Assessment of the effects of genetically modified potatoes on structure and functioning of soil fungal communities

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Content

1

Introduction

Although conventional breeding has been successful in developing plants with desired traits, transgenic techniques have recently extended these possibilities by allowing the transfer of interesting genes of other organisms (Jones, 2011). In the past decade transgenic techniques have become an increasingly accepted way of crop improvement and the number of fields allocated to transgenic crop production has increased each year worldwide; and currently approximately 9 % of all the arable land in the world is used to growing genetically modified (GM) crops (James, 2010). Yet, there is concern about the safety of GM plants for human consumption as well as about their impact on the environment (Dunfield and Germida, 2004; Lilley et al., 2006) including soil organisms (Wolfenbarger and Phifer, 2000).

The composition and activity of the communities of soil biota are strongly affected by intra-specific and inter-specific differences in the physiology and morphology of plants through changes in the quality of crop residues (Flores et al., 2005; Hättenschwiler et al., 2005; Berg and McClaugherty, 2008) and root derived compounds (Berg and Smalla, 2009; Hartmann et al., 2009) . This implies that the introduction of GM-crops might alter the composition and activity of soil biota. However, the direction and magnitude of these changes to soil borne microbes are largely unknown (Oger et al., 1997; Birch et al., 2007). Furthermore, when differences in effects on soil biota in the vicinity of the root of a modified cultivar and its parental isoline are observed, the relevance of these differences should be related to other factors affecting the soil biota.

The main objective of this thesis is to investigate the effects of starch-modified GM-potatoes on soil fungal communities via changes in root-exudates and litter composition, and to compare the observed differences between the GM- and its parental variety in the context of the 'normal' variation effected by conventionally produced cultivars.

1.1. Soil, rhizosphere and their inhabitants

1.1.1. Soil ecosystem

Belowground communities usually support much greater diversity than corresponding aboveground communities and soil is regarded to be one the most diverse ecosystems on earth. The soil ecosystem contains huge numbers of taxa and functional groups and our knowledge of many of these functions and interaction is still rudimentary (Lilley et al., 2006). It is estimated that a single gram of arable soil typically contains somewhere in the order of 107-109 bacteria and 105-106 fungal colony forming units (Murphy et al., 2003).

Each soil is presumed to harbour its own unique microbiota (Garbeva et al., 2004; Griffiths et al., 2008). Various factors such as soil type (texture), nutrient status, moisture, pH and in particular, plant related factors shape the soil microbial communities (Kent and Triplett, 2002; Lauber et al., 2008; Singh et al., 2009). Indeed, plants are the major drivers of the soil ecosystem. They provide the soil with the organic carbon which forms the basis for the below-ground food-web, as well as with energy resources and a habitat for root associated organisms such as root herbivores, pathogens and symbiotic mutualists (Wardle et al., 2004). The decomposer subsystem is responsible for the breakdown of dead plant material and thereby indirectly regulates plant growth and community composition by determining the availability of the nutrients. Root associated organisms and their consumers influence the plants and the energy and nutrients in the soil more directly. The area of soil around the root that is under the influence of living plant roots, is called the rhizosphere.

1.1.2. Rhizosphere

The term rhizosphere was first coined in 1904 by Hiltner (1904). Since then, our understanding of the rhizosphere processes have advanced tremendously, although we are still facing the very same challenges to understand the interactions between micro-organisms and roots in different soils (Smalla et al., 2006; Hartmann et al., 2009). The rhizosphere contains a distinct microbial population which is larger and more active than that found in the surrounding bulk-soil zone (Walker et al., 2003; Lilley et al., 2006). The composition and dynamics of the microbial community in the rhizosphere has as an important role in plant growth having either positive, negative or neutral influences on plants (Singh et al., 2004; Buée et al., 2009a; Raaijmakers et al., 2009). The importance of the microbes to the plants is due to their role in mobilisation of nutrients, the production of plant growth hormones and the protection they provide for the plants against pathogenic organisms (Bashan and Holguin, 1998). Pathogenic microbes can in turn display a severe negative impact on plant health (Raaijmakers et al., 2009).

While the microbes affect the plants, the plants in return influence the composition of the rhizosphere microbial community, in particular by the composition and quantity of rhizodeposition (Lynch and Whipps, 1990; Bais et al., 2006; Badri and

Vivanco, 2009; Dennis et al., 2010). It has been estimated that 20 to 50 % of the carbon obtained by the plant by photosynthetic assimilation is transferred to the roots and about half is further released into the soil (Kuzyakov and Domanski, 2000). Eventually, approximately 80-90 % of annual primary production enters the soil detrital food web in natural ecosystems (Christensen, 1989; Smalla et al., 2006). However, both the estimates of carbon economics and the composition of rhizodeposits are known to vary considerable between plants species and groups (Jones et al., 2004; Badri and Vivanco, 2009; Dennis et al., 2010). Furthermore, the quantity and quality of rhizodeposits can vary in time and space along the root system (Walker et al., 2003; Dennis et al., 2010). Root derived compounds can stimulate rhizosphere nutrient mineralization and are important for forming plant-specific rhizosphere communities (Lynch and Whipps, 1990; Kowalchuk et al., 2002; de Boer et al., 2006; Broeckling et al., 2008).

Historically, most attention has been paid to bacteria in the rhizosphere which was based on the assumption that bacteria monopolize the decomposition of simple organic substrates such as organic acids, sugars and amino acids which usually make up the majority of the actively exuded compounds, also called root exudates (Kent and Triplett, 2002). Indeed many papers have found bacteria as better competitors for these compounds (de Boer et al., 2005). However, other studies have demonstrated that fungi and especially so called sugar-fungi might be more important than assumed so far in rhizosphere processes (Butler et al., 2003; Drigo et al., 2010).

1.1.3. Fungi

The true fungi are ubiquitous in the environment and fulfil a range of ecologically important functions such as nutrient and carbon cycling in soil (Christensen, 1989). At a local scale, fungal diversity can have important consequences for plant communities and terrestrial ecosystems (van der Heijden et al., 1998). Despite their importance, the knowledge about functioning and structure of fungal communities is limited for many ecosystems (Anderson and Cairney, 2004), which is partly due to the lack of proper analysis tools. Until recently, the methods used to study fungi were based on microscopic counts of hyphae and spores. During the last decade, however, molecular identification techniques have become standard tools for fungal community analysis (James et al., 2006; Hibbett et al., 2007). The implementation of next-generation, high-throughput sequencing techniques have increased the possibilities to perform analysis of the function of fungi in soils (Buée et al., 2009b; Jumpponen and Jones, 2009; Öpik et al., 2009; Jumpponen et al., 2010).

The largest group of fungi present in soils are the higher fungi (Dikarya), comprising of Ascomycota and Basidiomycota (Carlile et al., 2001). Together with Glomeromycota, they are considered to be the most important phyla of fungi due to their role in decomposition activity and their ability to form symbiotic relationships with plants, called mycorrhiza. Fungi are better equipped for the degradation of plant materials than bacteria both through their mode of growth and their

enzymatic capabilities. Members of the phylum Basidiomycota are considered the most important decomposers. However, their role in agricultural soils remains unclear, especially given the saprotrophic capabilities of many Ascomycota (Lynch and Thorn, 2006; Lauber et al., 2009; Klaubauf et al., 2010). Besides their function as effective decomposers, fungi contain obligate mutualistic species (Glomeromycota) and pathogens (Ascomycota, Basidiomycota and Zygomycota) and thus form a very diverse group of organisms with many possible functions in the rhizosphere (Broeckling et al., 2008; Buée et al., 2009a; Raaijmakers et al., 2009). In agroecosystems, fungal diversity and community composition have been thought to depend on plant species and community diversity, as well as nutrient status of the soil, and management practices (Lynch and Thorn, 2006; Klaubauf et al., 2010).

Arbuscular mycorrhizal fungi (AMF) belonging to phylum Glomeromycota are of particular importance for functioning of plants due to their high capacity to increase plant growth and yield by improving the uptake of nutrients (Smith and Read, 1997). AMF have also been shown to improve soil structure (Sensoy et al., 2007). In addition, AMF enable plants to cope with both biotic and abiotic stresses: they may help fight diseases, alleviate certain nutrient deficiencies, improve drought tolerance, overcome the detrimental effects of salinity and enhance tolerance to pollution (Schreiner et al., 1997; Tonin et al., 2001; Lioussanne et al., 2008).

1.2. Influence of agricultural practices on soil fungi

Before considering the potential effects of genetically modified plants on soil fungi, it is necessary to consider the general effects of agriculture on fungi. Many studies have reported a reduction of fungal biomass under agriculture, and bacteria are thought to dominate in agricultural ecosystems (Kennedy, 1999; Berg and Smalla, 2009). This has been attributed both to the constant removal of crop plants, thereby reducing the input of litter (Berg and Smalla, 2009) and to mechanical actions such as plowing which can potentially break extensive hyphal networks (Wang et al., 2010). Indeed, it has been proven that agricultural management systems such as differential crop rotation (Larkin, 2003; Larkin, 2008) and biological versus conventional farming (Verbruggen et al., 2010), do affect the abundance and composition of soil organisms and consequently the soil functionality (Santos-González et al., 2011). It is known that especially AM fungi are strongly affected by agricultural practices such as fertilizer levels and changes in soil characteristics, thus representing potential key non target micro-organisms to be monitored in studies on environmental impacts of agricultural practices and of GM-crops (Helgason et al., 1998; Larkin, 2003; Turrini et al., 2004; Giovannetti et al., 2005; Verbruggen et al., 2010).

