Root-Associated Microbial Communities of Different Strawberry Cultivars as Influenced by Soil Type, *Verticillium dahliae* Kleb. and Biofumigation

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Strawberry (*Fragaria x anannasa*) Honeoye cultivar at its fruiting stage.(photo: Srivathsa Nallanchakravarthula)

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Abstract

Rhizosphere microorganisms and their interactions with plant roots play pivotal roles in controlling plant nutrition and health. Extremely high levels of soil microbial diversity, coupled with low levels of cultivability, complicate the study of these organisms but better mechanistic understanding of their interactions with each other and with plant roots is a prerequisite for development of sustainable management strategies to improve nutrient acquisition and control pathogens. This thesis describes different experiments designed to investigate how the community structure of fungi associated with rhizosphere soils and roots of strawberry plants are influenced by different soil types, different plant cultivars, the presence or absence of the soil-borne fungal pathogen *Verticillium dahliae* and biofumigation using plant residues of oilseed radish *Raphanus sativus oleifera*.

In an outdoor pot experiment, using cloning and Sanger sequencing, the community composition and overall levels of colonization by arbuscular mycorrhizal fungi appeared to be more strongly influenced by soil type than by different strawberry cultivars. In a similar experiment the effects of inoculation with *Verticillium dahliae* on the total fungal community structure were analyzed using high throughput 454-pyrosequencing. The inoculation with *V. dahliae* resulted in significant reduction in the numbers of operational taxonomic units (OTUs) associated with rhizosphere soil of four cultivars grown in a conventionally managed soil, but in an organically managed soil, no significant effects in two cultivars, and a large increase in numbers of OTUs in Florence, a tolerant cultivar. Non-metric multidimensional scaling (NMDS) analysis of rhizosphere communities in a less diverse peat-based soil revealed distinct clusters associated with *Verticillium* and non-*Verticillium* treatments but this effect was not visible in two more diverse field soils.

A third study of fungal communities associated with biofumigation treatments in a field soil, using 454 pyrosequencing, indicated significantly increased numbers of OTUs associated with biofumigation and *Verticillium*-inoculation in the absence of strawberry plants, suggesting a green-manuring effect of oilseed radish incorporation. Biofumigation did not affect total OTUs in the presence of strawberry plants but NMDS analysis showed a clear effect of all treatments on community structure. Complementary analyses of changes in bacterial community structure in the same experiments are in progress and will hopefully shed more light on possible functional interactions underlying treatment effects and enable construction of hypotheses that can be tested in further experiments.

Keywords: arbuscular mycorrhiza, biofumigation, denaturing gradient gel electrophoresis, microbial communities, *Raphanus sativus oleifera,* soil type, strawberry cultivars, pyrosequencing, *Verticillium dahliae*

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Om Asato Maa Sad-Gamaya |
Tamaso Maa Jyotir-Gamaya |
Mrtyor-Maa Amrtam Gamaya |
Om Shaantih Shaantih Shaantih ||

Meaning:

Lead us from unreality (of transitory existence) to the reality (of self), lead us from the darkness (of ignorance) to the light (of knowledge), and lead us from the fear of death to the knowledge of immortality. Peace, peace, peace.

Contents

List of Publications

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

I Santos-González JC, Nallanchakravarthula S, Alström S, Finlay RD (2011). Soil, but not cultivar, shapes the structure of arbuscular mycorrhizal fungal assemblages associated with strawberry. *Microbial Ecology* (62)25–35.

II Nallanchakravarthula S, Mahmood S, Alström S, Finlay RD (2013). Changes in fungal community structure in the roots and rhizosphere of different strawberry cultivars in response to *Verticillium dahliae.* Manuscript

III Nallanchakravarthula S, Mahmood S, Alström S, Finlay RD (2013). Effects of biofumigation on fungal community structure in the roots and rhizosphere of strawberry. Manuscript

Paper I is reproduced with the permission of the publishers

Paper not included in this thesis

IV Nallanchakravarthula S, Mahmood S, Alström S, Finlay RD (2013). Changes in bacterial community structure in the roots and rhizosphere of different strawberry cultivars in response to *Verticillium dahliae* and biofumigation.

The contribution of Srivathsa Nallanchakravarthula to the papers included in this thesis was as follows:

I Initiation, planning, research design, carrying out the outdoor experiment including all observations, sampling and partial analysis of the results after discussion with S. Alström and R. Finlay. Molecular work on AMF, advanced data analyses and writing by J. Santos-González together with the co-authors

II Initiation, planning, research design, carrying out of the outdoor experiment including all observations and sampling. All preparatory experiments, all molecular work including nucleic acid extractions, PCR amplicons preparations for pyrosequencing, major part of data analyses, writing together with the co-authors

III Initiation, planning, research design, carrying out of the outdoor and greenhouse experiments including all observations and sampling. All preparatory experiments, all molecular work including nucleic acid extractions, PCR amplicons preparations for pyrosequencing, major part of data analyses, writing together with the co-authors

Abbreviations

AMF	Arbuscular mycorrhizal fungi
BLAST	Basic local alignment search tool
CTAB	Hexadecyltrimethylammonium bromide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
GLS	Glucosinolates
ITCs	Isothiocyanates
ITS	Internal transcribed region
NCBI	National Center for Biotechnology Information
NMDS	Non-metric multi-dimensional scaling
OTUs	Operational taxonomic units
PAST	PAleontological STatistics
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
RNA	Ribonucleic acid
SCATA	Sequence Clustering and Analysis of Tagged Amplicons
SIMPER	Similarity percentage
TAE	Tris-acetic acid

Background

Plants allocate carbon below ground in the form of root exudates thereby influencing the structure of microbial communities (Morgan *et al.*, 2005; Drigo *et al.*, 2010). The term 'rhizosphere' was coined by Lorenz Hiltner (Hiltner, 1904) to define the volume of soil in close proximity to roots that is characterised by elevated microbial populations. The rhizosphere is under continuous influence of living roots and is a unique habitat for soil microorganisms. The rich nutrient supply and close contact to the living roots enables rhizosphere microorganisms to have a direct influence on plant growth and plant pathogens. The rhizosphere has been described as both a 'playground and a battlefield for soil-borne pathogens and beneficial microorganisms' (Raaijmakers *et al.*, 2008).

Root exudates regulate different kinds of associations between the plant and soil microorganisms. Such associations depend upon the physicochemical factors such as pH, moisture and soil type, which affect microbial composition and diversity. A wide range of microbial communities co-exist in the rhizosphere. Various interactions such as commensalism, symbiosis and mutualism exist between them. Rhizosphere-inhabiting microorganisms produce a range of compounds that can be antagonistic to plant pathogens or stimulate plant growth directly. Successful application of these microorganisms and their interactions requires better understanding of which microorganisms produce which compounds under different field conditions.

High input of chemical pesticides and fertilizers has led to the marginalization of the functions of the native communities. Increasing environmental concerns in many countries including Sweden, have led to a search for alternative strategies for sustainable management of agricultural systems. It is of fundamental importance to understand the various mechanisms and processes that regulate soil ecosystem functioning. For sustainable

agriculture it is crucial to understand the importance of soil microorganisms in enhancing nutrient acquisition and sustainable plant protection.

This thesis unravels the microbial associations in the rhizosphere of strawberry *Fragaria x ananassa* Duchesne, the host plant, the effect of cultivar interactions and soil type in shaping community structure and the changes induced by biological management practices such as biofumigation against soil-borne pathogens.

1 Introduction

> "The soil is the great connector of lives, the source and destination of all. It is the healer and restorer and resurrector, by which disease passes into health, age into youth, death into life. Without proper care for it we can have no community, because without proper care for it we can have no life."

This statement made by Berry (1977) suggests the overarching importance of the soil-air-water biosphere for the organisms which inhabit it, and the human beings who depend upon it. Soil microorganisms play an important role in soil fertility and plant health (Berg, 2009). A fertile soil consists of diverse forms of organisms such as archaea, bacteria, fungi, protozoans, insects and, nematodes. Many different plant-microbe interactions occur in the rhizosphere, including those involving pathogens and symbionts. Microorganisms associated with plants have been demonstrated to suppress plant pathogens or act as biofertilizers. When such microorganisms are used in a controlled manner, these can enhance overall soil fertility and plant health (Berg, 2009).

1.1 Microbial communities in the Rhizosphere

In the rhizosphere many groups of micro-organisms predominate, and among these arbuscular mycorrhiza fungi (AMF), nitrogen-fixing bacteria, soil-borne pathogens, free-living fungi and bacteria, antagonistic/plant growth stimulating fungi and bacteria are some of these that are commonly known to occupy a shared micro-habitat. AMF are important components of soil microbial communities that form symbiotic associations with most terrestrial plants and contribute to host nutrient acquisition and pathogen control (Newsham *et al.*, 1995; Whipps, 2004). They belong to the phylum Glomeromycota.

Non-symbiotic bacteria and fungi in the rhizosphere, as well as those living endophytically in the roots, are also known to increase plant growth, either by facilitating nutrient uptake and production of plant growth hormones, or through conferring plant protection against pathogens. Plant growth promoting rhizobacteria are one of the most commonly studied rhizosphere components in terms of direct plant growth promotion and biological control (Lugtenberg & Kamilova, 2009).

The harmful components of microbial communities in the rhizosphere can cause diseases in plants by disturbing their metabolism and absorption of nutrients from host cells. Intensive cultivation of agricultural crops is associated with a high risk for increased incidence of different fungal diseases. Fungal and oomycetous pathogens such as *Verticillium dahliae*, *Rhizoctonia solani* and *Phythophthora* spp. are soil-borne and known to cause diseases in several crops including strawberry. Different pathogens attack different crops at different developmental stages. They are difficult to control by current agricultural practices involving fungicide application. There is a growing interest in developing new strategies based on use of beneficial microbial components of the rhizosphere, selective rotation of crops and green manuring in combination with biofumigation and soil solarisation.

1.1.1 Microbe interactions with plants

There is accumulating evidence that different interactions of rhizosphere micro-organisms with each other and with plants influence plant health. Inter- and intra-specific variation between different plant hosts to the inoculation of AMF has been observed (Norman *et al.*, 1996; Scheublin et al., 2007; Picard *et al.*, 2008; Fan *et al.*, 2011). In a study by (Norman *et al.*, 1996), there were different responses to inoculation with *Glomus fasciculatum*, indicating that specific interactions occur between different AMF species and strawberry cultivars. Reduced sporulation by *Phytophthora fragariae* has been reported in AMF-colonised plants in comparison to that in non-mycorrhizal strawberry plants (Norman & Hooker, 2000). Bacteria are found in association with AMF and might help in certain functions, such as enhancement of AMF colonization of roots acquisition of nutrients and suppression of plant pathogens (Budi *et al.*, 1999; Xavier & Germida, 2003). In an *in vitro* experiment *Paenibacillius validus* supported the growth of *G. intraradices* up to spore formation (Hildebrandt *et al.*, 2006). The colonization of *Gigaspora rosea* has been shown to be promoted by *P. putida* UW4 (Gamalero *et al.*, 2008). Various synergistic effects of AMF and bacteria can also be exploited for pathogen control and nutrient acquisition in low input agricultural systems (Johansson *et al.*, 2004; Artursson *et al.*, 2006; Bharadwaj, 2007).

