III plants were harvested each week. The roots were washed as described in Papers I-III and used for plate counts, community-level physiological profiling (CLPP), denaturing gradient gel electrophoresis (DGGE) and 454-pyrosequencing.

4.3.4 Culture-dependent microbiological analyses (Papers I-IV)

Plate counts

Aliquots from the root wash solution and from the nutrient solution obtained from non-inoculated and *P. ultimum*-inoculated plants were dilution plated on different nutrient media targeting different microorganisms (Table 3). Viable bacteria and fungi were enumerated as colony-forming units (CFU) ml⁻¹ and g dw⁻¹ (Papers I-IV).

Community-level physiological profiling (CLPP)

In Paper I, microbial utilization of organic compounds was studied using Phenotype MicroArray (PM) panels PM1, PM2A and PM3B (Biolog Inc., Haywood, CA, USA). The PM panels are microtiter plates with 96 wells: one control well and 95 wells containing a single organic nutrient source. PM panels 1 and 2A contained different carbon compounds and panel 3B contained nitrogen compounds. A tetrazolium dye was added to the microtiter plates along with the inoculation of the root microbiota, which gave rise to a color change as the microbiota metabolized the organic compounds. The number of organic compounds and the level of utilization were read spectrophotometrically. For details concerning inoculation, incubation and data collection, see Paper I.

Table 3. Overview of plate count analysis media used for enumeration of different microbial groups

	R2A	Tryptic soy agar (TSA)	King's B agar	Corn meal agar (CMA)	Malt extract agar (MA)	Potato dextrose agar (PDA)
Target organism	total aerobic counts (nutrient solution)	total aerobic counts (rhizosphere)	fluorescent pseudomonas	<i>Pythium ultimum</i>	fungi	culturing <i>P. ultimum</i>
Product number	Difco 218263	Difco 236950		Difco 0386-17-5	Difco 218630	Difco 213400
Reference			King <i>et al.</i> , 1954			
Supplements				rifampicin 50 µg ml ⁻¹ ; kanamycin 200 µg ml ⁻¹	Bacto agar, 2 % (Difco 0140-01)	
Incubation time (h)	72	72	48	24	96	
Incubation temperature (°C)	25	25	25	22	25	Room temperature

4.3.5 Culture-independent microbiological analyses (Papers I, II and IV)

Denaturing gradient gel electrophoresis (DGGE)

The rhizosphere bacterial communities of non-inoculated and *P. ultimum*-inoculated tomato plants (Paper I) and of nutrient solution from a tomato culture with and without the addition of a fungicide (active substance fenhexamid) (Paper IV) were investigated using PCR-DGGE. Samples of the wash solution from roots and from the nutrient solution were processed as described in Papers I and IV respectively. The obtained pellets were used for extraction of genomic DNA (DNeasy Plant Mini Kit, Cat. No. 69104, Qiagen GmbH, Hilden, Germany). Bacterial amplifications were performed using the primers F968 and R1378 for primary PCR, and with the forward primer GC-F968 containing a GC-clamp for the secondary PCR (Muyzer *et al.*, 1993).

The PCR products were loaded on acrylamide gels (8 %) containing a linear gradient of 45-60 % denaturant. The gels ran at 70 V in 1xTAE buffer at 60 °C for 16 h, using a CTV 400 System (Cat. No. 700-71149, VWR International, Stockholm, Sweden). The gels were stained using 1xTAE buffer and SybrGold.

454-pyrosequencing

The rhizosphere bacterial communities of non-inoculated and *P. ultimum*-inoculated tomato plants were investigated using 454-pyrosequencing in Paper II. Samples from the wash solution of roots were processed as described in Paper II. The obtained pellets were used for the extraction of genomic DNA (DNeasy Plant Mini Kit, Cat. No. 69104, Qiagen GmbH, Hilden, Germany). For pyrosequencing, the DNA extractions of the five replicates from each treatment were pooled to form one 16S rRNA library for each treatment and plant stage (Paper II). Due to the low concentration of DNA in the samples, a multiple displacement amplification (MDA) was carried out on the pooled DNA samples using the illustra GenomiPhi V2 DNA amplification kit (GE Health Care Life Sciences, Uppsala, Sweden). After MDA, a DNA concentration of 415-1250 ng μL^{-1} was achieved, measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA).