1.3. The impact of genetically modified crops on the soil ecosystem

Common variables in agricultural practice such as fertilizer regimes, tillage, ap-

plications of pesticides and water/irrigation treatments do strongly affect the soil microbial community structure in fields with both GM and non-GM crops. Therefore, these variations due to agricultural management should be taken into account when evaluating possible side-effects of GM crops (Marschner et al., 2002 & 2003; van Overbeek and van Elsas, 2008; Berg and Smalla, 2009).

Due to their versatile relationship with the plant, fungi represent a group of import ant non-target organisms to be evaluated for these potential undesired side-effects. However, studies have indicated that both crop species and cultivars have an impact on the composition of soil microbial communities. Hence, effects of GM-crops should be considered within the range of microbial community compositions that can be found with non-GM crops and cultivars. The possible mechanisms how GM-crops could affect soil microbial and especially fungal communities are presented in Figure 1.1 and details of earlier research on effects of GM- crops on soil fungi are discussed further in chapter 2.

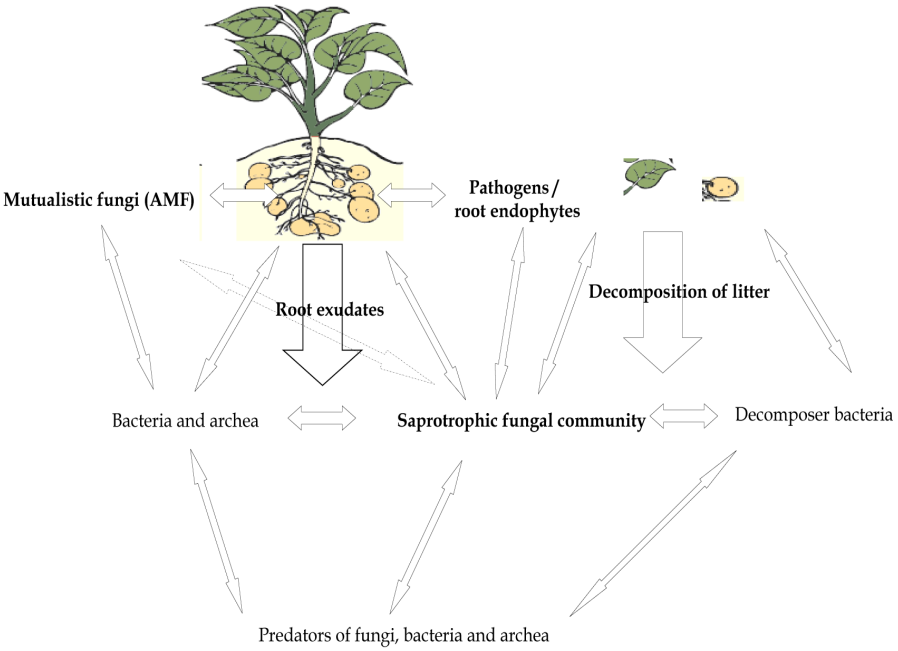


Figure 1.1. Possible mechanisms how GM-plants could affect soil fungi and soil functioning. The main mechanisms are the possible change in root exudation patterns affecting soil microbes and changes in litter quality or quantity, affecting the decomposer community. The groups and processes studied in this thesis are marked in bold.

The photosynthetically assimilated CO2 released via rhizodeposition is the primary carbon source in soils during the vegetation period. As there is so much variation in rhizodeposition patterns between different species (Walker et al., 2003; Dennis et al., 2010), it is fair to assume that genetic modification in plants, especially if the modification is targeting carbon related compounds, could result in a change in carbon allocation patterns and thus may affect soil microbial communities with a strong feedback on plant performance. Despite many articles providing information on the effects of modified crops on soil bacterial and fungal communities, only few have addressed the question from the carbon partitioning perspective (Wu et al., 2009; Gschwendtner et al., 2011).

Many approaches have been used to monitor the response of the rhizosphere microbial communities to root exudates (Kuzyakov and Domanski, 2000). One method that has proven very useful is the application of different carbon isotopes in tracking 13C in cellular components (e.g. lipids and nucleic acids) to determine which functional groups actively assimilate 13C labeled substrates (Boschker et al., 1998 ; Radajewski et al., 2000; Manefield et al., 2002; Mauclaire et al., 2003; Lueders et al., 2004; Rangel-Castro et al., 2005; Prosser et al., 2006; Bernard et al., 2007; Vandenkoornhuyse et al., 2007; Haichar et al., 2008; Rasche et al., 2009; Uhlik et al., 2009; Lee et al., 2011). These studies have provided valuable information concerning plant-microbe interactions, but most of them have not considered fungal community composition.

The second mechanism of how GM-crops could affect the soil fungal communities is via litter decomposition (Fig 1.1). The physiochemical environment, litter quality and the composition of the decomposer community itself are the three main factors identified to control litter decomposition (Hättenschwiler et al., 2005; Berg and McClaugherty, 2008). Furthermore, it is well known that litter-decay rates differ among plant species (Hobbie, 1992; Berg and McClaugherty, 2008). Though the mechanisms by which plant diversity and species identity can affect ecosystem functioning are well documented, associations between plant genotype (and GM-trait) and litter decomposition remain elusive (Bernard et al., 2007).
During harvest, parts of GM-crops are left behind in the fields and thus could potentially impact the fungal diversity and consequently influence ecosystem functioning via decomposition (Deacon et al., 2006). So far, studies on decomposition of GM-residues have been mostly done with maize cultivars (Flores et al., 2005; Daudu et al., 2009). Earlier studies have found differences in microbial communities associated with GM-potatoes mostly at the senescent growth stage (Lottmann et al., 1999; Lottmann et al., 2000; Lukow et al., 2000). This increases the likelihood of observing effects of GM-crop residues. Possible effects of GM-crops on soil fungal decomposers are further discussed in chapter 2.

1.3.1 GM potato with altered starch metabolism

In this thesis, a GM potato (*Solanum tuberosum* L.) with altered starch metabolism is studied as a model system for the assessment of the ecological impacts of GM crops

on soil fungal communities. Starch consists of two polysaccharides, amylose and amylopectin and, because of their different physico-chemical properties, they need to be separated for industrial use. In order to avoid the high production and energy costs of the separation process (Visser et al., 1991), the production of potato tubers composed solely of amylopectin is an important aim in potato crop breeding, notably for the paper and textile industries. The GM potato used in this thesis was constructed by the marker-free introduction of an antisense granule-bound starch synthase gene (gbss), which encodes key enzymes for amylose production (de Vetten et al., 2003). Consequently, tuber starch consists of virtually pure amylopectin which can be more readily used in paper, textile and food industries.

GM starch composition has been shown to influence microbial community structure in potato rhizosphere (Milling et al., 2004). Despite the importance of fungi in the soils (Carlile et al., 2001) and the potential effects of modification on carbon related compounds such as starch especially on fungi, the effect of this modification on specific fungal communities and their function has not been investigated. In a recent paper, information was provided that fungal gene abundance (specifically Trichoderma) was not different between parental and starch-modified potato although there were some differences between growth stages and cultivars (Gschwendtner et al., 2010).

1.4. Baseline approach and the need for indicators

It is well established that the microbial communities in the rhizosphere and bulk soil are immensely diverse and dynamic, showing changes in relation to many abiotic and biotic variables. Hence, in order to evaluate the consequences of GM-crops on soil fungal community composition, it is important to first establish the composition of the fungal community and its fluctuations under normal operating conditions. Previously, the assessment of the potential effects of GM-crops has been hampered by the lack of temporal baseline data, e.g. the lack of knowledge on the seasonal dynamics of microbial communities and the influence of normal agricultural practices (Milling et al., 2004; van Overbeek and van Elsas, 2008). The variability in traits of both soil and plants have to be taken into consideration in a case-by-case manner so that the knowledge of different properties of soil (e.g. pH, level of different nutrients, water content and in some cases the types of soil animals present) is combined with information about the past and present land use and agricultural practices (i.e. used fertilizers and crop rotation). Only with proper knowledge about these characteristics, we can conclude whether observed variations in fungal community structure and diversity under transgenic potato is within the normal operating range or if transgenic potatoes do induce changes in the community that are beyond the normal variation (van Overbeek and van Elsas, 2008).

However, generating such data is labour intensive which explains the low number of studies in the scientific literature covering all aspects presented in figure 1.2. Moreover, as it is not feasible to monitor all components of a soil ecosystem for

their response to a GM crop, the use of keystone indicators can be a suitable approach (Kowalchuk et al., 2003). The focus in such an approach should be on organisms that are expected to be (1) important for soil functionality, (2) relevant for the modification studied and (3) responsive to perturbations.

So far, a baseline of variation for fungi has not been well defined. One of the objectives of this thesis is therefore to establish and validate a set of advanced tools, which together will constitute a protocol, for the assessment of the normal operating range of soil fungal functioning and community structure in agricultural soils under a set of different crop cultivars as presented in Fig 1.2.