Plants may influence rhizosphere microbial communities to inhibit pathogens in their vicinity (Berg *et al.*, 2006). Interactions between plant associated rhizosphere microorganisms with plant pathogens have revealed different mechanisms of antagonism. Antagonism may be caused by different metabolites or by mycoparasitism or competition for space, or by enzymes or through induced systemic resistance (Raaijmakers *et al.*, 2008; Raaijmakers & Mazzola, 2012). Some studies have demonstrated some of the detailed molecular mechanisms underlying antagonism of plant associated rhizosphere microorganisms towards soil-borne pathogens (Tjamos *et al.*, 2005; Mendes *et al.*, 2011; Berendsen *et al.*, 2012).

1.1.2 Factors affecting the structure of soil microbial communities

Plants invest carbon in the form of various organic compounds thereby influencing the community structure of rhizosphere microorganisms (Morgan *et al.*, 2005; Drigo *et al.*, 2010). Different plants influence the structure of soil microbial communities by selecting specific micro-organisms in their rhizosphere (Costa *et al.*, 2006; Haichar *et al.*, 2008). Soil characteristics are also known to influence the microbial community structure in the rhizosphere (Berg *et al.*, 2006; Berg & Smalla, 2009; Santos-González *et al.*, 2011; Peiffer *et al.*, 2013). When soil samples across two American continents were analysed for their bacterial communities they were unrelated to site, temperature, latitude, soil moisture or carbon:nitrogen ratio, among other variables, but were affected by pH (Fierer & Jackson, 2006) as also established by (Lauber *et al.*, 2009). A higher abundance of functional genes was shown to be expressed in organically managed strawberry fields than in adjacently located conventional soils, indicating diversified functions of the soil microbial community in the organic soil (Reeve *et al.*, 2010). A separate study conducted (Reganold *et al.*, 2010) on the same sites with an aim to compare the fruit quality in relation to soil type, showed that strawberry fruit quality was better from the organic soils and there was no significant difference in pH between the two types of fields.

Other than edaphic factors, biotic factors such as effect of plant and cultivar have also been studied (Sieling *et al*., 1997; Neupane *et al.*, 2013). Berg *et al.*, (2005) compared the strawberry and oilseed rape rhizospheres in *V. dahliae*-infested fields, and found antagonistic *Pseudomonas* spp. to be specific to strawberry at Rostock and Braunschweig soil sites, while *Serratia* spp. were found to be oilseed rape-specific at a Berlin soil site. (Costa *et al.*, 2006) also compared the bacterial profiles of oilseed rape and strawberry rhizospheres from different soil sites and found *Streptomyces* sp. and *Rhizobium* sp. to be

strawberry-specific and *Arthrobacter* to be oilseed rape-specific. A study using stable isotope probing confirmed that different plant species select particular bacterial communities (Haichar *et al.*, 2008). Strawberry rhizosphere harboured distinct microbial communities with respect to *Streptomyces*, *Rhizobium* and *Nocardia* in the presence of *Verticillium dahliae*, the bacterial community was dominated by *Pseudomonas* spp. populations (Smalla *et al.*, 2001; Berg *et al.*, 2005; Costa *et al.*, 2006).

In some studies, the effect of soil type on microbial community structure has been found to be more pronounced than the effect of cultivars (Wang *et al.*, 2008; Santos-González *et al.*, 2011). AMF sub-groups and beneficial bacteria, such as nitrogen fixers, 2, 4-diacetylphloroglucinol- and pyrrolnitrin-producers were different in parents compared to in the hybrid maize cultivars (Picard *et al.*, 2008). Studies using pyrosequencing and phylo-chips have also shown that potato cultivars can select specific bacterial communities (İnceoğlu *et al.*2011; Weinert *et al.*, 2011).

Different plant genotypes have recently been demonstrated to influence their associated microbial communities (Weinert *et al.*, 2009, 2011; Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Peiffer *et al.*, 2013) reported that significant differences occurred more frequently for fungi, especially *Ascomycetes*, than for bacteria. When all seven plant genotypes that were compared by the researchers, different cultivars had a greater effect on both bacterial and fungal communities than genetic modification (Weinert *et al.*, 2009). Knowledge of the different components of soil microbial communities and their functions in different crops, such as oilseed rape, potato, strawberry, their cultivars grown in different soils, is fundamental for designing strategies for sustainable plant protection.

1.2 Strawberry cultivation

Strawberries were first described by the Roman senator Cato around 200 BC. Strawberry (*Fragaria x ananassa* Duch) belongs in the family Rosaceae and is a cross between two American varieties *F. virginiana* and *F. chiloensis*. Strawberry is an important cash crop, which is cultivated in most parts of the world. It is a perennial crop. According to FAO statistics in 2011 the total global land area used for strawberry cultivation was 244283 hectares (http://faostat.fao.org). In Sweden alone, in 2011 strawberry cultivation accounted for nearly 1800 hectares of land with a yield of more than 7200 Kg/ha (http://faostat.fao.org). Strawberries are usually cultivated in open fields as well as in greenhouses and plastic tunnels. They grow best in sunny locations with well drained, sandy loam soils with an optimum pH range of

5.5-7.0 (*Cornell guide to growing fruit at home*, 2003; Lola-Luz, 2003). Strawberries are grown outdoors in matted rows and raised bed systems but also on table top systems in tunnels or greenhouses (Hochmuth *et al.*, 1998; Daugaard, 2008; Shiigi *et al.*, 2008).

Many cultivars including Honeoye, Senga Sengana, Zephyr, Bounty, Elsanta, Korona, Polka and Pegasus are cultivated in Sweden (http://sv.wikipedia.org) of which Honeoye, Zephyr and Korona are preferred for their early production or high productivity and/or tolerance to fungal diseases grown for their berry quality yield (Davik *et al.*, 2000). The cultivars can be classified into three categories; a) short day June bearers that can grow and initiate flower buds during short daylight seasons, giving a single but large yield b) long day ever bearers insensitive to light, producing fruits 2-3 times per year and c) day-neutral, insensitive to light, continuously producing fruits under favourable conditions (Guerena & Born, 2007). Some of these cultivars have been developed by breeding programs in Sweden (Hjalmarsson & Wallace, 2004).

Strawberries are high in vitamin C, phenolic compounds (flavonoids e.g. anthocyanins) and minerals such as potassium and manganese. The red colour of strawberries is due to the anthocyanins, pelargonidin-3-glucoside and cyanidin-3-glucoside (*Berries and their role in human health*, 2005). Their phenolics are reported to have anti-cancer, antioxidant, and anti-inflammatory effects as well as having effects against type 2 diabetes and obesity (Hannum, 2004; Giampieri *et al.*, 2012).

1.2.1 Diseases in Strawberry

About 50% of the diseases of soft fruits are caused by fungi (Sigee, 2005). Strawberries are prone to attacks by various pests and pathogens. Among fungal pathogens, *Botrytis cinerea* (causes grey mould), *Phytophthora cactorum* (causes crown rot), *Phytophthora fragariae* var. *fragariae* (causes red stele or red core), *Verticillium dahliae* (causes Verticillium wilt), *Colletotrichum acutatumi* (causes black spot) and *Sphaerotheca macularis* (causes powdery mildew) are the major pathogens reported to occur in strawberry cultivations in Europe (Parikka, 2004). Different control measures such as crop rotation, cultural measures and chemicals are practised to reduce the damage caused by these pathogens (Guerena & Born, 2007). Attempts have been made to test the efficacy of some biological control agents such as *Trichoderma* spp, arbuscular mycorrhizal fungi (AMF) against grey mould and *Phytophthora* spp. respectively (Lola-Luz, 2003; Guerena & Born, 2007). The results with their applications have been shown to be promising. *Phytophthora* spp are soil-borne that form root rot complex with other important fungal

pathogens, *V. dahliae*, *Rhizoctonia* spp. and *Pythium* spp. These affect many crops including strawberry crop world-wide.

According to European and Mediterranean Plant Protection Organization, *Verticillium* spp. has been listed as a 'principal strawberry pest'. The pathogen has a broad host range and can infect nearly 400 plant species. It forms conidia and microsclerotia. They germinate in the presence of root exudates and enter the plant through primary roots or wounds. Subsequently, the pathogen colonises the vascular system by forming conidia which accelerate the secondary infections. The symptoms on strawberry include outer leaves drooping, wilting and or become reddish-yellow, few new leaves develop and curl up along the mid vein. The pathogen overwinters in the soil in the form of microsclerotia on dead plant tissues or in the soil. Alternative strategies are required to control this pathogen because its total control is difficult without soil fungicides and disease resistant cultivars (Klosterman *et al.*, 2009).

Use of antagonistic micro-organisms has been attempted to reduce the damages caused by soil-borne fungal pathogens including *V. dahliae* (Elad *et al.*, 1981; Berg *et al.*, 2005; Tjamos *et al.*, 2005). Biological agents such as *Trichoderma*, *Serratia* and *Pseudomonas* different plant extracts and biofumigation are some of the alternative strategies that have been explored to reduce/number of microsclerotia/wilt symptoms (Berg *et al.*, 2001; Kurze *et al.*, 2001; Steffek *et al.*, 2006; Tahmatsidou *et al.*, 2006; Meszka & Bielenin, 2009).

1.3 Biofumigation

The earliest concept of biofumigation was documented by Theophastrus in 300BC when he observed that the odours of cabbage were causing harmful effects on vines (Willis, 1985). After the ban of noxious chemicals for soil fumigation including methyl bromide, alternatives to pesticides have been increasingly explored (Duniway, 2002; Porter & Mattner, 2002). Biofumigation can also be considered as a form of green-manuring where the plant material is incorporated in the soil before planting of the main crop. With an aim to provide different alternative control strategies, effect of biofumigation have been studied against soil-borne fungal pathogens e.g. *Rhizoctonia, Verticillium, Colletotrichum, Fusarium, Pythium, Phytophthora* spp. (Zurera *et al.*; Steffek *et al.*, 2006; Mattner *et al.*, 2008; Friberg *et al.*, 2009). Glucosinolate-containing *Brassica* spp. is known to release volatilic isothiocyanates (ITCs) which are toxic to different pathogens (Kirkegaard *et al.*, 1993; Matthiessen & Kirkegaard, 2006).