The DNA samples were sent to LGC Genomics GmbH, Berlin, Germany for pyrosequencing analysis where the Roche 454 FLX-Titanium chemistry system was used (Roche/454 Life Sciences, Bradford, CT 06405, USA). The primers used by LGC were GM3 and 926R, targeting the V1-V5 region of the 16S gene.

4.3.6 Statistical analyses

Basic statistical analyses (Papers I-IV) were performed using Minitab Statistical Software version 16.1.0 (Minitab Inc., USA). PCR-DGGE gels were analyzed with the GelCompar II program versions 4.1 (Paper I) and 6.5 (Paper IV) (Applied Maths, Sint-Martens-Latem, Belgium). Discriminant analysis of data from the CLPP in Paper II was carried out with SAS v. 9.2 (Institute Inc., Cary, NC, USA). Analysis of the 454-pyrosequencing data was performed by the bioinformatics services at LGC Genomics using QIIME Virtual Box v. 1.6.0. The PAleontological STatistics software package (PAST) version 3.0 was used for additional calculations on the pyrosequencing data. A detailed description of the statistical methods used is given in each individual paper.

5 Results and Discussion

5.1 Impact of a root pathogen on root-associated microbial communities

The rhizosphere is a place of great microbial activity closely linked to the status of the plant (Mendes *et al.*, 2013; Hinsinger *et al.*, 2009; Jones & Hinsinger, 2008). Damage to plants caused by necrotrophic pathogens, such as *P. ultimum*, have been shown to activate the jasmonic acid defense pathway. This activation alters the composition of the root exudates and consequently the rhizosphere microbial communities (Carvalhais *et al.*, 2013). A range of studies have compared the rhizosphere microbiota of diseased and non-diseased plants in soil and compost (Zhu *et al.*, 2013; Yu *et al.*, 2012; Zhang *et al.*, 2011; Hagn *et al.*, 2008; Fillion *et al.*, 2004; Gardener & Weller, 2001), and found differences in the microbial communities due to pathogen attack. For soilless cultures, few studies deal with both quantitative and qualitative differences related to pathogen infection.

In the present thesis, the effect of pathogen attack on microbial communities associated with the roots of tomato plants in soilless culture was examined. Differences were seen in the microbial community structure between non-inoculated and *P. ultimum*-inoculated tomato plants through DGGE, CLPP (Paper I) and 454-pyrosequencing (Paper II). There were, however, significant individual differences between the replicates, which were clearly seen in the DGGE results (Paper I). When the replicates were run individually on the gel, there was no clear treatment effect. However, when the replicates for each treatment were pooled, the distinct treatment effects became evident. The pooling of samples has been shown to omit many species and thus comparisons of individual samples portray a more accurate picture of the species' diversity (Manter *et al.*, 2010). It should, however, be borne in mind that DGGE is a method that has been estimated to detect only those microbial

community members that represent $\geq 1-2$ % of the population in an environmental sample (Muyzer *et al.*, 1993). This implies that even with replicates and pseudo-replicates, some of the microbial community will still remain undetected. The differences seen between treatments of the pooled samples are therefore most likely to reflect differences among the species of highest abundance.

With CLPP it was found that the presence of *P. ultimum* did change the utilization of different carbon sources, and significant differences between the treatments were found in this respect. Despite considerable differences between replicates with this method as well, there was an abundance of carbon sources with significantly different utilization on a biochemical basis (Table 2, Paper I). This fact provides good evidence that the microbial communities did indeed change due to pathogen attack. Considering the results from DGGE of the individual samples, the change in the microbial communities might not have been structural, but could have been in relation to activity and function. According to Blagodatskaya and Kuzyakov (2013), the active part of the total microbiota is approximately 0.1-2 %, while the fraction of potentially active microorganisms is 10-60 %. The potentially active microorganisms have a quick response to the influx of new substrates, such as a potential difference in root exudates due to pathogen attack. DGGE and other DNA-based methods cannot differentiate between active and non-active microorganisms (Bodrossy *et al.*, 2006).