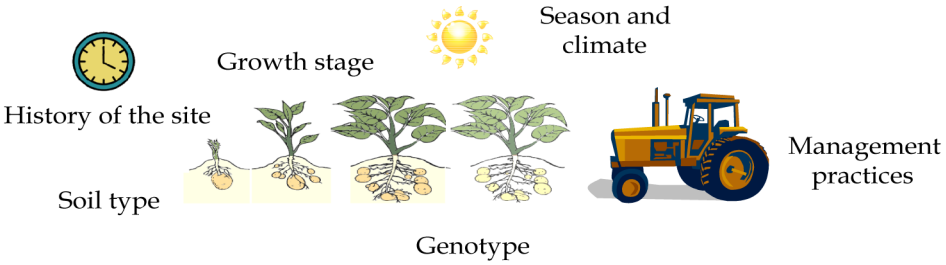


Figure 1.2. Abiotic and biotic factors affecting potato plants in field sites. Besides the factors mentioned in the figure, soil biota and plant species have an impact on soil fungal communities.

In order to evaluate the baseline for the GM-cultivar, a field trial was designed. In chapters 3 and 4 results are described from an experiment performed in two fields (history of the site and soil type) with a total of six genotypes of potato (which of one modified for its tuber starch quality) during three years and a total of 15 sampling time-points. The approach designed for the studies is depicted in figure 1.3.

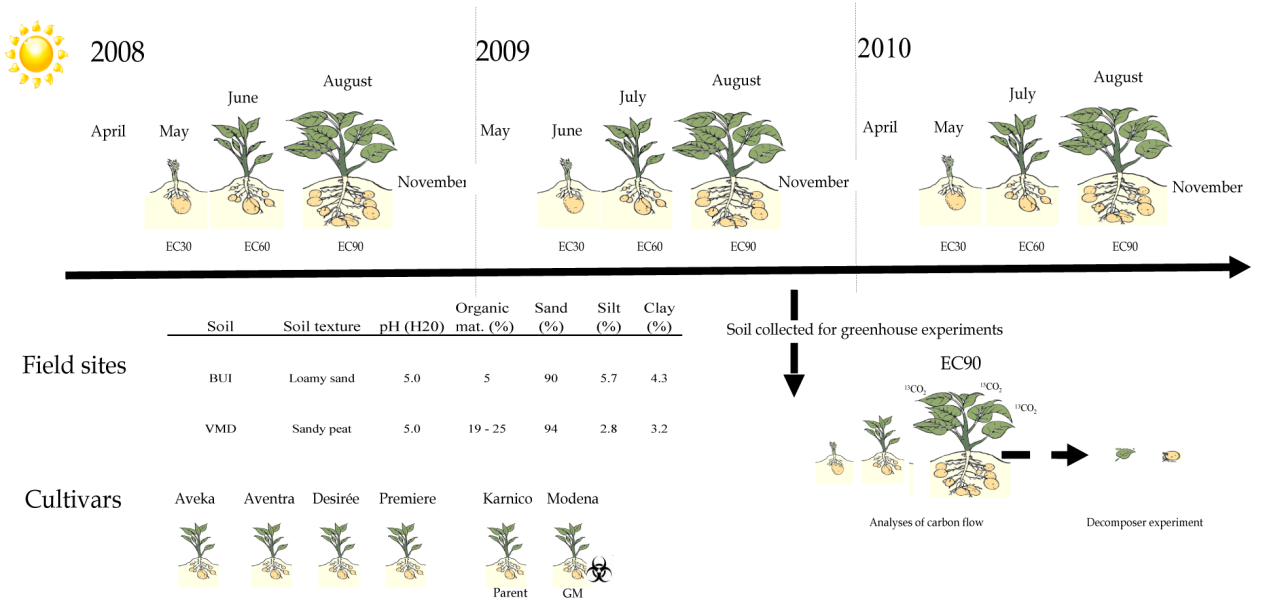


Figure 1.3. Approach designed to investigate the effects of multiple factors on soil fungal communities in potato fields.

1.5. Aim and thesis outline

The major aim of this thesis is to investigate the effects of GM-potato on soil fungal communities and to establish a baseline of normal variation in fungal abundance and community composition for potato farming systems. This is done with a three year field experiment in which community function, diversity and structure of three phyla were evaluated. In addition, greenhouse experiments are performed using stable isotopes ($^{13}CO_2$) to gain insight into the carbon flow from plant to the rhizosphere fungal communities.
My specific research objectives are:

1. Develop robust methods to evaluate soil fungal community structure and function

2. Establish which fungi and fungal-related functions are most sensitive to the plant genotype and possibly the GM-modification

3. Evaluate to what extent the fungal communities in the rhizosphere of potato fluctuate between years, growth stages and fields in both function and structure and thus provide 'baseline' information for the evaluation of GM-potatoes

4. Investigate if the genetic modification in the starch content in tubers affects the carbon flow from the plant to soil fungal communities and thus shape the rhizosphere fungal communities in the potato rhizosphere.

5. Investigate the decomposer community involved in the degradation of potato plant material and evaluate the possible differences in decomposition between the GM- and its parental variety.

These objectives are addressed in 7 chapters.

In CHAPTER 2 an overview of earlier studies on the relationship between soil fungal communities and GM-crops is made. Furthermore, the approaches suitable to obtain baselines and methods available to detect these effects are evaluated.

In CHAPTER 3 the methods which can be used in evaluation of GM-crops on soil fungal community and functionality are established. Furthermore, the dynamics of fungi in fields cropped with GM-potato cultivars are evaluated in one-year field experiment and the effect of different cultivars and GM-trait on rhizosphere are investigated.

In CHAPTER 4 the concept of baseline in a 3 year field study is addressed and the results from chapter 3 are re-evaluated.

In CHAPTER 5 insight in the carbon flow from GM potato plants and parental isoline to soil microbial and fungal communities is provided in a greenhouse experiment using stable isotopes.

In CHAPTER 6 the effects of a GM-trait on the decomposition rate of potato residues and fungal decomposer communities are described in a 6 months decomposer experiment.

Finally, in CHAPTER 7 main findings of this thesis are combined and their implications for evaluations of risk-assessment of GM-crops are discussed.

2

Effects of GM-crops on non-target soil fungi

Emilia Hannula, Wietse de Boer & Hans van Veen

2.1. Introduction

Scientific as well as ethical concerns about the implementation of transgenic crops have been discussed in many public forums and have spurred scientific discussions regarding their safety to the environment (Jones, 2011). Despite these concerns, the advantages (such as possibility to transfer interesting genes from other organisms) seemed to have outweighed the concerns and the number of fields allocated to transgenic crop production has increased each year worldwide, and currently approximately 9 % of all the arable land in the world is used to growing genetically modified (GM) crops (James, 2010). Big part of discussion have involved the risks that occur when genetically modified plants are grown in uncontrolled environments such as agro ecosystems (Dunfield and Germida, 2004). Among these concerns are the possibility of unintended transgene flow to indigenous plants, development of super pests, and the effects of transgenic plants on non-target organisms, including soil microbial communities (Wolfenbarger and Phifer, 2000).

Effects on composition and activity of soil biota could occur via changes in the quality of crop residues and root exudates as a result of crop modification. However, several studies have shown that growing of different crop species is also coinciding with changes in the soil microbial communities making the interpretation of the results of GM-crops versus parental crops more complicated (Bruinsma et al., 2003; Kowalchuk et al., 2003; Liu et al., 2005). The majority of the studies on effects of GM-crops on soil microbes have been investigating bacterial numbers, activities and community composition whereas only relatively few have studied the impact on fungi in similar detail despite the importance of fungi for the functioning of soil ecosystem (Carlile et al., 2001). In 2003, it was thought that the remaining gaps regarding the impact of GM-crops on soil microbes were (1) incomplete knowledge of the functional aspects of the microbial community in soil, (2) poor understanding of the structural and functional responses of the microbial community to "normal" variation in soil systems (such as due to season, weather, and agricultural management practices including fertilizer use, crop rotation, pesticide use, etc.), and (3) inability to transform complex laboratory procedures into practical assays that are easy to perform and interpret (Bruinsma et al., 2003). Few of these knowledge gaps, in particular in the area of effects on fungi, have been filled since.

In this article, we will summarize published studies which have examined the effect of GM-plants on non-target soil fungi. We pay special attention to methods recently developed such as next-generation sequencing and stable isotope probing which have the potential, both in their own way, to facilitate and improve the evaluation of the response of soil fungi to GM-crops.

2.2. Mechanisms by which GM-plants can affect soil fungi

GM crops can potentially influence soil ecosystems positively, negatively or neutrally, (Oger et al., 1997; Birch et al., 2007a). The potential impacts of GM crops on soil ecosystem can be (1) direct (e.g. toxicity of an expressed new protein on key non-target species), (2) indirect (e.g. effects via trophic interactions), (3) caused by unintended changes in the metabolisms of the plant and thus altering root exudation and/or (4) effects caused by changes in the management regime used to cultivate GM crops (Birch et al., 2007b). The possible effects discussed in this review are effects of different genetic modifications on non-target fungi via changes in root-exudation patterns and on decomposition processes via unintentional changes in the chemical composition of the GM-plants.