The chemistry involved in the biofumigation can be attributed to the action of myrosinases on the Glucosinolates (GLS) thereby releasing ITCs, thiocyanates, nitriles, oxalidine, dimethyl sulphide, methanethiol among other compounds (Matthiessen & Kirkegaard, 2006; Gimsing & Kirkegaard, 2008). About 20 different GLS have commonly been found depending upon the side organic chain. Their concentrations vary with the age of the plant and conditions in which they are grown (Sarwar & Kirkegaard, 1998). GLS are generally found in members of Tovariaceae, Resedaceae, Capparaceae, Moringaceae, and Brassicaceae (Brown & Morra, 1997), however ITCs remains the prime choice of interest for research because they are the main hydrolytic products of GLS compared to e.g. thiocyanates or nitriles (Gimsing & Kirkegaard, 2008). Concentrations of ITCs have been shown to decrease by 90% within 24 hours of incorporation of *Brassica* residues (Brown *et al.*, 1991). Their persistence up to 45 days has also been demonstrated (Gimsing & Kirkegaard, 2008; Poulsen *et al.*, 2008).

Isothiocyanates are toxic to wide range of microorganisms (Walker *et al.*, 1937), they react with sulphur-containing proteins by a nonspecific and irreversible reactions (Brown & Morra, 1997). The bioactive compounds released during biofumigation suppress pathogens, weeds and influence rhizosphere microbial communities (Matthiessen & Kirkegaard, 2006; Hoagland *et al.*, 2008). *Brassica* sp. as plant material or its seed meal has been tested by several researchers for green-manuring and was found to influence microbial community structures (Vera *et al.*, 1987; Williams-Woodward *et al.*, 1997; Mazzola *et al.*, 2001; Cohen & Mazzola, 2006; Hoagland *et al.*, 2008; Friberg *et al.*, 2009; Omirou *et al.*, 2010).

The incorporation of brassica plant material for biofumigation has been shown to increase or decrease the population of the rhizosphere micro-organisms such as *Trichoderma* spp., *Pythium* spp., fluorescent pseudomonads, *Streptomyces* spp, actinomycetes and other antagonists of soil-borne pathogens depending on the plant species and soil type (Mazzola *et al.*, 2001, 2012; Cohen & Mazzola, 2006; Perez *et al.*, 2007; Mazzola & Zhao, 2010). Steffek *et al.*, (2006) reported decreased number of microsclerotia in infested strawberry fields as a result of biofumigation with different glucosinolate-containing *Brassica* spp. The decrease of numbers varied between 0-30% depending on the field and the biofumigant. Biofumigation has also shown to reduce the nematode populations (Henderson *et al.*, 2009; Zasada *et al.*, 2010).

Effects of *Brassica* spp. for biofumigation have been studied by several researchers with respect to soil microbial community dynamics as well as on pathogens (Steffek *et al.*, 2006; Mattner *et al.*, 2008; Friberg *et al.*, 2009; Omirou *et al.*, 2010). Their studies revealed that biofumigation affects number

of microsclerotia of *V. dahliae*, decreases growth of *Phytophthora cactorum* upto 20%, increases Ascomycetes community but confers no effect on ammonia-oxidising bacteria and the population of *R. solani* was found to be resilient. In these studies, the effect of biofumigation on the microbial community structure in the roots/rhizosphere of the main crop was not included.

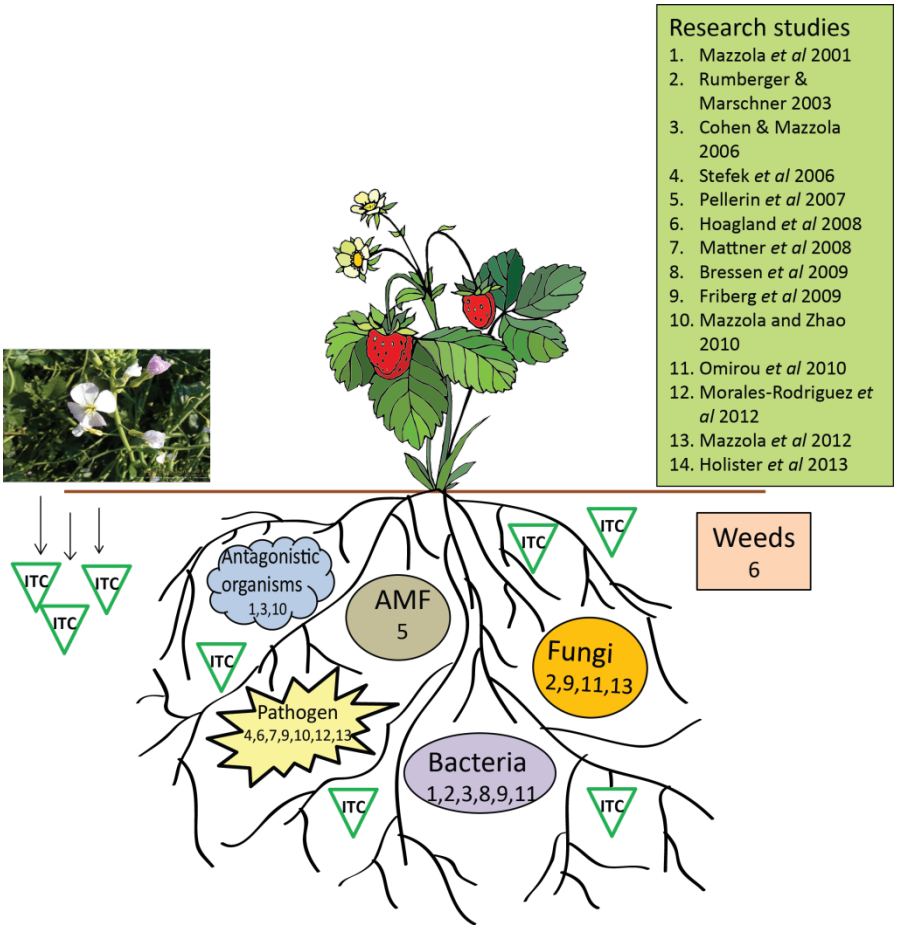

Figure 1. Studies published with regard to *Brassica* spp. plant incorporation (biofumigation).

1.4 Analysis of community dynamics

Cultivation-dependent methods reveal only a fraction of the soil microbial diversity (Torsvik & Øvreås, 2002). With the use of molecular methods for soil community analysis, it has been possible to discover the untapped components of the communities. The commonly used phylogenetic markers are e.g 16S

rRNA genes (for bacteria) and internal transcribed spacer regions (ITS, for fungi) as well as functional genes e.g. *nif*, *amoA, gac*A (Mahmood *et al.*, 2006; Weinert *et al.*, 2009; Xuan *et al.* 2012). Other phylogenetic markers such as RNA polymerase beta subunit (rpoB), gyrase beta subunit (gyrB), recombinase A (recA) and heat shock protein 60 (hsp60) have also been used to study microbial communities (Woese & Fox, 1977; White *et al.*, 1990; Dahllof *et al.*, 2000; Ghebremedhin *et al.*, 2008). These methods focus on the conserved regions of the genomes. Most microbial community studies are based on DNA and provide information on total and/or abundant members of the community. In contrast, RNA-based analysis provides information on active members of the community (Duineveld *et al.*, 2001; Norris *et al.*, 2002; Griffiths *et al.*, 2003; Nicol *et al.*, 2003; Mahmood *et al.*, 2005; Hoshino & Matsumoto, 2007). Many of these cultivation-independent molecular techniques have limitations (Rastogi & Sani, 2011; Lee *et al.*, 2012).

1.4.1 Denaturing gradient gel electrophoresis (DGGE)

DGGE method has been used widely for fingerprinting of environmental microbial communities (Muyzer *et al.*, 1993; Mahmood *et al.*, 2005; Costa *et al.*, 2006). The principle of DGGE involves separating DNA fragments (PCR products) of the same length in presence of a chemical denaturant across a polyacrylamide gel under constant temperature. This separation is based on melting behaviour of double stranded DNA that depends on the base pair content. The use of a 5' end attached 'GC' (Guanine and Cytosine) clamp helps in preventing complete melting of double strands during electrophoresis. When a PCR product migrates in the gel matrix with low-to-high denaturant gradient, it starts melting depending on the denaturant concentration at various points and thus leaves behind several fragments of DNA varying in 'GC' content. The banding patterns thus produced represent a community profile and generally it is assumed that each band on the gel represents a unique member of the microbial community. DGGE has better resolution than T-RFLP as it allows sequencing bands of interest to identify members in a complex microbial community. Due to technological advancement in recent years it has become possible to study microbial communities using high throughput methods.

1.4.2 Pyrosequencing

Pyrosequencing technology was invented by (Ronaghi *et al.*, 1998). It has become one of the large-scale sequencing methods (e.g. Illumina, 454, Ion torrent) that allow studying microbial communities in depth. It is based on the principle that synthesis of the complementary DNA takes place by addition of

one nucleotide at a time and during this process, pyrophosphate gets released and transformed into adenosine tri-phosphate (ATP). ATP reacts with luciferin, which generates light in an amount that is proportional to the amount of ATP (Ronaghi, 2001). The light thus emitted is captured and analysed. Pyrosequencing is currently being used to reveal the microbial associations in terrestrial systems, aquatic systems and in medical sciences. (Hewson *et al.*, 2009; Rastogi & Sani, 2011; van Boheemen *et al.*, 2012; Xuan *et al.*, 2012; Zaki *et al.*, 2012).