When the same samples were subjected to 454-pyrosequencing, clear differences in bacterial community structure were seen between the non-inoculated and *P. ultimum*-inoculated tomato plants. This is illustrated in Figure 3. The root-associated microbial communities were highly dominated by Proteobacteria. This has also been found by Calmin *et al.* (2008) and Deery (2012) in the rhizosphere of tomato, and in the rhizosphere of many other crops by Filion *et al.* (2004), Hagn *et al.* (2008), Zhang *et al.* (2011), and Zhu *et al.* (2013). Despite a high number of reads for each sample, ranging from 11, 283 to 51,453, the results do not reflect an entirely accurate view of the bacterial communities. Replicates are essential due to the heterogeneity of microbial communities, and without an estimate of variability no conclusion can be drawn (Prosser, 2010). However, the present results give a general understanding and a trend analysis of the microorganisms present in the rhizosphere of non-inoculated and *P. ultimum*-inoculated tomato plants.

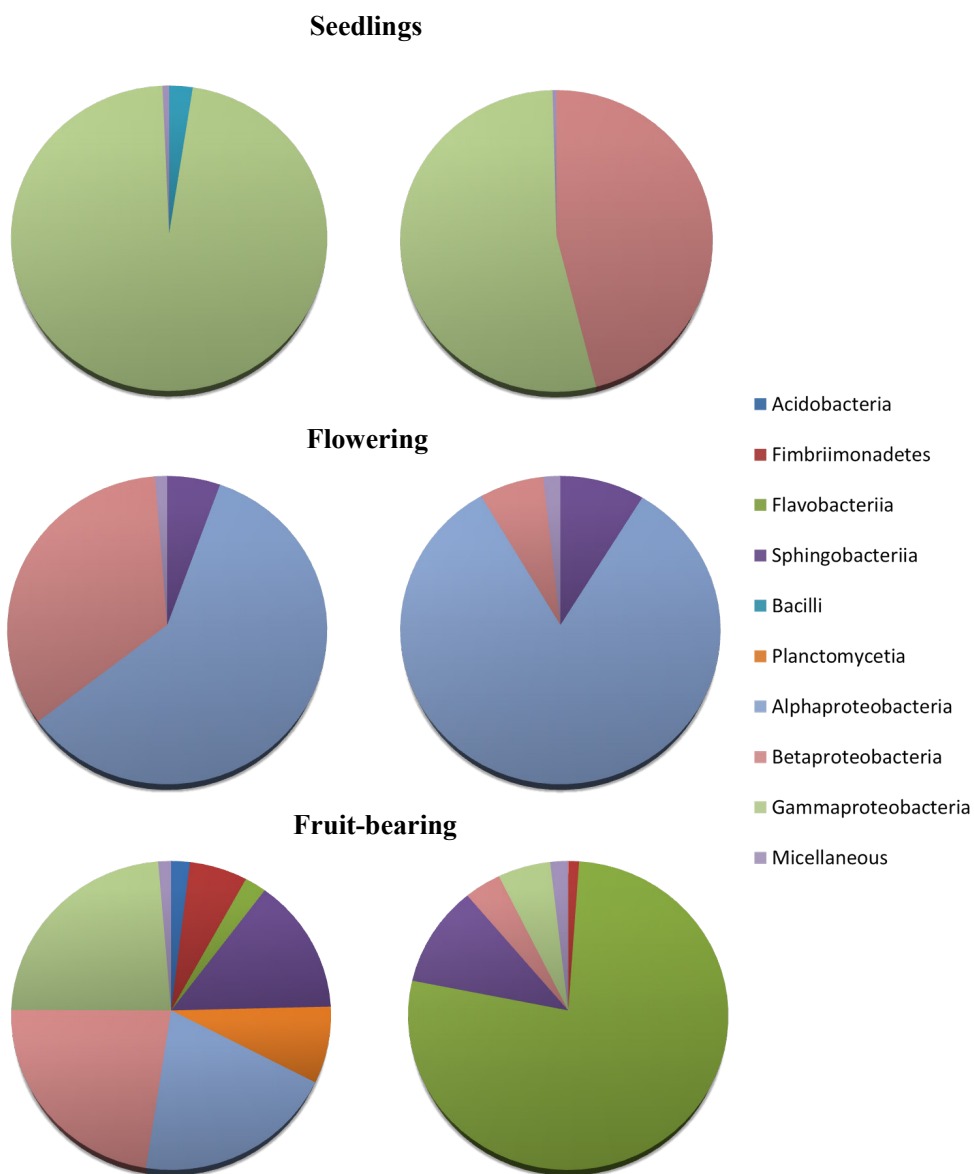


Figure 3. Results from 454-pyrosequencing of the bacteria present in the rhizosphere of non-inoculated and *P-ultimum*-inoculated tomato plants, at class level. Left column: non-inoculated treatment; right column: *P. ultimum*-inoculated treatment.

5.2 Impact of plant age on root-associated microbial communities