2.2.1. Root exudation and soil fungal communities

Root exudates (rhizodeposition) have been indentified as an important factor for the development of rhizosphere microbial communities (Lynch and Whipps, 1990; Berg and Smalla, 2009). A substantial amount of photosynthetically fixed carbon is released into the rhizosphere by roots, but composition and quantity differs among plant species (Berg and Smalla, 2009). Therefore, the first mechanism by which GM-crops can affect soil fungal communities is via intentional or unintentional changes in root-exudation quantity and quality. The latter does not only include changes in composition of well-known root-exudates (sugars, organic acids and amino acids) but also the presence of toxins, introduced via GM, into the soil via exudation. It has been shown that the presence of a novel compound in root exudates of a transgenic plant conferred a selective advantage to a specific group of soil bacteria which are able to utilize this compound (Savka and Farrand, 1997). However such specific process has not yet been demonstrated for fungi. The effects of toxin release in root exudates from Bt-crops and its persistence in the soils has been discussed in detail in an earlier review (Icoz and Stotzky, 2008) and thus we will not further discuss it here.
Several studies have investigated the effect of GM-crop in comparison to its parental isoline and other varieties of the same crop species in field and greenhouse experiments. Most of these studies have shown that the GM-crop does not affect soil fungi differently than its parental variety. Only in five studies significant differences between the GM-variety and its parental isoline (Cowgill et al., 2002; Xue et al., 2005; Götz et al., 2006; Wei et al., 2006; Kremer and Means, 2009) were observed (Fig. 2.1A). The reason why these studies, and not others have found differences between GM- and their parental varieties remains unclear as there is little common in these studies. Two of the mentioned studies were carried out with potatoes (nematode and pathogenic bacterial resistant) (Cowgill et al., 2002; Götz et al., 2006), one with both maize and potato (Bt and viral resistance) (Xue et al., 2005), one with soybean and maize (herbicide tolerance) (Kremer and Means, 2009) and the last one with viral resistant papaya (Wei et al., 2006); the methods used to assess the fungal community ranged from quantifying fungi with fatty acid methyl esters (FAME) biomarkers (Xue et al., 2005) to specific endophyte denaturing gradient gel electrophoresis (DGGE) (Götz et al., 2006).

Remarkably, other studies carried out with the same crops and modifications showed no effect on fungi (Fig. 2.1A). For example, a study by Kremer & Means (2009) found that frequency of Fusaria colonizing glyphosate resistant maize roots was higher than in the roots of the parental cultivar, whereas in the same year Hart et al. (2009) found no difference in abundance or community structure of rhizosphere fungi between the same parental and GM-varieties. In seven studies differences were found between GM- and parental varieties, but due to the large variation in time (Donegan et al., 1996; Dunfield and Germida, 2003; Icoz et al., 2008; Oliveira et al., 2008) and space, these effects were deemed transient. These 'transient' effects are discussed later in this review.

Despite the importance of arbuscular mycorrhizal fungi (AMF) in plant-soil systems, only in rather few studies the non-target effects of GM-crops on AMF colonization and community structure have been evaluated (Liu, 2010) (Fig 2.1B). AMF are ubiquitous soil microorganisms, existing in almost all types of soil ecosystems and form symbioses with more than 80% of plant species (Smith and Read, 1997) also with many important crop species. As plants vary naturally in their AMF-hosting ability, GM trait in a crop might, in some cases, alter their relationship with AMF. Furthermore, because AMF are obligate symbionts and thus require the plant host for nutrition and reproduction, they may be more sensitive to changes in the physiology of the host plant than other fungi (Liu, 2010; Cheeke et al., 2011).

Earlier, it has been shown that AMF are sensitive to different agronomic practices such as tillage and fertilization (Oehl et al., 2010) and are thought to be especially important in low input agro-ecosystems (Verbruggen and Kiers, 2010). Thus, it is crucial to know what are the impacts of GM-traits on functioning and diversity of AMF. Only in one study a reduction in mycorrhizal colonization potential of Glomus mosseae in the roots of a Bt-cultivar of maize was reported (Castaldini et al., 2005). Transient effects of GM-crops on soil AMF colonization or community structure have been reported in 3 studies done to investigate Bt-maize and herbicide tolerant soybean (Turrini et al., 2004; Powell et al., 2007; Cheeke et al., 2011). In one of these studies, different levels of rhizobium and mycorrhizal colonization were observed between conventional and GM- soybeans (Powell et al., 2007). However, these differences could be attributed to variation found between different cultivars and not the GM status of the plant (Fig. 2.1B). Other studies did not find any effect of the GM-modification in any aspect of AM biology studied. For instance, four different modifications introducing insect resistance or herbicide tolerance in cotton had no effect on AMF colonization (Knox et al., 2008). In addition, de Vaufleury et al. (2007) did not find any significant effect of *Cry*1Ab modification of maize on AMF colonization. However, the total number of studies about effects of GM-crops on AMF is rather low (n = 10) and certain traits such as the herbicide tolerance and resistance to pathogens have been studied only in two studies (Fig.2.1b) which makes it difficult to come to a conclusion about the effects of GM-crops on the AMF community.

2.2.2. Effect of unintentional changes in composition of plants on decomposition and decomposer fungi

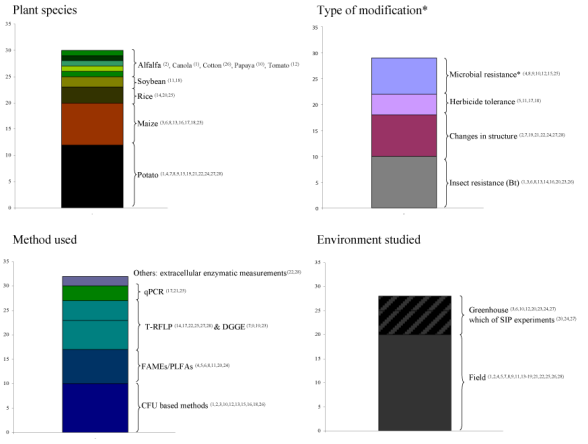
Decomposition of litter is a key function in soil carbon cycling and thus any change in plant litter composition may potentially affect soil functioning (Deacon et al., 2006; Berg and McClaugherty, 2008). In general, fungi are more significant as litter-decaying agents than bacteria and thus more studies have been conducted on fungi compared to bacteria in this field (Berg and McClaugherty, 2008). Nevertheless, the large bulk of the relevant studies have addressed litter decomposition as a functional response to GM-traits without referring explicitly to the fungal communities involved.

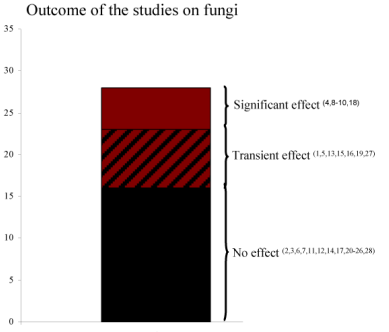
The Bt-varieties of corn, cotton and rice have been the most studied modifications in litter decomposition studies due to the observed unintended effect of *Cry*1Ab on the lignin content of corn and possibly also cotton (Saxena and Stotzky, 2001). Slower decomposition resulting from this altered lignin concentration has been reported in few studies (Castaldini et al., 2005; Flores et al., 2005) while a greater number of studies did not find a difference between Bt and non-Bt corn (Jung and Sheaffer, 2004; Fang et al., 2007; Zwahlen et al., 2007; Daudu et al., 2009; Zurbruegg et al., 2010). An early study concerning *Cry*1A expression in cotton found more fungi based on plate counts in the soils incubated with transgenic leaves than in the soil incubated with leaves from the parental variety (Donegan et al., 1995). However, this study seemed to be an exception, and the only one in which significant differences which could not be explained by other factors than the genetic modification, were detected.

The majority of studies on fungi in decomposing plant material have showed no effect (Fig. 2.1c) or only a transient effect of genetic modifications on certain aspects of fungal community composition and functioning (Wu et al., 2004b; Castaldini et al., 2005; Naef and Defago, 2006; Lu et al., 2010a; Xue et al., 2011). One of these studies involving Cry3Bb expressing Bt-corn in a field experiment revealed no difference in decomposition rate of roots, stalks, cobs or leaves between the Bt- and its parental variety at different locations but did detect a significant difference in fungal community composition (determined by T-RLFP) in one of the soils tested and in one year revealing the transient nature of the observed effect (Xue et al., 2011). Other studies detected effects on one or more time points during decomposition but not at the overall community or in the end result of decomposition (Wu et al., 2004a; Castaldini et al., 2005; Lu et al., 2010a).