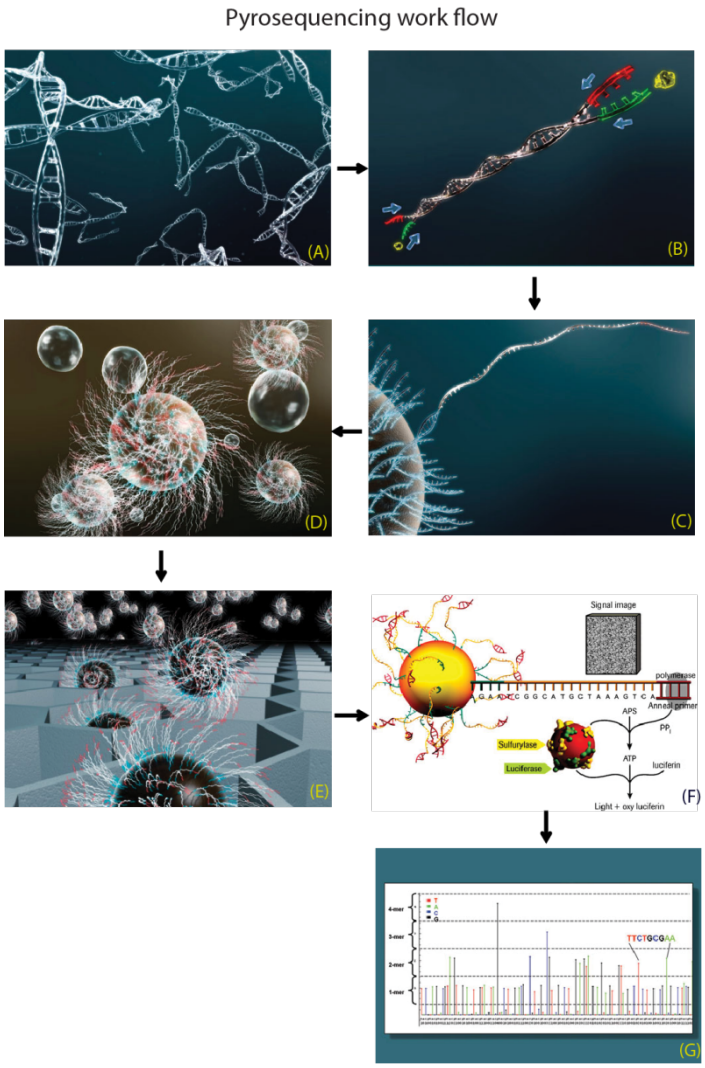

Figure 2. Pyrosequencing work flow. (A) Start of sequencing of samples such as PCR products including genomic DNA, and cDNA. (B) Library preparation using specific adapters to the samples (C) Attach library to DNA capture beads (D) Amplify the entire emulsion in parallel to create millions of clonally copies of each library fragment on each bead (E) Load the beads onto the PicoTiterPlate device, where the surface design allows for only one bead per well. The PTP Device is then loaded in instrument for sequencing. Individual nucleotides are flowed in sequence across the wells. Each incorporation of a nucleotide complementary to the template strand results in a chemiluminescent light signal recorded by the camera. (F) Pyrosequencing reaction of millions of copies of a single clonal fragment is contained on each DNA capture bead. (G) 454 sequencing data analysis software uses the signal intensity of each incorporation event at each well position to determine the sequence of all reads in parallel (adapted from http://454.com/products/technology.asp).

1.5 Aims of the study

- To explore the cultivar type and soil interaction effects on arbuscular mycorrhiza fungal communities (**Paper I**).
- To explore the pathogen/cultivar/soil interaction effects on fungal communities. If presence of a soil-borne fungus, *V. dahliae* pathogenic to strawberry affects soil microbial community, if there are any cultivar-specific responses. (**Paper II**)
- To explore and biofumigation/pathogen interaction effect on changes in fungal communities and if bio-fumigation effect is due to build-up of antagonistic fungi. (**Paper III**)

Strawberry was used as the host plant and oilseed radish was used as biofumigant plant species. Strawberry was grown in soils with different cultivation management practices.

Cultivation-independent molecular techniques cloning and sequencing, DGGE and 454-pyrosequencing were employed to study changes in microbial communities as a result of different interactions in the rhizosphere.

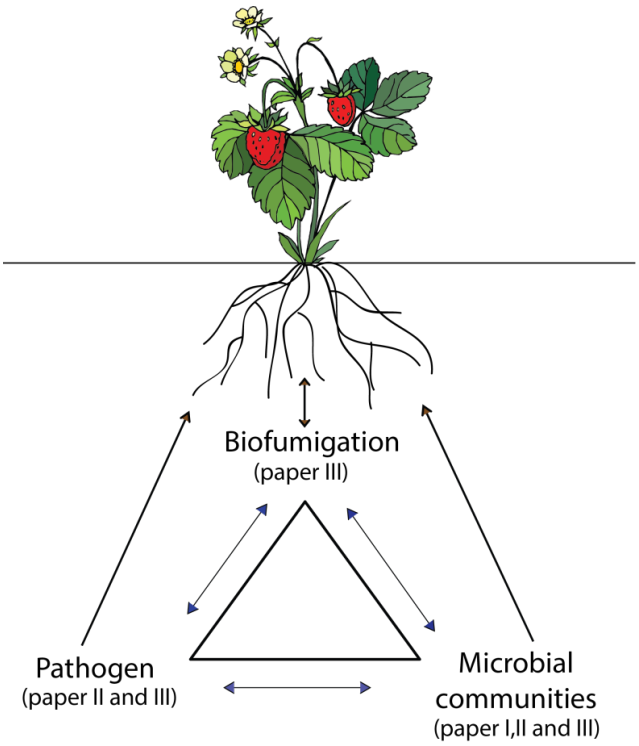

Figure 3. Overview showing the relationship between the studies performed.

2 Materials and Methods

2.1 Soils and plant material

Physico-chemically different soils were selected for the studies, three were field soils and one was a greenhouse commercial soil (peat-based, Hasselfors, Sweden). Physical and chemical characteristics of the field soils in papers I and II were the same and the peat-based soil was used only in paper II. The field soils were collected from two agricultural fields located in Hörby (55° 50′N, 13°35′E) and Kristianstad (56° 06′N, 14° 01′E), Southern Sweden. These fields are situated 38 km apart from each other and are differently managed. The field in Hörby is an arable soil that is organically managed with tilling, yearly crop rotation since 1983 and pre-cropped with potatoes prior to sampling. The field in Kristianstad is conventional managed and pre-cropped with strawberry. In this thesis Hörby soil is referred to as an 'organic' soil and Kristianstad soil as 'conventional' soil. The field soil in Paper III had pH 6.2 and contained carbon 2.15%, nitrogen 0.22% and phosphorus16.3 mg/100 g.

Twenty soil cores (10 cm diameter and 30 cm deep) were collected at random locations from each field and thoroughly mixed. The soil in paper III was collected an agricultural field north of SVA, SLU, Uppsala (59° 48N, 17° 39′E) and it had no history of strawberry cultivation. Four replicated samples of each soil were analysed for physico-chemical characteristics (Agrilab AB, Uppsala, Sweden).

Plantlets of four strawberry cultivars ('Honeoye', 'Senga Sengana', 'Florence' and 'Zephyr') were planted in organic and conventional soils. Senga Sengana and Zephyr (denoted as the 'old' cultivars in this thesis) All plantlets were obtained from Elof Dahlén AB, Vara, Västergötland, Sweden. All plantlets were of the type frigo A+ (plants that were pulled out carefully from the field during winter and stored at −2 °C).

2.2 Experimental design, pathogen introduction

In paper II and III, the soil-borne pathogen, three isolates of *Verticillium dahliae* originating from strawberry plants were obtained from C. Dixelius, Department of Plant Biology and Forest Genetics, SLU, Uppsala. The isolates were purified and grown in potato dextrose broth as stationary cultures for inoculum preparation. Preparatory studies were conducted to confirm their pathogenicity on strawberry plants. The isolate, *V. dahliae* 12086 was selected for this study. The isolate was grown as stationary culture for inoculum preparation. The plant roots were injured to stimulate the pathogen infection. The control plants were treated in a similar manner but with the suspension medium.

An outdoor pot experiment (paper II) was set up using four different strawberry cultivar Honeoye, Senga Sengana, Florence and Zephyr plantlets (n=7) in three different soils. Four weeks after planting *V. dahliae* was inoculated at the base of strawberry crowns and their controls were inoculated with suspension medium but without pathogen. Root and rhizosphere samples were collected after 12 and 14 weeks after planting and analysed for respective microbial communities.

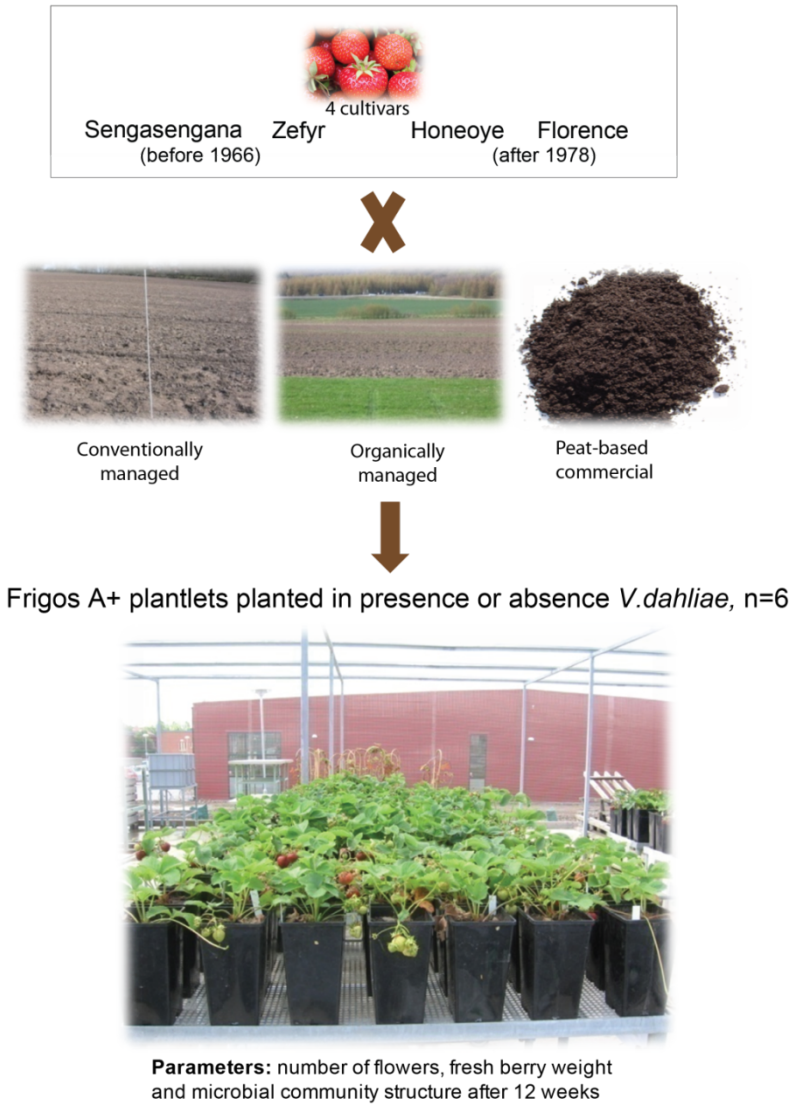

Figure 4. Study design of paper I and II.

A greenhouse pot experiment (paper III) was set up using oilseed radish as a biofumigant (n=6) in a field soil. In one of the treatments *V. dahliae* was also inoculated where a biofumigant crop was sown. Fourteen weeks after planting, the biofumigant crop was mulched into the soil (sample 0 h). Soil samples were collected regularly after incorporation. Eighteen days after oilseed radish incorporation, strawberry plantlets of Honeoye cultivar were planted (n=10).