The impact of plant age on rhizosphere microbial communities has been shown by several authors (Micallef *et al.*, 2009; Houlden *et al.*, 2008; Marschner *et al.*, 2002). It is a well-established fact that root exudation changes with plant age (Rovira, 1959), hence changes in microbial community structures are most likely caused by changes in root exudation patterns (Micallef *et al.*, 2009; Marschner *et al.*, 2002).

In the present thesis, the effects on the microbial communities related to plant age were found using DGGE, CLPP (Paper I) and 454-pyrosequencing (Paper II). In contrast to the effect of a pathogen, the effect of plant age was clearly seen with DGGE when individual replicates were compared (Paper I). At the seedling and flowering plant stage, clear clusters were formed. At the flowering stage, however, the replicates were more diverse. Postma *et al.* (2000) demonstrated that the complexity of bacterial communities increased with plant age, resulting in a decrease of replicate similarity. This also seems to be the case in the present thesis.

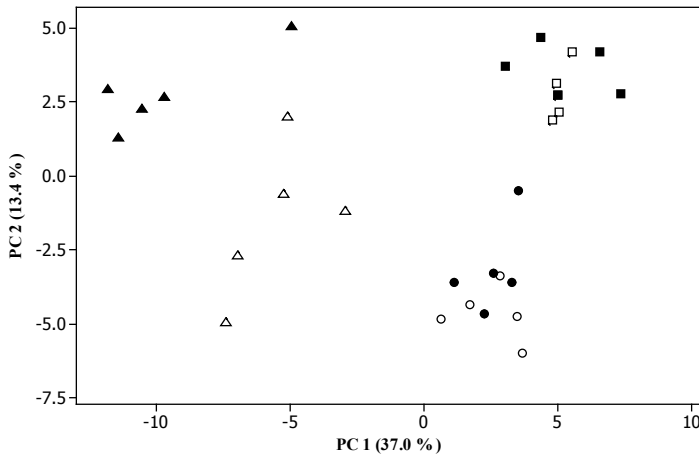


Figure 4. Ordination plot of principal components (PC) 1 and 2 from community-level physiological profiling (CLPP) of root microorganisms on tomato seedlings (Δ, ▲), and flowering (○, ●) and fruit-bearing (□, ■) plants. CLPP was established from carbon substrate utilization patterns obtained using PM panel 2 (Biolog). Filled and open symbols indicate inoculated (*P. ultimum*) and non-inoculated plants respectively.