It should be noted that effects of genetic modifications on decomposer fungi have only been addressed for a limited number of modifications (Fig. 2.1c) and most of the studies have investigated the effects of Bt-modifications (Donegan et al., 1995; Wu et al., 2004b; Castaldini et al., 2005; Flores et al., 2005; Naef et al., 2006; Lawhorn et al., 2009; Lu et al., 2010a; Lu et al., 2010b; Tan et al., 2010; Xue et al., 2011). Because modifications of pathogen resistance and structural changes of plant parts would be the most obvious GM-traits to affect the non-target decomposer fungal communities, it is surprising that no studies have been done with modifications enhancing pathogen resistance and only two dealt with structural changes of plant (lignin syn-

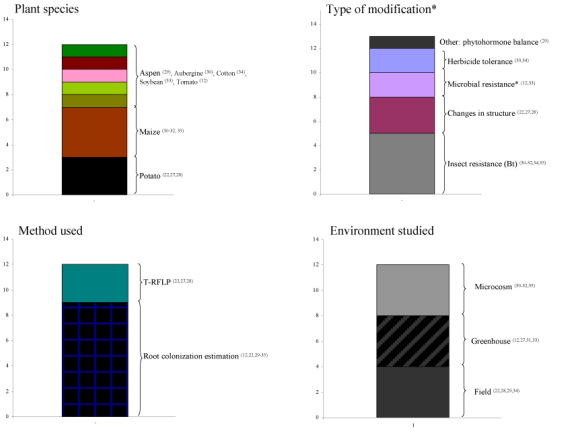
A (n = 28)

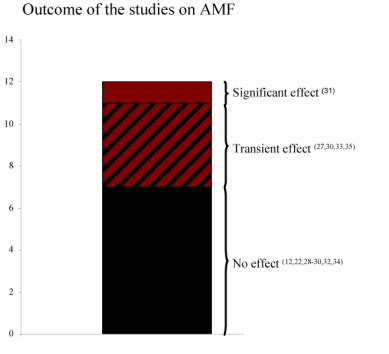




1.Donegan et al., 1996; 2.Donegan et al., 1999; 3.Saxena and Stotzky, 2001a; 4.Cowgill et al., 2002; 5.Dunfield & Germida, 2003; 6.Blackwood & Buyer, 2004; 7.Milling et al., 2004; 8.Xue et al., 2005; 9.Götz et al., 2006; 10.Wei et al., 2006; 11.Weaver et al., 2007; 12.Girlanda et al., 2008; 13.Icoz et al., 2008; 14.Liu et al., 2008; 15.O'Callaghan et al., 2008; 16.Oliveira et al., 2008; 17.Hart et al., 2009; 18.Kremer and Means, 2009; 19.Weinert et al., 2009; 20.Wu et al., 2009; 21.Gschwendtner et al., 2010; 22.Hannula et al., 2010; 23.Tan et al., 2010; 24.Gschwendtner et al., 2011; 25.Lee et al., 2011; 26.Li et al., 2011; 27.Hannula et al., 2012a; 28.Hannula et al., 2012c

B (n = 10)





12.Girlanda et al., 2008; 22.Hannula et al., 2010; 27.Hannula et al., 2012a; 28.Hannula et al., 2012c; 29.Kaldorf et al., 2002; 30.Turrini et al., 2004; 31.Castaldini et al., 2005; 32.de Vaufleury et al., 2007; 33.Powell et al., 2007; 34.Knox et al., 2008; 35.Cheeke et al., 2011

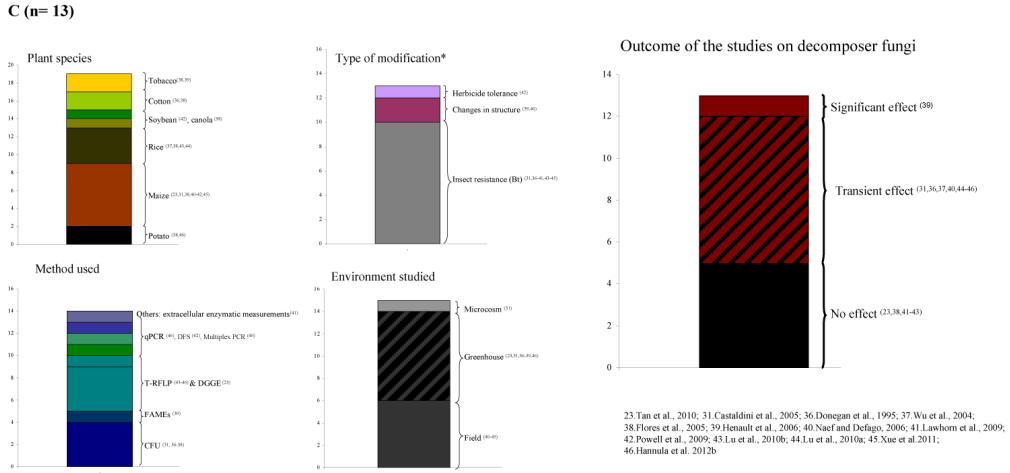


Figure 2.1. Studies on the effect of GM-crops on (a) general fungal communities, (b) arbuscular mycorrhizal fungal (AMF) communities and (c) decomposer fungal communities. This classification was done for convenience and because of the large differences between the types of studies and organisms. The results are categorized by plant species studies, type and method of study and type of modification studied. The observed effect of GM crop was categorized as i) a (lasting) effect, ii) a transient effect or iii) no effect on fungi. In the mycorrhizal studies, only effects on AMF were included; the few studies addressing effects of lignin-modified trees on ectomycorrhiza were not included. Despite the presumed role of fungi in decomposition processes, only studies actually measuring fungal activity were included. Further, studies based on fatty acid analysis (FAME, PLFA) were not included in AMF studies.
*Only non-target effects were included – pathogen resistance marks thus resistance to viruses, bacteria or nematodes

thesis in tobacco and chitinase in birch (Henault et al., 2006; Seppänen et al., 2007) even though there are already several of these modifications in use in field-grown plants. In the case of plants with genetic modifications to structural parts such as lignin synthesis or starch quality, risk assessment studies taking into account the effects on soil microbes and processes are fundamental because of the importance of carbon stock available in regulating the rate of decomposition rate of plant material and the decomposer community.

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2.3. Baseline of normal variation

A common issue in the debate on possible harmful side-effects of GM-crops is the difficulty to discern the effects of the modification from all the other factors e.g. effects of different crop species. This is also the case for effects of GM-crops on soil fungal communities (Fig.2.1). Factors such as season, weather, agricultural management and plant developmental stage might affect the outcome of the experiments more than the actual modification (Griffiths et al., 2000; Lukow et al., 2000; Dunfield and Germida, 2001). Decomposition studies are further also affected by the soil type and burying depth of the tested plant material (Holland and Coleman, 1987; Burgess et al., 2002; Powell et al., 2009; Xue et al., 2011).

The first variables to consider are site related variables. In general, soil type and field conditions, including the history of the site are considered to be among the most influential factors governing soil fungal communities (Costa et al., 2006; Singh et al., 2007; Wang et al., 2009). In case of Bt-modifications it is known that the physicochemical and biological characteristics of soils are likely to influence the persistence of Cry class proteins (Icoz and Stotzky, 2008) in the environment thus influencing the outcome of the studies. Moreover, especially AMF are known to respond strongly to soil type, agronomic practice and soil fertility level (Helgason et al., 1998; Jansa et al., 2002). Unfortunately, only relatively few studies addressing possible effects of GM crops on soil fungal communities have included more than one soil type (Dunfield and Germida, 2003; Blackwood and Buyer, 2004; Weinert et al., 2009; Li et al., 2011). For instance, Blackwood & Buyer (2004) investigated the effects of Bt-modified maize on soil fungi in three soils and found the soil type, but not the modification, to have a significant effect on the fungi. Even fewer studies have compared agricultural management practices (Weaver et al., 2007; Hart et al., 2009; Kremer and Means, 2009) (Fig.2.2). Results from these studies, however, point out that soil type is one of the principal factors affecting soil fungal communities. Cheeke et al (2011) showed in a study with artificial inoculation of AMF Glomus mosseae in Bt- maize and parental roots that there was a significant interaction effect of cultivar and fertilizer level. The effect of the GM-trait could only be seen in the low or no fertilizer treatments but not in the high fertilizer treatment highlighting the important role of the soil environment in modulating the interaction between the GM-trait and fungi. Furthermore, the decomposition of litter is found to be site and soil dependent and, indeed, studies on Bt maize and rice have shown that both the site and the burying depth are very important factors governing the decomposer processes and associ-

ated fungal communities (Cortet et al., 2006; Lu et al., 2010a; Lu et al., 2010b; Xue et al., 2011). For example Lu et al. (2010b) found that the decomposition dynamics and fungal communities associated with decomposition were strongly affected by the placement of the litter bags (top soil and buried) and temporal factors but did not find significant difference between Bt- and non-Bt rice.

The growth stage of the plant is a second factor determining the activity and community structure of fungi in soil. Jones et al. (2004) indicated that the amount and composition of root exudates change during plant development and that this will have important consequences for microbial activity and community composition. Indeed, plant growth stage and sampling time were found to have the largest effect on activity and composition of both general fungi and AMF in many experiments (Dunfield and Germida, 2003; Liu et al., 2008; Li et al., 2011). The effect of growth stage was not seen in the bulk soil (Milling et al., 2004) or in the AMF community under a tree (aspen) (Kaldorf et al., 2002) but in all other studies in which the stage was evaluated. The effect of growth stage was found to affect general fungi (18 studies) and AMF (6 studies) (Fig. 2.2). For example studies on genetically modified potatoes (Donegan et al., 1995; Cowgill et al., 2002; Weinert et al., 2009; Gschwendtner et al., 2010) have showed that growth stage is the single most important factor affecting the outcome of the study. The effects of sampling date on decomposer fungal communities were also clear (Fig. 2.2).