Soils in all pots were sampled by destructive sampling at 12, 14 and 16 weeks after strawberry planting and analysed for microbial communities.

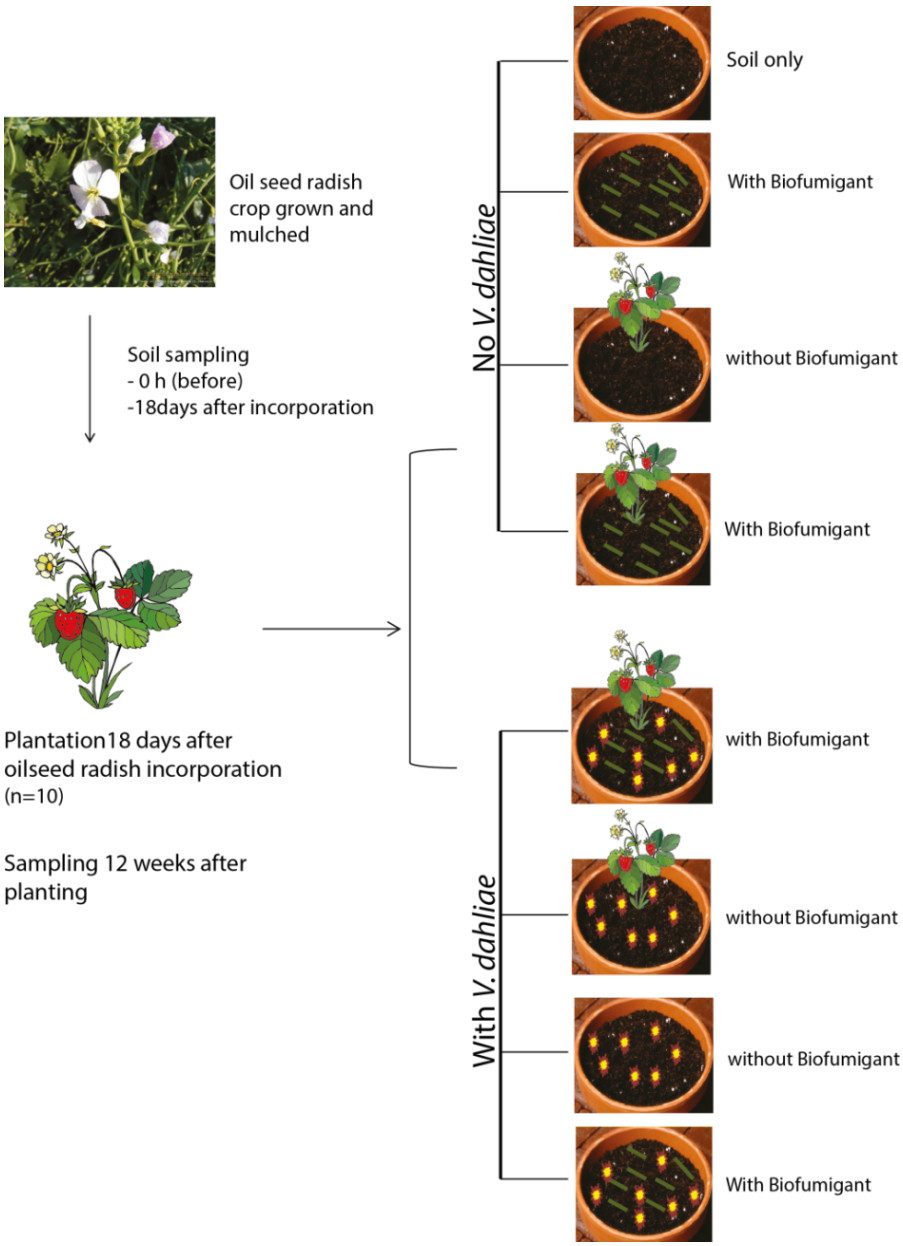

Figure 5. Study design of paper III.

2.3 Sampling strategy

The rhizosphere soils and roots were sampled 12 and 14 weeks after strawberry planting for paper I, II and III. In paper III, soil was also sampled before incorporation (=zero hour), and 2, 4, 6, 8, 10, 12, 24, 48, 72 hours after incorporation of biofumigant plant material, 18 days after incorporation/before planting and 12,14 and 16 weeks after planting strawberry plantlets. Three replicate pots per treatment were sampled destructively. The soil samples were stored at −20 °C and the roots stored in glycerol at −20 °C prior to community analyses.

2.4 The microbial community analyses

Bacterial and fungal community analysis was performed with DGGE and pyrosequencing except for AMF communities that were analysed using cloning and sequencing.

2.4.1 AMF community analysis (Paper I)

The roots were washed, processed for microscopy and DNA extraction as described in paper I. Microscopy was done after staining the roots with trypan blue and AMF colonisation was quantified using an intersection method. The DNA was extracted using DNeasy plant kit (Qiagen, Crawley, UK). The primer pairs, AML1 and AML2 used in the amplification are described in Lee *et al.*, (2008). The amplicons were cloned into One Shot™ TOP10 chemically competent *Escherichia coli* (Invitrogen, California, USA) following the manufacturer's instructions. Using AML1 as sequencing primer, the sequencing reactions were carried out in an ABI 3100 Sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing data were subjected to phylogenetic analysis.

2.4.2 Microbial community analysis (Paper II and III)

Nucleic acids were extracted from the rhizosphere soil samples using CTAB (hexadecyltrimethyl ammonium bromide) as described by Griffiths *et al.*, (2000). Using this approach, the pathogen-inoculated samples did not give reproducible results for the triplicates. Hence, another nucleic acid extraction method was employed to test the reproducibility. This method was based on a commercial kit, 'RNA power soil total RNA isolation and DNA elution accessory kits (MOBIO laboratories, California, USA) (paper II). Using the 'MOBIO' kit, the reproducibility improved significantly between the replicates.

DGGE analysis using nucleic acids from Griffiths *et al.*, (2000) method and the MOBIO kit revealed significantly different community fingerprints (paper III) and therefore, nucleic acid extracts from both methods were pooled for 454-pyrosequencing (Paper II and III).

The nucleic acids were subjected to DNase treatment (Promega, USA) according to manufacturer's instructions. To target active components of bacterial communities, cDNA was generated as described by Mahmood *et al.*, (2005) and analysed on a DGGE gel following PCR amplifications using 357f and 518r primer sets (Muyzer *et al.*, 1993). The 'cDNA' was also generated using IScript^{TM} cDNA synthesis kit (BIORAD, USA) and the comparison of results showed no difference between the two methods. The method of iScript^{TM} cDNA synthesis was decided to be used for bacterial community analysis with pooled nucleic acid extracts (data not included).

Attempts were made to target active fungal community using cDNA from the iScript^{TM} synthesis kit targeting the 'ITS' region. No reproducibility was found between the replicates using primers targeting ITS region. Primers targeting 18S rRNA region were not tested because they are known to amplify non-target eukaryotes in addition to fungi, (Anderson *et al.*, 2008) DNA-based approach was therefore implemented for fungal community analysis.

DGGE

PCR products generated by using either fungal or bacterial universal primers were run on a DGGE gels. A nested approach (DNA) was employed for both bacterial and fungal amplifications. Bacterial amplifications were carried out using the primers 27F and 1492R for primary PCR, 357F with a 'GC' clamp and 518R for secondary PCR (Muyzer *et al.*, 1993). Fungal amplifications were carried out using ITS1F and ITS4 followed by a secondary PCR using ITS1F with a 'GC' clamp and ITS2.

The PCR products were analysed using DGGE which was performed on Dcode^{TM} universal mutation detection system (BIORAD, USA). Acrylamide gels (8%) were prepared with urea (Sigma-Aldrich, USA) and formamide as a denaturant with a gradient of 20%-50% for fungal community analysis (Figure 10) and 35%-65% for bacterial community analysis (Figure 6). Equal volumes of PCR products were loaded in the wells and ran at 75 V in 1X TAE buffer at 60°C, for 16 h. The gels were stained and developed as described by Mahmood *et al.* (2006) and scanned. Numerical analysis of the gels was performed using TotalLab software (TotalLab, Newcastle, U.K.).

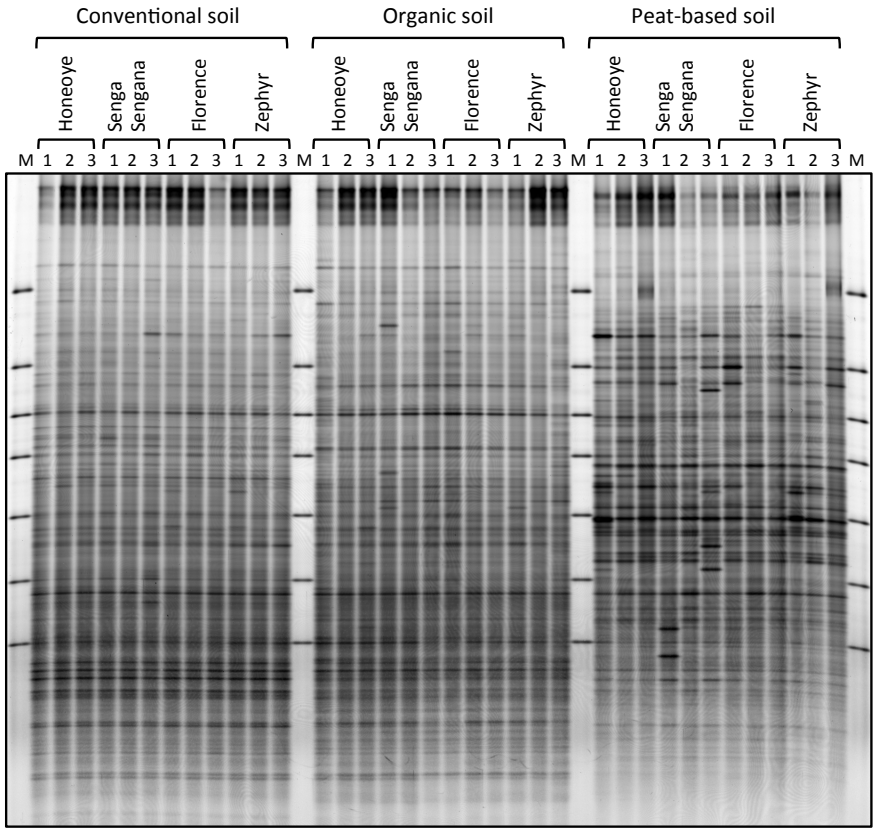

Figure 6. Denaturing gradient gel electrophoresis (DGGE) banding profiles of rhizosphere bacterial communities of four strawberry cultivars (Honeoye, Senga Sengana, Florence, Zephyr) when grown in three different soils. Lanes 1-3 relate to triplicate samples that were harvested destructively from the strawberry rhizosphere at week 12.