When the results of the CLPP were subjected to PCA, clear clusters with respect to plant physiological stages became apparent (Figure 4). With this analysis the microbial community structure of seedlings showed the highest

diversity between replicates. This high variability between seedling replicates could be due to the fact that each replicate, consisting of 100 seeds, was grown in a sealed Petri dish, inhibiting the migration of microorganisms from the surrounding environment. The microbiota developing in each Petri dish was therefore a result of microorganisms already present on the seeds and in the water. At the flowering and fruit-bearing stages, however, the plants were grown in open containers and subjected to a nutrient solution. Despite the large number of carbon sources for which the utilization was significantly different between the treatments, few of these carbon sources reoccurred in two plant stages, and none reoccurred in all three. This implies that the microbial community structure, activity or function was different at the different plant stages.

With 454-pyrosequencing the differences in bacterial communities between the plant stages became apparent. The microbiota formed clear groups with respect to plant age in the PCA. Similar findings were made by Zhang *et al.* (2011) who saw clear differences in bacterial communities at the different plant physiological stages of cotton. Bacterial diversity initially increased and then decreased at plant maturity, while in the present thesis the bacterial species' richness and diversity increased with plant age. As illustrated in Figure 3, not only the classes of bacteria differed, but the species' richness also increased with plant age. Although there was a high number of reads per sample, rarefaction analysis showed that the samples from the fruit-bearing stage did not reach saturation. Hence it can be assumed that the species' richness and the complexity of the microbial communities are greater than the result suggests.

5.3 Impact of available nutrients on nutrient solution-associated microbial communities

The first two sections of this chapter have focused on the root, which is the main producer of carbon in the hydroponic cropping systems used in the present thesis. The following two sections will focus on the microorganisms present in the nutrient solution (Papers III-V). These are the main consumers and transformers of the carbon provided by the roots. As seen in Figure 5, the carbon in hydroponic cropping systems is provided by the macrophytes, *i.e.* plants, as well as by phytoplankton and biofilms that develop on surfaces such as pipes and growing containers (Paper V). If a growing medium is used, then depending on its origin, this could also act as a supplier of carbon. The main users of the carbon pool are free-living microorganisms and microorganisms associated with biofilms.

Organic carbon is the dominant energy source in the root environment that is available to heterotrophic microorganisms, and is also the major constituent of the microbial cell (Madigan *et al.*, 2009). Organic carbon, however, is not the only nutrient needed for microbial growth. Mineral nutrients, such as nitrogen and phosphorous, as well as micronutrients are also required (Duffy & Defago, 1997; Fagerbakke *et al.*, 1996). The number of microorganisms inhabiting the rhizoplane and nutrient solution fluctuates during the growing season in hydroponic cropping systems with tomato (Khalil & Alsanius, 2001). There are few explanations as to why these fluctuations occur, other than the impact of plant physiological age discussed in the previous section.

In the present thesis, a study was conducted regarding the interactivities between available organic carbon and mineral nutrients in the nutrient solution from microbially-colonized hydroponic cropping systems (Paper III). Regression analysis indicated that the level of organic carbon was highly correlated with the number of bacteria, fungi and fluorescent pseudomonads. The correlation values were particularly high in the non-inoculated treatment, with R^2 values as high as 95.9 for the correlation between organic carbon and fluorescent pseudomonads. Despite differences in the concentration of TOC and DOC, both fungi and fluorescent pseudomonads were equally correlated with TOC and DOC. The concentrations of TOC and DOC were quite similar (Paper III; Table 3) but in the last five weeks of measurements, DOC did not increase at the same rate as TOC. While TOC consists of plant organic matter, microorganisms and DOC, DOC contains the low-molecular weight compounds readily available for the microbiota, as well as some high-molecular weight compounds of hydrophilic and acidic origin (Wetzel, 1983). The increasing difference in TOC and DOC therefore indicates that a larger part of the organic carbon became organically bound to roots, microorganisms and microfauna, for example. The high level of DOC measured means that carbon alone was not sufficient as a nutrient source, but that other nutrient sources had a limiting effect on the number of microorganisms. The high levels of DOC could also be an indication that the available organic carbon compounds were in a form that the resident microbiota could not degrade (Alsanius & Jung, 2004). It has been found that carbon-to-nitrogen ratios (C:N) are important for microbial growth, with a C:N ratio of 15 preferred by bacteria and a C:N ratio of 75 by fungi (Rousk & Bååth, 2007). In the present study, the C:N ratios were more favorable to fungi. This could be seen in the numbers of CFU, which increased over time for fungi but stayed at a relatively stable level for the bacteria.