However, in field trials effects of growth stage can be enhanced by coinciding changes in temperature and water availability, which are both important determinants of microbial growth. To add further evidence, even greenhouse experiments have shown that there is an effect, although smaller than in the field, of plant growth stage on soil fungal communities (Powell et al., 2007; Girlanda et al., 2008; Wu et al., 2009; Gschwendtner et al., 2010; Gschwendtner et al., 2011). Finally, there is emerging evidence that plant parts collected at different stages of growth, also would decompose differently (Zurbruegg et al., 2010) and might , thus, also have an effect on fungal communities.

Annual variation including climatic factors such as precipitation and temperature can often also explain a large part of the variation in decomposer experiments (Naef and Defago, 2006; Powell et al., 2009; Xue et al., 2011) (Fig. 2.2). From 11 studies on GM-crops and fungi in which annual variation was accounted for, 9 observed differences in fungal community composition or abundance between years whereas in 2 studies no annual variation was apparent (Milling et al., 2004; Li et al., 2011) . An elegant field study during 3 years revealed that the year was the strongest explaining factor for changes in decomposition rate and associated fungal communities making it much more important factor than the Bt-trait of maize (Xue et al., 2011). In a four year field study on Bt-corn, year was shown to be a highly significant explanatory factor while the Bt-and its parental variety differed only one of the years and thus had a transient effect on culturable soil fungi (Icoz et al., 2008). Furthermore, none of the studies showed long term effects of GM-variety after the harvest of the GM crop (Oliveira et al., 2008; Powell et al., 2009).

The last important factor that should be considered when evaluating the effects of

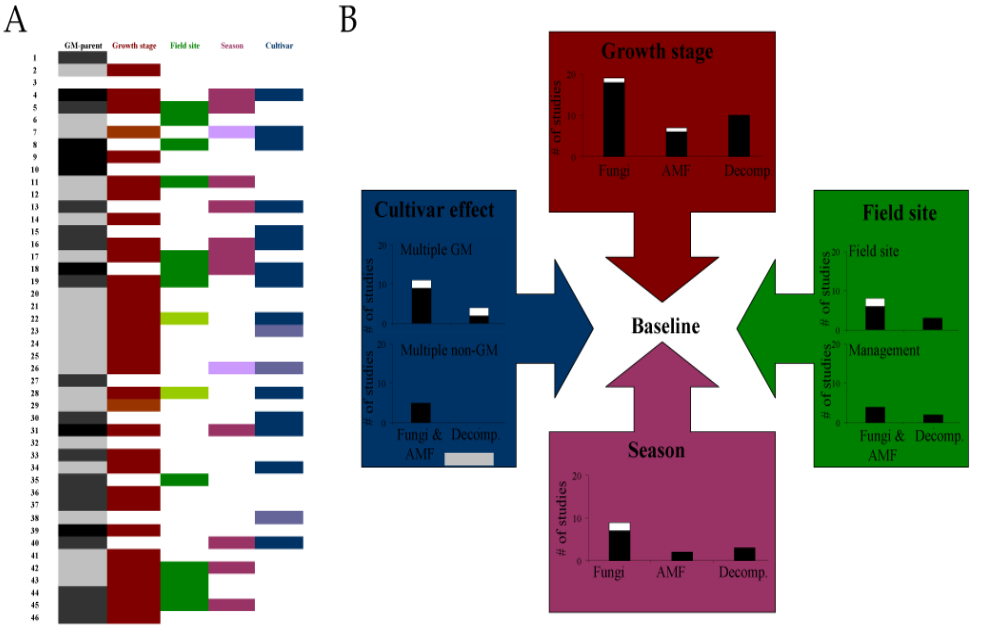

Figure 2.2. Published studies on GM that have included the effect of other parameters than GM-trait on fungal community composition. (A) List of studies which investigated each parameter and either found an effect (darker color) or did not detect an effect (lighter color). In the first row the studies detecting a significant effect of GM are marked with black, no effect with light gray and 'transient' effect with dark gray. (B) 'Transient' effects divided between the different 'baseline' factors. An observed effect of these parameters on soil fungi is marked as the black part of the bars; no effects are marked as the white part of the bars.

The studies referred in the column (A) are: 1.Donegan et al., 1996; 2.Donegan et al., 1999; 3.Saxena and Stotzky, 2001a; 4.Cowgill et al., 2002; 5.Dunfield & Germida, 2003; 6.Blackwood & Buyer, 2004; 7.Milling et al., 2004; 8.Xue et al., 2005; 9.Götz et al., 2006; 10.Wei et al., 2006; 11.Weaver et al., 2007; 12.Girlanda et al., 2008; 13.Icoz et al., 2008; 14.Liu et al., 2008; 15.O'Callaghan et al., 2008; 16.Oliveira et al., 2008; 17.Hart et al., 2009; 18.Kremer and Means, 2009; 19.Weinert et al., 2009; 20.Wu et al., 2009; 21.Gschwendtner et al., 2010; 22.chapter 3 (this thesis); 23.Tan et al., 2010; 24.Gschwendtner et al., 2011; 25.Lee et al., 2011; 26.Li et al., 2011; 27.chapter 5 (this thesis); 28.chapter 4 (this thesis); 29.Kaldorf et al., 2002; 30.Turrini et al., 2004; 31.Castaldini et al., 2005; 32.de Vaufleury et al., 2007; 33.Powell et al., 2007; 34.Knox et al., 2008; 35.Cheeke et al., 2011; 36.Donegan et al., 1995; 37.Wu et al., 2004; 38.Flores et al., 2005; 39.Henault et al., 2006; 40.Naef and Defago, 2006; 41.Lawhorn et al., 2009; 42.Powell et al., 2009; 43.Lu et al., 2010b; 44.Lu et al., 2010a; 45.Xue et al.2011; 46.chapter 6 (this thesis)

GM–traits on soil fungi is the normal variation among cultivars that exists due to their long history of breeding. This may explain some of the transient effects observed when studies have compared multiple GM-varieties (Cowgill et al., 2002; Turrini et al., 2004; Castaldini et al., 2005; Xue et al., 2005; Powell et al., 2007; Icoz et al., 2008; Knox et al., 2008; Oliveira et al., 2008; Kremer and Means, 2009) or multiple 'normal' varieties against the GM (Milling et al., 2004; Weinert et al., 2009). In most cases it was found that the normal variation among cultivars was larger than the difference between GM-variety and its parental cultivar (Fig.2.2). Icoz et al. (2008) compared 4 Bt-varieties and their corresponding parental isolines and observed that the Bt-modification did not have an effect on numbers of fungi while crop variety had a significant albeit transient effect on the soil microbial community.

In conclusion a relevant effect of the GM-trait should exceed normal operating variables and the commonly accepted changes due to environmental factors presented above. Thus, a proper assessment of the effects of GM-crops should include, all factors mentioned in Fig. 2.2.

2.4. New methods and new possibilities in GM-research

Traditionally most of the studies dealing with effects of GM-crops on fungi have used cultivation based methods and colonization counts for AMF (Fig. 2.1) while only few used DNA based fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) (Milling et al., 2004; Götz et al., 2006) or terminal restriction length polymorphism (T-RFLP) (Hart et al., 2009). However, in order to answer fundamental questions still open in this field such as the effects of GM-crops on soil fungal diversity as well as the impact of changes in root exudation patterns, new methodology is ready to be adopted. In this chapter we will discuss methodologies that could help answering these key questions.

2.4.1. Monitoring differences in root exudation patterns with stable isotope probing (SIP)

Many studies have reported on the differences in the community composition of rhizosphere fungi between cultivars and have hypothesized this would have been the result of changes in rhizodepositions. However, only few studies have actually measured root exudates or monitored carbon flow from the plant to the rhizosphere for instance using stable isotope probing (SIP) where the whole plant is (pulse) labeled with $^{13}CO_2$ and the incorporation of ^{13}C in microbes is followed into the endosphere and rhizosphere. SIP has been used to study for example effects of soil management (Rangel-Castro et al., 2005; Lu et al., 2007) and climate change (Drigo et al., 2010), and is proven to be a reliable technique which can provide a quantitative insight into the fungi that metabolize root-derived materials. In combination with phospholipid fatty acid (PLFA-SIP) analyses it has been used to evaluate the effects of GM-plants on carbon partitioning to different groups of soil organism (Wu et al., 2009; Gschwendtner et al., 2011). Both of these studies have shown how important

both fungi and especially AMF are in the rhizosphere. Neither of these studies did find significant differences between the GM-and its parental cultivar, although Wu et al. (2009) found significant differences between the Bt and parental rice in the amount of ^{13}C distribution at the seedling, booting and heading stages. A study done with DNA-SIP revealed cultivar dependent distinctions in ^{13}C-label flow to endophytic bacteria of potato (Rasche et al., 2009). However, in these studies the baseline of environmental variation was not investigated and thus it is not clear whether these differences are ecologically relevant. SIP methodology will, nevertheless, offer a great opportunity to study the effects of GM-varieties on active members of rhizosphere communities.