Pyrosequencing

The DNA from the two extraction methods was pooled and used as a template for generating PCR amplicons for pyrosequencing. Prior to pyrosequencing, the control samples were subjected to DGGE-based analysis following PCR amplification. The samples were amplified using primers, fITS7 and ITS4 (Ihrmark *et al.*, 2012). Each sample was tagged with ITS4 primer with a unique sample identifier consisting of eight bases. The samples were purified, quantified, pooled and freeze-dried prior to pyrosequencing, which was carried out by LGC Genomics (Berlin) using Roche 454/GS-FLX+ Titanium technology.

2.4.3 Data analyses (Papers I, II and III)

Most data were subjected to non-parametric analysis. Microbial community analyses were conducted with respect to cultivars/soils, characteristics of field soils, dynamics in OTUs diversity in absence/presence of *V. dahliae*, the relative abundance and number of OTUs with respect to different phyla.

Multivariate methods were employed to analyse the structure of microbial communities in pyrosequencing studies. Non-metric multidimensional scaling (NMDS) is known to be used in various community analyses (Ramette, 2007). NMDS uses ranks for mapping the objects by several iterations in order to obtain the lowest stress value possible (Shepard's plot stress value) in a two-dimensional ordination space. Different distance measures can be employed for computing distances in NMDS. In the ordination, the proximity between the treatments corresponds to their similarity. The ordination distances do not correspond to the original distances among treatments but to their ranked order. However tests such as similarity percentage (SIMPER, Papers II and III) and multi-response permutation procedure (MRPP, Paper I) reveal the observed difference or similarity between the treatments. SIMPER is used for assessing the taxa that are primarily responsible for an observed difference between groups of samples. A pairwise comparison of the samples and also a pooled sample can be used for the analysis.

2.4.4 Advantages and limitations of the methodology

A wide array of methods is used for microbial community analyses. Both cultivation-dependent and cultivation-independent methods are being still employed. Since the advent of cultivation-independent methods, the knowledge about microbial communities is constantly increasing. Molecular methods including DGGE, cloning and sequencing, pyrosequencing have allowed detection of the changes in communities with a higher resolution in comparison with cultivation-dependent methods, however newer high throughput methods such as pyrosequencing can detect many more taxa simultaneously. Differences in community fingerprints were observed in this study depending on the nucleic acid extraction method, the MOBIO kit seemed to work better than the CTAB-method, depending on the soil type and detected other taxa. In order to maximise coverage of community templates, the nucleic acids from the two methods were pooled prior to pyrosequencing.

3 Results and discussion

3.1 Influence of soil type on AMF community in strawberry cultivars (Paper I)

Arbuscular mycorrhizal fungi play important roles in nutrient acquisition in different soils and strawberry is known to respond to inoculation with AMF. Cultivars have shown to respond differently to AMF inoculation in several experimental studies (Azcon & Ocampo, 1981; Vestberg, 1992; Ross & Hoover, 2004), but no study has addressed whether different cultivars associate preferentially with different AMF taxa in different soil types.

In this study, the effects of soil type on AMF communities colonising the roots of different strawberry cultivars grown in two different field soils were evaluated. Cloning and Sanger sequencing were employed. The field soils displayed significant differences in mull content, pH, total carbon, nitrogen and phosphorus.

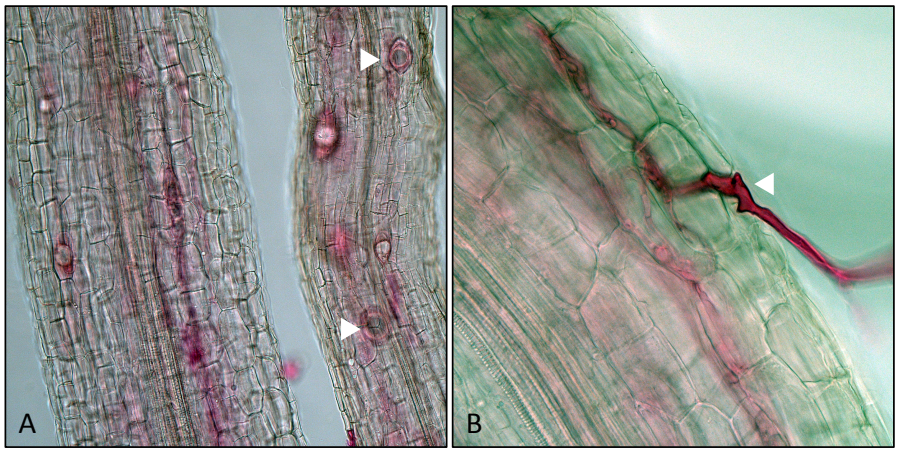

Figure 7. Strawberry roots colonized by AMF: (A) vesicles, (B) hypha entering the root.

Total AMF root colonisation was higher in an organically managed soil (Hörby, referred to as 'organic' in this thesis) than in a conventionally managed soil (Kristianstad, referred to as 'conventional') (Figures 7 and 8).

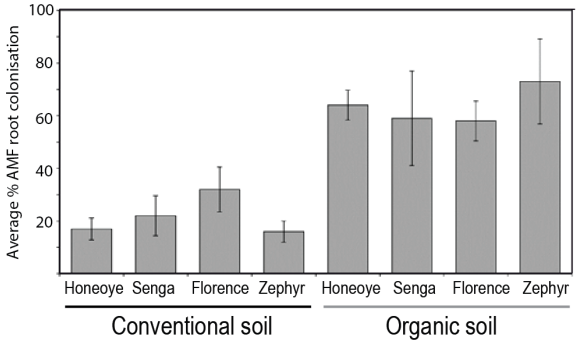

Figure 8. Percentage of root length colonised by AMF in four strawberry cultivars grown in two agricultural soils. Columns represent mean values, and error bars show ±SD (n=3).

No significant difference in total AMF colonisation could be detected between the cultivars (Figure 9). The relative abundance of *Glomus* spp. was higher in conventional soil than in organic and *Acaulospora* spp. dominated the AMF assemblages in the organic soil. The latter was not detected in the conventional soil. These results suggest that physico-chemical characteristics and management can play a role in determining the identity and structure of root-associated microbial communities in agricultural systems.

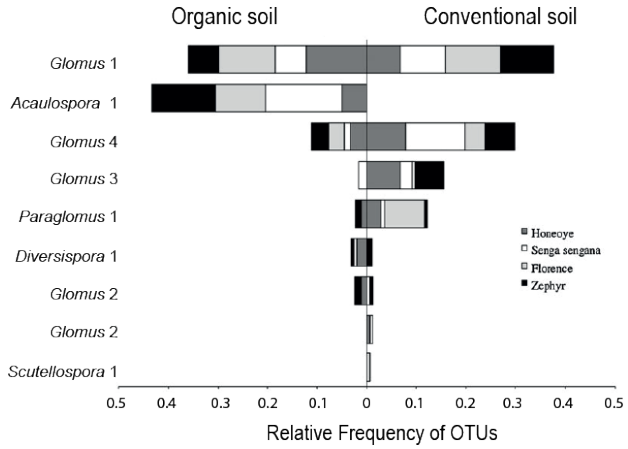

Figure 9. Distribution and proportions of different AMF taxa on strawberry roots from different cultivars when grown in different soils. The x-axis indicates the relative frequencies of OTUs within each soil. Relative frequencies were first calculated for each OTU within samples since the number of sequenced clones is not the same across the samples, and afterwards referred as proportions for each soil.

No significant differences between the structures of AMF communities colonising the roots of different cultivars could be detected after 12 weeks using the cloning and sequencing. However, analyses of additional samples collected at different time points may have revealed differences due to different growth rates of the strawberry cultivars. Other studies (Picard *et al.*, 2008; Weinert *et al.*, 2009) have demonstrated cultivar-specific effects with respect to fungal communities and it is possible that use of high-resolution sequencing technologies such as pyrosequencing might have also revealed AMF-based differences between the cultivars.

The exclusive appearance of *Acaulospora* spp. in the organically managed soil is consistent with the suggestion of Oehl *et al.*, (2004) that this genus may play an important role in organic farming where P values may be low. However the P concentration in organic soil was almost double that in the conventional soil suggesting that some other explanation contributes to its dominance.

3.2 Influence of soil type and *V. dahliae* inoculation on rhizosphere fungal community structure in strawberry cultivars (Paper II)

Selection of cultivars with better resistance to pathogens is an important tool in breeding for improved plant health. Improved resistance may depend upon, a) direct alteration of physical and chemical plant characteristics, b) indirect interactions with pathogens and/or c) modification of the rhizosphere microbial communities that have an indirect effect on plant health through their antagonism towards plant pathogens. Although there have been many investigations of the effects of inoculated biocontrol microorganisms on the structure of rhizosphere microbial communities, there have been fewer detailed examinations of the effects of plant pathogens on these communities. The roles of cultivar specificity and soil type in this respect are still poorly known. The diversity of these communities is exceptionally high and cultivation-independent methods with high taxonomic resolution provide better understanding of these interactions. In this study, we examined the effects of inoculation of *V. dahliae* on rhizosphere fungal community structure of four strawberry cultivars, Florence, Honeoye, Senga Sengana and Zephyr, cultivated in three different soils, conventional, organic and peat-based.

Rhizosphere soils from uninoculated (control) samples from 12 and 14 weeks were subjected to DGGE-based community finger-printing following PCR amplification. The results showed greater changes in community structure in response to the cultivar differences observed at the first sampling, 12 weeks after planting, than at 14 weeks (Figure 10). Pyrosequencing was then used to analyse the 12 week samples.