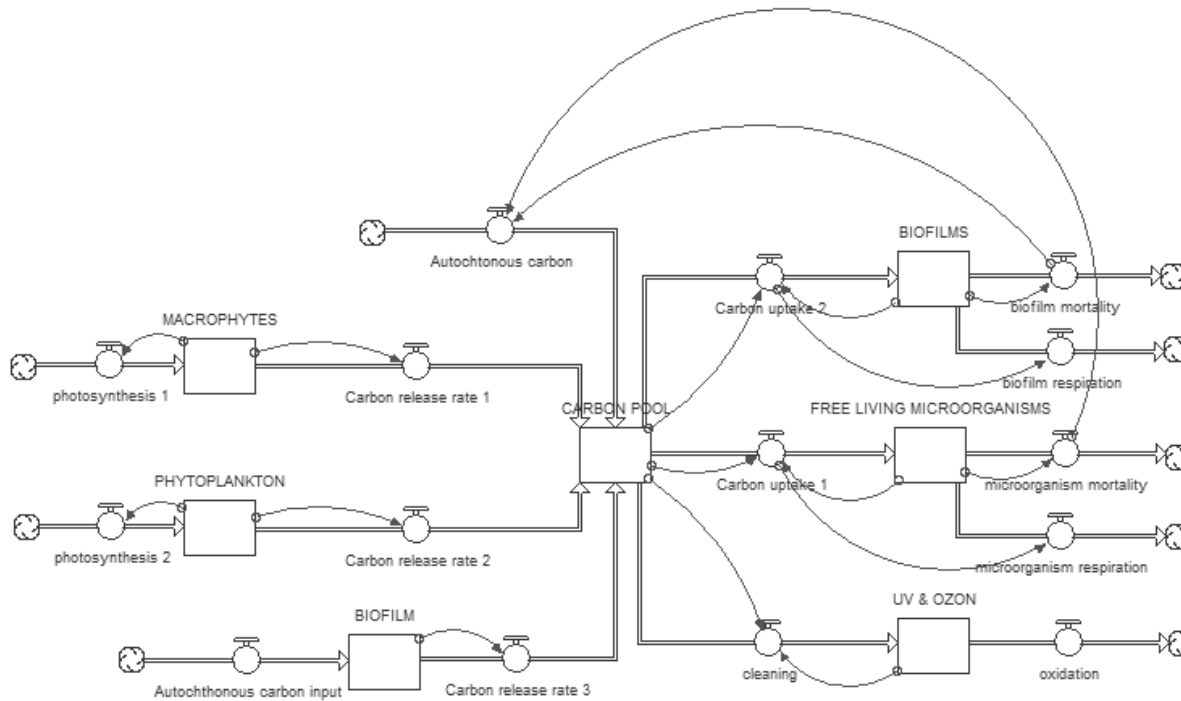


Figure 5. Flow of organic carbon in a hydroponic growing system where the autochthonous and allochthonous carbon is provided by macrophytes, phytoplankton, biofilms and carbon sources associated with the cropping system. The consumers of carbon are biofilms and free-living microorganisms, while the use of UV or ozone leads to a removal of carbon from the system (Paper V; Figure 28.2).

The quantity and quality of nutrients are both important. Hultberg and Alsanius (2008) saw that production of the antibiotic 2,4-diacetylphloroglucinol (DAPG) by the biocontrol agent *Pseudomonas fluorescens* Pf-5 was highly dependent on the type of nitrogen source provided. Similarly, micronutrients are known to act as catalysts for the production of secondary metabolites. It has been shown that production is highly sensitive to fluctuations in micronutrient availability (Zweifel *et al.*, 1993).