2.4.2. Possibilities of high-throughput sequencing to reveal fine scale differences in diversity between GM- and parental variety

It has been recognized that in addition to using broad scale keystone indicators such as fungal biomass and community composition, there is a need to improve sensitivity of detection methods for detailed analyses of the impacts of GM-crops on soil microbial communities (Lilley et al., 2006). This should further target relevant species and functions for each combination of modification and species. Earlier, microbial biodiversity has been thought to be a very sensitive parameter to perturbation and thus it can be considered as a good indicator for soil functioning (van der Heijden et al., 1998; Kennedy, 1999; Garbeva et al., 2004). Thus, modern molecular methods might be useful in evaluating the effects of GM-crops on soil diversity and functioning. A study on bacteria (and fungi) in the GM-rice rhizosphere using pyrosequencing as a fingerprinting method for bacteria (Lee et al., 2011) showed that deeper sequencing revealed higher diversity but the overall trend was the same as assessed with T-RFLP and no significant differences were found between the GM crop and its parental variety. One problem in the deep sequencing approach is that the increased sensitivity can produce significant differences between GM- and its parental line without having any significance to ecosystem functioning. Therefore, function based sequencing or meta-transcriptomics of soil fungi could be a better approach when evaluating the effects of the GM-crops.

2.4.3. Future prospects of GM-research

The results available on the impact of GM plants on natural and agricultural ecosystems show that specific effects of single transformation events should be tested on a case-by-case basis in a natural setting where the baseline factors are all taken into the consideration, including biochemical, physiological, and molecular parameters. Further, there is a need for statistical methods which can evaluate the effects of GM-trait in order to properly assess the baseline noise in the system. The new techniques such as SIP-experiments and high throughput sequencing and metatranscriptomics should be used in parallel with well designed field experiments considering all the 'baseline' factors.

3

In situ dynamics of soil fungal communities under different genotypes of potato, including a GM- cultivar

Emilia Hannula, Wietse de Boer & Hans van Veen

Soil Biology and Biochemistry (2010)

Chapter 3

Fungi are key to the functioning of soil ecosystems, and exhibit a range of interactions with plants. Given their close associations with plants, and importance in ecosystem functioning, soil-borne fungi have been proposed as potential biological indicators of disturbance and useful agents in monitoring strategies, including those following the introduction of genetically modified (GM) crops. Here we report on the impact of potato crop varieties, including a cultivar that was genetically modified for its starch quality, on the community composition of the main phyla of fungi in soils, i.e. Ascomycota, Basidiomycota and Glomeromycota in rhizosphere and bulk soil. Samples were collected at two field sites before sowing, at three growth stages during crop development and after the harvest of the plants, and the effects of field site, plant growth stage and plant cultivar (genotype) on fungal community composition were assessed using three phylum-specific T-RFLP profiling strategies and multivariate statistical analysis (NMDS ordinations with ANOSIM test). In addition, fungal biomass, arbuscular mycorrhizal colonization of roots and activities of extracellular fungal enzymes (laccases, Mn-peroxidases and cellulases) involved in degradation of lignocelluloses-rich organic matter were determined. Fungal community compositions, densities and activities were observed to differ significantly between the rhizosphere and bulk soil. The most important factors determining fungal community composition and functioning were plant growth stage for the rhizosphere communities and location and soil properties for the bulk soil communities. The basidiomycetes were the most numerous fungal group in the bulk soils and in the rhizosphere of young plants, with a shift toward greater ascomycete numbers in the rhizosphere at later growth stages. There were no detectable differences between the GM cultivar and its parental cultivar in terms of influence on fungal community structure of function. Fungal community structure and functioning of both GM- and parental cultivars fell within the range of other cultivars at most sampling moments.

3.1. Introduction

One of the concerns surrounding the cultivation of GM crops is the possible impact on plant-soil ecosystems, including the soil-borne biota. Numerous studies have investigated the effects of GM-crops on soil bacterial community structure and functioning (Savka and Farrand, 1997; Dunfield and Germida, 2001; Rasche et al., 2006; LeBlanc et al., 2007; Weinert et al., 2009). While some studies reported effects of modified crops on soil bacterial numbers (Siciliano and Germida, 1999; Dunfield and Germida, 2001), others have documented only minor or transient effects (reviewed by Kowalchuk et al. (2003)). So far, the effect of the GM crops on soil fungi has received much less attention, despite the importance of fungi in terrestrial ecosystems (Carlile et al., 2001). A few studies have addressed the effects of GM-crops on general fungal community structures (Milling et al., 2004; Turrini et al., 2004; Götz et al., 2006; Hart et al., 2009; Wang et al., 2009). However, detailed studies on the effects of GM crops on the abundance, composition and functioning of fungi have not yet been reported. Moreover, most studies to date have focused on one time point and one field situation. Yet, effects of factors such as plant growth stage (Sessitsch et al., 2004; Hart et al., 2009), the plant community (Berg et al., 2002; Viebahn et al., 2005; Berg and Smalla, 2009) and tillage (Griffiths et al., 2007) are known to affect the microbial community considerably.

The 'true' fungi are ubiquitous in the environment and fulfil a range of important terrestrial ecological functions e.g. mineralization of soil organic matter and facilitation of plant nutrient acquisition (Christensen, 1989). Yet, the interactions between plants, plant residues and the soil fungal community and activities are not fully understood (Carlile et al., 2001). The most important fungal groups in most soils are the *Ascomycota, Basidiomycota* (Carlile et al., 2001) and *Glomeromycota*, comprising the arbuscular mycorrhizal fungi (AMF). Like all organotrophic soil microbes, fungi are influenced by plants. AMF as well as pathogens are in direct contact with plants, and saprotrophs are also influenced by the plant either directly via root exudates or indirectly via decomposition of litter and crop residues (Christensen, 1989; Buée et al., 2009). Members of the phylum *Basidiomycota* are perhaps the most important fungal decomposers, at least in forest soils, due to their ability to produce enzymes such as lignin peroxidases, manganese peroxidases and laccases that break down lignin-rich recalcitrant components of the litter (Lynch and Thorn, 2006). The relative importance of basidiomycetes in decomposition processes in agricultural soils is, however, not clear (Lauber et al., 2009), especially given the saprotrophic capabilities of many members of the phylum *Ascomycota*.

There are several mechanisms by which plants can influence soil-borne fungi. The chemical composition of root exudates, litter and other plant debris can vary strongly between plant species and even between cultivars of the same species (Lynch and Whipps, 1990; Kabouw et al., 2010). Effects of plant species composition on bacterial community composition and functioning are well known, and some similar evidence has been reported for fungi (Lynch and Whipps, 1990; Kowalchuk

et al., 2002; Bais et al., 2006; Broeckling et al., 2008; Badri and Vivanco, 2009).
In addition to the direct effects of plant residues on soil communities, there are many other factors that may affect soil-borne fungal communities, including soil type, past and present land use, management practices and crop species and -cultivars (Milling et al., 2004). Knowledge of these sources of natural variation in fungal communities is critical for the assessment of the relative effects of specific potential perturbations, such as transgenic crop cultivation. In this paper, we describe the development of fungal abundance, fungal community composition and fungal-related ligno-cellulolytic enzyme activities in two agricultural field sites planted with six different varieties of potato, including a GM-variety with modified starch quality. This approach facilitated an evaluation of the normal variation in fungal communities over time, between soils and under different cultivars, thereby providing the necessary baseline for assessing the potential impact of the GM variety. In order to provide a high resolution of site- and crop-related effects on fungal community composition, we adopted a terminal restriction fragment length (T-RFLP)-based approach to examine the composition of ascomycete, basidiomycete and glomeromycete communities separately. Resulting community profiles were related to environmental factors via multivariate statistics to determine their relative importance in driving fungal community composition and function.

3.2. Materials and methods

3.2.1. Experimental design.

The experiments were carried out during the 2008 growing season at two field sites in the north-eastern part of Netherlands, which is the main starch potato-producing region of the country. The sites VMD and BUI were located 10 km from each other and differed considerably in their soil characteristics: site VMD is characterized by a high organic matter content (average of 19 %) and is categorized as sandy peat (silt fraction 2.8 %, sand fraction 94.3 %) whereas site BUI is a loamy sand (silt 5.7 %, sand 90.5 %) soil with an organic matter content of around 5 %. pH of both soils was similar, around 5. Both fields had been under crop rotation and conventional agricultural practices for many decades. Six cultivars of potato (Solanum tuberosum) were grown in a randomized plot design consisting of four replicate plots per cultivar, each containing 28 plants. These cultivars comprised one modified potato line ('Modena') with altered starch quality used for industrial purposes, its parental cultivar ('Karnico') and four additional non-modified cultivars ('Aveka', 'Aventra', 'Désirée' and 'Premiere'). The altered starch composition was created by complete inhibition of the production of amylose via introduction of a RNAi construct of the granule-bound starch synthase gene inhibiting GBSS and amylose formation, which yields pure amylopectin. The modification was made without a marker gene as described by de Vetten et al. (2003). Cultivars 'Aventra', 'Aveka', 'Karnico' and 'Modena" produced tubers with relatively high starch content and had a low to medium growth rate, whereas cultivars 'Désirée' and 'Premiere' had lower starch content in the tubers and higher growth rates.