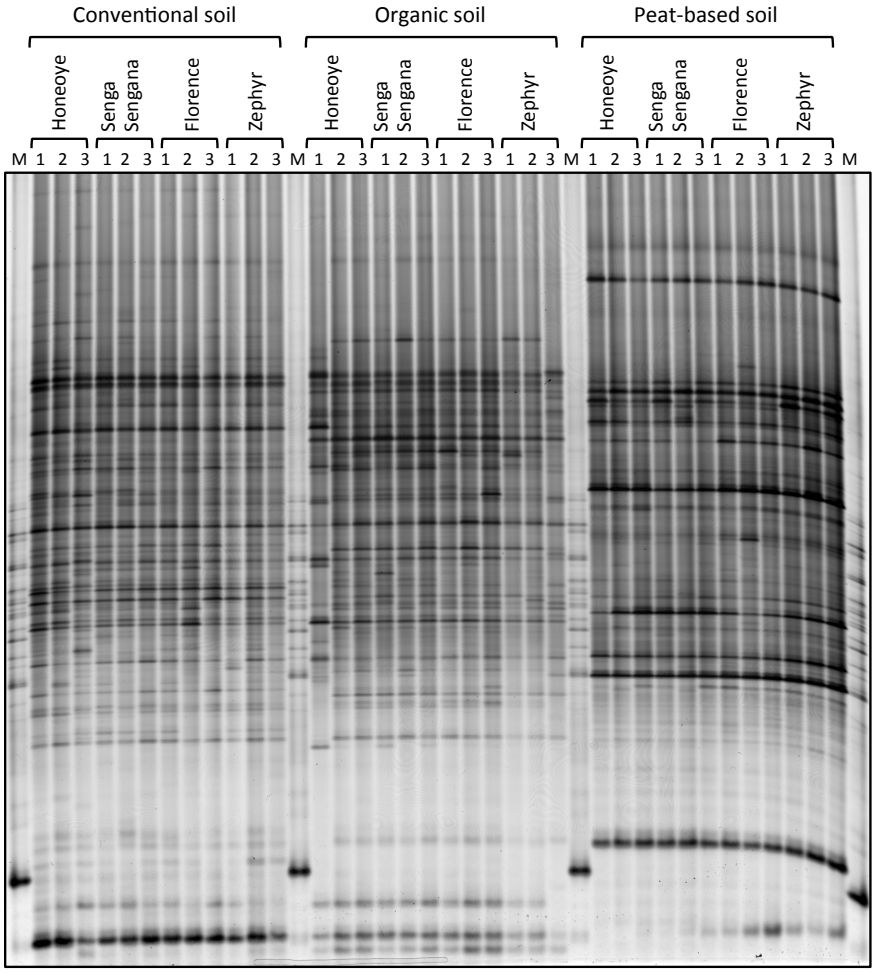

Figure 10. Denaturing gradient gel electrophoresis (DGGE) banding profiles of rhizosphere fungal communities of four strawberry cultivars (Honeoye, Senga Sengana, Florence, Zephyr) when grown in three different soils. Lanes 1-3 relate to triplicate samples that were harvested destructively from the strawberry rhizosphere at week 12. Marker in lane M consisted of banding profiles of an unrelated fungal community with known electrophoretic behavior on DGGE gels.

In the absence of *V. dahliae*, the strawberry yield differed significantly between the cultivars in all three soils (conventional p=0.0014, organic p=0.0087 and peat p=0.0022). Yield of the Senga Sengana cultivar was consistently low in all soils. In the presence of *V. dahliae*, the yield levels differed depending on both soil and cultivar type. Honeoye and Zephyr had low berry yield in peat-based soil and a similar trend was observed in the other two soils, with one exception (Honeoye in organic soil) but it was not significantly different. Florence and Senga Sengana are known for their tolerance to *V. dahliae*. Interestingly, Florence yielded significantly lower in organic soil than in conventional soil inoculated with *V. dahliae*, indicating that its tolerance is soil type dependent.

Bioinformatic analysis of rhizosphere soil revealed 16923 pyrosequencing reads that passed the quality control checks revealed 589 clusters, 86% of which were of fungal origin. In general, lower numbers of reads were observed for most cultivars grown in the three pathogen-inoculated soils, the exception being Florence in organically managed soil. Bioinformatic analysis of the molecular data from root samples revealed 47153 reads passing quality control, grouped into 312 clusters. Dominant taxa in roots belonged to the genera *Leptodontidium*, *Entrophospora*, *Ilyonectria*, *Exophiala*, *Scytalidium*, *Acremonium*, *Fusarium*, and *Cephalosporium*. The fourteen most abundant taxa constituted >50% of the reads.

In the absence of *V. dahliae*, the number of OTUs in rhizosphere soil of four cultivars, in general, was highest when grown in conventional, intermediate in organic and lowest in peat-based soil indicating that the soil type has a strong effect on the fungal diversity. The lowest fungal diversity in all cultivars grown in the peat-based soil is most likely due to its relatively less complex biogeochemical characteristics compared to that of the field soils. The higher diversity in the rhizosphere of cultivars in conventional soil could be attributed to differences in soil biogeochemical characteristics including (pH), which was significantly lower in conventional (5.97) than in organic soil (6.36) (Santos-González *et al.*, 2011). In general, organically managed soils have been reported to have higher functional diversity and microbial biomass than conventionally managed soils (Oehl *et al.*, 2004; Reeve *et al.*, 2010).

In general, the treatments caused changes mainly in the proportions of Ascomycota and Basidiomycota in the rhizosphere. The relative abundance of Basidiomycota was found to be much higher than that of Ascomycota in all treatments in organic soil. In two field soils numbers of OTUs belonging to Basidiomycota were only half than those belonging to Ascomycota, but this difference was not evident in peat-based soil.

Surprisingly only three reads of *V. dahliae* were detected in the rhizosphere in Verticillium-inoculated treatments despite the fact that the strawberry yield was affected negatively. However over 560 reads of *V. dahliae* were detected in root material. Preliminary light microscopy of cleared and stained roots of strawberry plants grown in the presence of *V. dahliae* showed microsclerotia-like structures that seemed to be absent in controls (Figure 11). Further molecular analysis based on FISH (Fluorescent *in situ* Hybridisation) is needed to confirm the true identity of the structures.

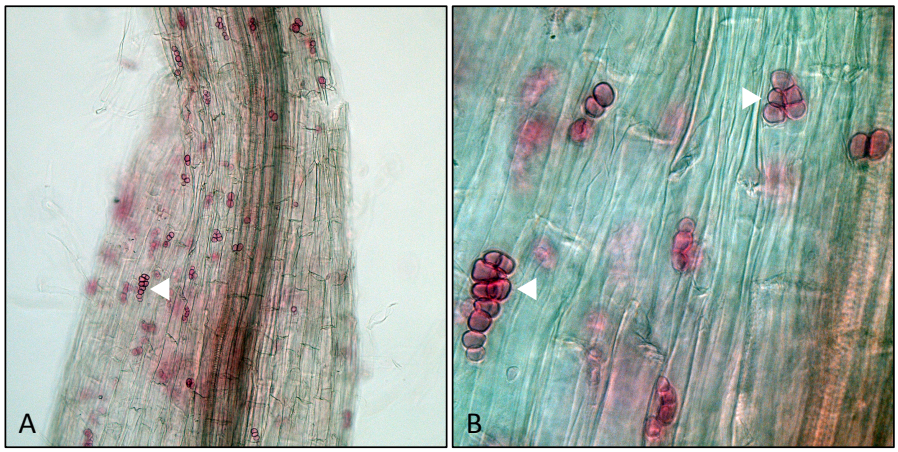

Figure 11. Strawberry roots showing *V. dahliae*-like microsclerotia structures.

The effect of *V. dahliae* inoculation was more pronounced in the four cultivars grown in peat-based soil, where the relative abundance of Ascomycota increased and Basidiomycota decreased. Furthermore, the relative abundance of Ascomycota increased in the rhizosphere of Florence and Zephyr, whereas no differences were evident in Honeoye and Senga Sengana in conventional soil in the presence of *V. dahliae*. No such clear trend in relative abundance of the two phyla was observed in relation to the cultivars in the organic soil, except that Ascomycota decreased in Florence and Basidiomycota decreased in Zephyr in the presence of *V. dahliae*.

Evidence is available on the effects of interactions of cultivars with pathogens on endophytic bacterial communities (e.g. Reiter *et al.*, 2002). However information on the effects of cultivar-soil type-pathogen interactions on fungal communities does not appear to be available in the literature.

NMDS ordination depicted a clear separation of communities with respect to soil type. In the two field soils, *V. dahliae* inoculation appeared to have little effect on rhizosphere fungal community structure, however more pronounced effect of *V. dahliae* inoculation was observed in peat soil. SIMPER was

performed using abundance data for all taxa with more than 50 reads to assess which particular taxa were primarily responsible for the observed differences between all treatments. Senga Sengana and Zephyr ('old' cultivars) showed greater dissimilarity in all three soils indicating differences in composition of communities associated with 'old' cultivars compared to 'new' cultivars. Further experiments are needed to explore the basis of this dissimilarity.

In order to detect the taxa that responded to the treatments, the OTUs with >50 reads were selected for cell plot analysis. Fungal community structure was found to be more affected by *V. dahliae*-inoculation than by plant cultivar.

3.3 Influence of biofumigation on rhizosphere soil fungal communities in presence and absence of *V. dahliae* (Paper III)

Biofumigation has been investigated as an alternative method of plant protection, especially for soil-borne pathogens. Biofumigation typically releases isothiocyanates which may have a direct antagonistic effect against pathogens; however these chemicals may have an indirect impact on plant health through modification of rhizosphere communities which may influence the structure and/or activity of antagonists. The residues of biofumigant crops may also act as green manure, causing further effects on microbial community structure through stimulation of decomposers.

The impact of biofumigation on structure of soil microbial communities and soil-borne plant pathogens prior to the main crop cultivation has been studied but its effects on the dynamics of the community composition in the rhizosphere of the main crop is not yet fully elucidated. In this study, we used 454-pyrosequencing to examine possible changes in fungal community structure in the rhizosphere of strawberry following incorporation of oilseed radish plant material in the presence and absence of a soil-borne pathogen; *V. dahliae*. The study was carried out using a field soil different from the field soils in Papers I and II.

Four weeks after strawberry planting, reduction in plant growth was observed in the presence of *V. dahliae*, irrespective of the biofumigation treatment. In absence of *V. dahliae*, biofumigation induced early flowering in >50% of the strawberry plants, lower numbers of buds and delayed flowering were observed in control plants. Furthermore, a significant decrease in berry yield was observed in response to biofumigation. In the presence of *V. dahliae*, a similar effect of biofumigation was observed but it was not statistically significant. These results are consistent with those reported by Vera *et al.* (1987) who studied the effects of incorporation of different types of cruciferous

plant material on the stand establishment and yield of the five crops and demonstrated negative effects of the treatments on both parameters in barley, flax, oilseed rape and wheat. Biofumigation effects have been attributed to changes in the structure of the native microbial communities (Mazzola *et al.* 2012; Hoagland *et al.* 2008; Sarwar & Kirkegaard 1998; Mazzola *et al.* 2001; Rumberger & Marschner 2003; Matthiessen & Kirkegaard 2006; Lu *et al.* 2009).

Prior to pyrosequencing, samples from different time points; 0 h, 24 h and 18 d after biofumigant incorporation, and 90 d and 120 d after strawberry planting was subjected to DGGE-based analysis following PCR amplification. Based on the findings that the maximum effect after strawberry planting on fungal community composition occurred after 90 d, further analysis using pyrosequencing did not include samples from 120 d. Furthermore, DGGE-based analysis did not reveal any difference in community structure between 0 h and 24 h and hence only samples from 0 h were included in the analysis. Bioinformatic analysis of 622 clusters obtained from 28971 reads revealed also 86% clusters of fungal origin.