The type of carbon source available is also important. From the CLPP study in the present thesis (Paper I), it was seen that not all carbon sources available were utilized at the same rate. This could be an effect of adaptation by the microorganisms to the carbon sources to which they had not previously been exposed. Given time, all sources were used to some degree. There were differences in the utilization patterns, both with respect to treatment and to plant age. This implies that there were changes in the microbial community structures or that the active parts of the communities changed due to differences in root exudation patterns as a result of pathogen infection and the ageing of the plants.

5.4 Impact of plant protection products on nutrient solution-associated microbial communities

Disturbances of rhizosphere microbial communities through the use of plant protection products have been shown in several studies (Spyrou *et al.*, 2009; Borzi *et al.*, 2007; Engelen *et al.*, 1998). Depending on the pesticide used, the effect has been found to act either to eliminate certain organisms or to stimulate their growth. The use of fenhexamid in soil has been shown to exhibit a selective pressure on some microbial communities where a number of microorganisms disappeared and a few increased. It was hypothesized that the species increasing in numbers after the addition of the pesticide were those able to degrade xenobiotics (Borzi *et al.*, 2007).

In the present study (Paper IV), the number of CFUs obtained through plate counts differed between untreated and fenhexamid-treated nutrient solutions. With the presence of fenhexamid, the number of bacterial CFUs started to decrease after just two days. Without fenhexamid, differences in plate counts were first seen after eight days. However, at the end of the exposure time, the number of bacterial counts was higher in the fenhexamid-supplemented medium compared to the control. For the fungal flora, counts dropped immediately after exposure to fenhexamid and did not recover for the length of the experiment. This was to be expected since fenhexamid is a fungicide with

Botrytis as the main target. Through DGGE analysis, a shift in the bacterial community structure was seen after eight days.

Pesticides which are dissolved in water are available as substrates for the microorganisms, and are metabolized through enzymatic hydrolysis, oxidation and reduction. Both free-living microorganisms and those associated with biofilms are involved (Katagi, 2013). The level of fenhexamid in the nutrient solution was unaffected after 21 days of incubation. Hence no degradation by the microorganisms occurred. The increase in bacterial counts was therefore not due to the utilization of fenhexamid, but was more likely an effect of mineralization of the organic carbon from the microorganisms that did not survive the pesticide treatment, predominantly fungi. Previous studies have demonstrated similar findings where a lack of degradation was revealed after 112 days of incubation in natural river water (Abbate *et al.*, 2007). In the same study, fenhexamid had a DT_{50} of 1.2 days in the presence of an active microbiota, while in the absence of microorganisms, fenhexamid proved to be stable. The degradation of the insecticide fenthion increased in the presence of plants and plant-associated microorganisms (Cripe *et al.*, 1989). The absence of plants in the experiments of Paper IV might have been a factor limiting the degradation of fenhexamid.

6 Conclusions

In the present thesis, it has been demonstrated that root-associated and nutrient solution-associated microbial community structures are affected by plant age, pathogen attack, the availability of organic and inorganic nutrients, and the use of plant protection products. Plant age in particular seemed to have the greatest impact on community structure.

The combined use of culture-dependent and culture-independent techniques proved successful, since different parts of the microbial communities were visualized with different methods. The methods used, however, did not target the active parts of the communities. There should be a focus in future studies on microbial activity and function through the use of metabolomics and metaproteomics. Changes induced by abiotic and biotic factors are more likely to be seen in microbial activity rather than in qualitative, structural alterations. The activity and function of the microbial communities were partly seen with the use of CLPP, where it was demonstrated that the utilization of sole carbon sources differed between treatments and between plant physiological stages. This could imply changes in the activity of different microbial species caused by pathogen inoculation.

The rhizosphere is one of the most complex ecosystems on earth. Hence, additional environmental factors, such as temperature, light intensity and oxygen saturation of the nutrient solution, should be monitored closely in future studies in order to understand their combined effect on microbial communities.

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