Bioinformatic analysis of the molecular data from root samples revealed 47433 reads passing quality control, grouped into 986 clusters. Dominant taxa in roots belonged to the genera *Leptodontidium, Tetracladium, Leptosphaeria, Cephalosporium, Setophoma, Ilyonectria, Phomopsis and Chaetomium*. The 20 most abundant taxa constituted >50% of the reads.

The estimated richness showed significantly higher numbers of OTUs in the rhizosphere of strawberry plants than in the bulk soil, in the absence of biofumigation and *V. dahliae*, demonstrating the positive effects of strawberry plant root exudates on the diversity and abundance of fungal communities (Hilton *et al.* 2013).

Biofumigation with oilseed radish in itself increased fungal OTUs in the soil indicating its green-manuring effect in this study. Combined treatments of biofumigant and inoculation of *V. dahliae* also increased number of OTUs significantly in the soil in absence of strawberry plants confirming the green-manuring effect of the oilseed radish incorporation on fungal community structure. Our results contrast with those of Hollister *et al.* (2013) who reported a reduction in numbers of OTUs in mustard-amended soil compared to the numbers in un-amended soil. However, they used mustard meal while the present study was based on incorporation of fresh plant material. Biofumigation did not appear to have any significant effect on the number of OTUs of the strawberry rhizosphere fungal communities.

The above OTUs were grouped into Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, early diverging fungal lineages,

environmental samples and uncultured fungi (unidentified fungal sequences according to the NCBI database) and similar to the results in Paper II, the three most abundant groups were Ascomycota and Basidiomycota. In addition, OTUs of early diverging fungal lineages (e.g. *Mortierella* spp.) were also abundant compared to the other phyla detected.

Overall, the number of OTUs representing Ascomycota was nearly two times higher than that of Basidiomycota. In the absence of biofumigation and *V. dahliae*-inoculation, their OTUs increased significantly in the rhizosphere of strawberry plant suggesting an effect of strawberry root exudates and that the increase in total fungal OTUs can be attributed to the increase in OTUs of these two phyla. In presence of biofumigation only, an increase in OTUs of all three groups, Ascomycota, Basidiomycota and early diverging fungal lineages was also evident in the absence of strawberry indicating an effect of green manuring. No effect of treatments with biofumigant and/or inoculation with *V. dahliae* was observed on numbers of OTUs of the three fungal phyla in strawberry rhizosphere compared to that in the corresponding control soil.

The results indicated that the inoculation with the pathogen isolate in this study may not influence the structure of abundant groups of fungal communities in the strawberry rhizosphere may depend on the soil type. The field soil and the method of pathogen inoculation used in this study were different from those used in Paper I and II. Mazzola *et al.*, (2012) showed that the soil-type affects the composition of different *Pythium* populations that were recovered as a result of mustard seed meal incorporation.

NMDS analysis carried out on all fungal OTUs 12 weeks after strawberry planting separated all the treatments significantly. *Olpidium brassicae,* an obligate plant pathogen was detected in all treatments subjected to biofumigation. The presence and absence of different pathogens was not known for the field soil used for the study. The OTUs of this fungus seemed to be present before growing the oilseed radish and its incorporation. Interestingly, its population was shown to decrease in rhizosphere soil of strawberry. This can be explained by strawberry being a non-host of *O. brassicae*. Surprisingly *V. dahliae* was not detected in any of the Verticillium-inoculated treatments despite the fact that the strawberry plant growth was affected negatively. Possible explanations are inherent PCR bias of the primers chosen for the study or insensitivity of their specificity to detect the pathogen (Hong *et al.*, 2009; Prosser *et al.*, 2011; Schloss *et al.*, 2011; Peiffer *et al.*, 2013).

Mortierella spp. usually a non-pathogenic soil fungus has been considered to be the first organism growing on root was detected in all treatments. The fungus is a saprophyte and is known to produce extracellular hydrolases such

as chitinases (Boer *et al.*, 1999). Certain members of *Mortierella* have been demonstrated for their potential as bio-control agents of phytopathogens such as pathogenic oomycetes and Streptomyces spp. (potato scab) (Wills & Lambe, 1980; Wills, 1989; Tagawa *et al.*, 2010). Oomycetes were marked by their absence in all the treatments in this study, the same were detected in the three soils in Paper II. Interestingly, relative abundance of *Mortierella* seemed to increase in presence of *V. dahliae* regardless of the presence or absence of strawberry plant or biofumigant incorporation. Further studies will reveal the role of *Mortierella* in influencing the population of oomycetous pathogens of strawberry and its potential role as a bio-control agent against Verticillium wilt disease.

4 Conclusions

This is, to the best of our knowledge, the first study describing the effect of soil type-cultivar-pathogen-biofumigation interactions on the structure of rhizosphere fungal communities in strawberry, using high resolution 454 pyrosequencing.

- Verticillium has often been mentioned in connection with diseases of strawberry but the studies described in this thesis are the first of their kind in Sweden. Inoculation with *V. dahliae* resulted in decreased berry yield in a number of cases, but increased berry yield in the Florence cultivar grown in conventional soil.
- For unknown reasons, Senga Sengana had much lower yields than three other cultivars in the three soils tested, although the numbers of flowers were not the lowest of all cultivars.
- The numbers of fungal operational taxonomic units (OTUs) were consistently higher in the conventional soil, lower in organic soil and lowest in peat-based growth substrate.
- In the conventional soil, the number of fungal OTUs showed a consistent decrease in response to *V. dahliae* inoculation. However, the number of fungal OTUs increased in response to *V. dahliae* inoculation in the rhizosphere of the Florence cultivar in organic soil. *Speculation*: Is the decreased yield of berries in Florence in organic soil due to carbon allocation to extra fungal OTUs in the rhizosphere soil in the presence of *V. dahliae*?
- There was a strong effect of soil type on total rhizosphere soil fungal community composition in all treatments.
- In the two field soils, there was no visible effect of *V. dahliae* inoculation on total rhizosphere soil fungal community structure; however its inoculation resulted in distinct fungal communities in the species-poor peat-based growth substrate.

- The cultivar effect on rhizosphere fungal community structure varied in different soils.
- Biofumigation induced early flowering and a decrease in berry yield.
- There were clear effects of biofumigation and strawberry plant root exudates on total numbers of rhizosphere soil fungal OTUs.
- Significantly increased numbers of OTUs were associated with biofumigation and Verticillium-inoculation in the absence of strawberry plants, suggesting a green-manuring effect of oilseed radish incorporation.
- In the absence of biofumigation and *V. dahliae* inoculation, the total fungal diversity estimates revealed significantly higher numbers of OTUs in the presence of strawberry plants than in their absence, indicating a stimulatory effect of their root exudates on fungal taxa.
- Biofumigation did not affect total OTUs in the presence of strawberry plants but NMDS analysis showed a clear effect of all treatments on community structure indicating the interaction effects of biofumigation, *V. dahliae* inoculation and strawberry root exudates.
- The relative abundance increased in the presence of *V. dahliae* of *Mortierella* spp. irrespective of biofumigation and presence of strawberry plant indicating its potential role in biocontrol of *V. dahliae*.
- *Acremonium and Coniothyrium* two fungi with known biocontrol potential, were detected at low levels of abundance in the rhizosphere soil but were the sixth and eleventh most abundant fungi respectively in the roots.
- The abundance of *Olpidium brassicae* decreased in all treatments after planting of strawberry suggesting the role of strawberry as a precrop to *Brassica* spp.
- Depending on the soil type, the degree of arbuscular mycorrhizal colonisation and assemblage structure changed in the strawberry roots. No clear differences were observed between the cultivars.
- *Acaulospora* spp. seems to be exclusively associated with organic soils. Members of this genus and its microbial associates may be explored for plant growth stimulation through improved nutrient acquisition and biocontrol.
- Nucleic acid extraction methods influenced the outcome of results on fungal community structures suggesting that utmost care should be taken to choose the appropriate method for community analysis.
- Further studies are needed to study the functional aspects of the fungal communities reported in this study.

5 Future perspectives

Plant-microbe interactions play an important role in plant protection and plant health. Many studies have examined the effects of biological control agents on pathogen populations and other microbial communities; however effects of plant pathogens on microbial communities have not normally been the focus of most studies. This study is one of the first describing such interactions with respect to host variation. Many interesting observations in relation to this study have revealed number of thought-provoking ideas and questions such as

- Single inoculation studies of the taxa that were found responding to certain treatments should be explored to investigate their potential as biocontrol agents and/or their capacity to improve plant nutrient acquisition.
- The taxa that were found inhabiting the roots and rhizosphere might play different roles either in protecting the plants from pathogens or in biogeochemical cycling of nutrients. Further studies are required to investigate the functional complementarity between the root and rhizosphere microbial communities.
- Do endophytes respond more strongly to a pathogens presence than the rhizosphere microbial community?
- Are bacteria more prone to stress caused by pathogens presence than fungi?
- How to distinguish between true endophytes and opportunistic taxa entering the roots through wounds?
- What role does soil-type play for production of plant growth stimulators or pathogen antagonistic substances?
- There are indications of pathogen helper taxa (PHT) which could be beneficial to a pathogen as well as plant-induced antagonists

(PIA) which may provide protection to plant. Further investigations are needed to conceptualize such plant-pathogen-microbial community interactions. To have a concrete evidence, the results of this study should be validated in field trails and substantiated further by more molecular studies of plant-microbial community interactions.

- With respect to plant-microbe-pathogen interactions in rhizosphere, the nutrient use efficiency of different plant cultivars should also be investigated in such studies.
- Does biofumigation favour glucosinolate-degrading microorganisms and can such organisms improve disease resistance in plants or act as antagonists against soil-borne pathogens?
- In the presence of a pathogen and/or a biocontrol agent in the rhizosphere, do carbon allocation patterns below-ground change?
- What is the functional role of *Acaulospora* in plant protection and/or nutrient acquisition?
- Further studies based on soil metagenomics should be carried out in order to find the functional roles of the microbial communities that respond to pathogen inoculations. This would enable us to understand the mechanisms underlying the changes in community composition.

The importance of the soil- air-water biosphere for sustainability has been summarised succinctly by Eliot Coleman….

> "The only truly dependable production technologies are those that are sustainable over the long term. By that very definition, they must avoid erosion, pollution, environmental degradation, and resource waste. Any rational food-production system will emphasize the well-being of the soil-air-water biosphere, the creatures which inhabit it, and the human beings who depend upon it."
> — *The New Organic Grower: A Master's Manual of Tools and Techniques for the Home and Market Gardener*

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