Soil samples were collected at five time points namely one day before planting, at three crop growth stages and after harvest. The growth stages sampled were: young plants (EC30), flowering plants (EC60) and senescent plants (EC90) (Hack et al., 2001). The bulk soil samples were collected from 0-15 cm depth, and 5 cores per plot were used to form a composite sample. Four plants per plot were used for a composite sample of the rhizosphere soil. In order to collect rhizosphere soil, the plants were shaken to remove the excess soil and the soil tightly adhering to the roots was collected by brushing. Bulk soils were homogenized and sieved (4 mm mesh) to remove possible root fragments and stones. Soil water content was determined from fresh material as weight loss after overnight drying at 105 °C.

3.2.2. Fungal biomass and enzyme activities

Quantification of ergosterol, via the alkaline extraction method, was used as an estimate of fungal biomass (de Ridder-Duine et al., 2006). Analyses of activities of enzymes involved in decomposition of lignocellulose-rich organic matter, i.e. laccase, cellulase and Mn-peroxidase were performed according to van der Wal et al. (2006).

3.2.3. Assessment of root colonization by AM fungi

Levels of mycorrhizal colonization and arbuscule and vesicle abundances were determined microscopically according to McGonigle et al. (1990). Briefly, randomly chosen 2 cm fine root pieces were cut, washed with water, cleared for 30 min at 90°C in 10% KOH, incubated overnight in 1% HCl, subsequently stained with 0.05% tryptan and methyl blue in lactic acid: glycerol: water (1:1:1) and mounted onto slides. One hundred intersections per slide were counted.

3.2.4. Extraction of DNA from soil

DNA extractions were carried out using fresh soil material and the remaining soil was stored at -20 °C for enzymatic analyses. DNA was extracted from soil (0.5 g wet weight) with a Power Soil DNA isolation kit (MOBIO Laboratories, Inc.) using a bead beating system. Yields of genomic DNA were checked on 1 % agarose gel and visualized under UV after ethidium bromide staining.

3.2.5. T-RFLP analyses

Terminal restriction fragment length polymorphisms (T-RFLP) combined with the construction of a small library of the most dominant operational taxonomical units (OTUs) was used to determine the fungal community compositions. Previously, T-RFLP has been used successfully to study total fungal communities or separate phyla like basidiomycetes or AMF in a variety of environments (Brodie et al., 2003; Edwards et al., 2004; Koide et al., 2005; Anderson et al., 2007; Hart et al., 2009). The structures of the three fungal phyla studied, ascomycetes, basidiomycetes and glom-

eromycetes, were assessed separately. For the analysis of ascomycete and basidiomycete communities, internal transcribed spacer (ITS) regions were used as target regions. For both groups, the ITS1 and ITS2 regions and 5.8S rRNA gene were amplified using the fungus-specific forward primer ITS1F in combination with the reverse primer ITS4B (Gardes and Bruns, 1993) for basidiomycetes and ITS4A (Larena et al., 1999) for ascomycetes. FAM was used as a label for ITS1F primer and NED for the reverse primers (Applied Biosystems). The reaction mixtures of 25 µl were composed of 1 µl of template DNA (3-5 ng), 2.5 µl 10x PCR buffer with MgCl2, 0.2 mM dNTPs, 25 µM MgCl2, 1 % BLOTTO and 1.25 U Fast Start DNA polymerase (Roche). PCR conditions for ascomycetes and basidiomycetes were as follows: denaturation at 95 °C for 5 min, followed by 32 cycles of 95 °C for 30 s, 62 °C / 55 °C (for *Ascomycota* / *Basidiomycota*, respectively) for 30 s and 72 °C for 1 min 30 s. The final extension step was 72 °C for 10 min. Glomeromycetes were analyzed using a nested PCR approach targeting the nuclear large ribosomal sub-unit (LSU) using primers LR1- FLR2 for the first PCR and FAM-labeled FLR3 and NED-labeled FLR4 for the second PCR round (Gollotte et al., 2004). The PCR mixture for both reactions was the same as described earlier, except for the second PCR when no BLOTTO was added and 1:100 diluted PCR product from the first reaction served as a template. The conditions for the first PCR were: initial denaturation in 95 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 40 s and 72 °C for 1 min 10 s. The final extension step at 72 °C was 7 min. The second PCR included 27 cycles of the same conditions except for the annealing temperature, which was 56 °C. After verifying the presence of amplicon of expected sizes (approximately 670, 650 and 400 bp for *Basidiomycota*, *Ascomycota* and *Glomeromycota*, respectively) via agarose gel electrophoresis, PCR products were subjected to restriction digestion. The restriction enzymes were selected on the basis of their ability to produce on average one fragment per OTU both in silico and with pure cultures of fungi. Virtual restriction digests suggested that HaeIII and HinfI would be suitable for both basidiomycetes and ascomycetes and MboI and AluI for glomeromycetes. PCR products were digested with restriction enzymes (New England BioLabs) at 37 °C for 3 hours in an appropriate buffer with bovine serum albumin (BSA). After restriction, the products were purified using ethanol precipitation in a microtitre-plate format. Appropriate dilutions based on test runs of TRFs were analyzed with an ABI 3130 sequencer using GeneScan™ -500 LIZ (Applied Biosystems) as a size standard. The dilution factors of runs were standardized with the highest peaks and by comparing different dilutions thus making the number of peaks (TRFs) comparable between samples. All steps of the T-RFLP were performed with negative and positive controls (fungal pure cultures).

Identification of the TRFs

In order to identify TRFs, PCR was performed with non-labeled primers (ITS1F-4A, ITS1F-4B and FLR3-FLR4) for all the fungal groups. PCR products of 8 samples per time and type of soil showing the peaks of interest in earlier analyses were purified with Qiaqen PCR purification kit and pooled. The pooled fragments were cloned into Escherichia coli JM109 using the pGem-T Easy System II cloning kit (Promega, UK) with a vector : insert ratio of 3:1. Approximately 40 successful trans-

formants per time and soil compartment i.e. bulk and rhizosphere were selected for amplification, restriction digest and identification with labeled primers as described above. The clones producing unique fragments with both restriction enzymes were amplified using vector-based M13 primers and sequenced. Out of the 96 clones from the *Ascomycota*-specific libraries, 38 unique restriction patterns were identified and sequenced. The primer pair ITS1F-4A yielded only ascomycete sequences: 11 sequences could not be assigned to any order or class (with >97 % similarity to a class), but did show highest similarity to ascomycete sequences. Out of 96 clones from the *Basidiomycota*-specific libraries, clones representing the 30 detected restriction classes were sequenced. In order to evaluate the method, some sequences showing a similar restriction pattern were sequenced as duplicates. The ITS1F-4b primer pair yielded 15 unique basidiomycete sequences and one ascomycete sequence (>97% similarity). Due to the lower diversity estimates, only 48 *Glomeromycota* clones were tested for their restriction patterns and only 12 were sequenced as most of the clones seemed to belong to only a few dominating types. Out of these, all but one were assigned to be members of phylum *Glomeromycota*.

3.2.6. Data analyses and statistics.

The enzymatic data were analyzed with SPSS for windows (Release 17.0.) using the univariate regression within the general linear mode (GLM) procedure. The assumption of normality was tested with Shapiro-Wilk statistics and homogeneity of variances was assessed with Levene's test. Differences between fields, time points, type of the soil and cultivar were tested with Tukey's HSD test, or, when variances were unequal, with Tamhane's T2 test. All values were initially expressed per dry weight of soil. The number of TRFs was used as a rough estimate of the number of OTUs within a phylum. Total numbers of TRFs in a treatment was not significantly affected by the combination of restriction enzymes used (HinfI / HaeIII and MboI / AluI) and the number of TRFs produced with the pair of restriction enzymes significantly correlated with each other (spearman 2-tailed <0.01). Therefore, the average of both enzyme combinations was used for further analyses. The differences in the quantity of TRFs were analyzed in a similar way as for the enzyme activities.

The quality of T-RFLP data was first visually inspected in GeneMapper Software v4.1 (Applied Biosystems) and then transferred to T-Rex (Culman et al., 2008a). True peaks were identified for both labels as those of which the height exceeded the standard deviation (assuming zero mean) computed over all peaks and multiplied by two (Abdo et al., 2006). Both Additive Main Effects and Multiplicative Interaction Model (AMMI) and Non-Metric Multidimensional Scaling (NMDS) with Jaccard as distance measure were used to assess the similarity of the fungal communities in different fields, growth stages, soil compartments and between potato cultivars. Although clustering was very similar with both methods, we present the NMDS analysis as this method is thought to be better for datasets with relatively high beta diversity in the matrix (i.e. greater than 2) as is the case here (Table 3.2) (Culman et al., 2008b). Goodness of fit between similarity rankings and ordination distances

was analyzed using Kruskal's stress value (<0.2) (Bennett et al., 2008). The effect of the treatments was tested using one- or two-way ANOSIM in PAST (Hammer et al., 2001) with Jaccard as a distance measure. Only presence-absence data were used. For NMDS plots, a simplification was made by combining peak data from replicates, and the full dataset was used for all other analyses. The assignment of the peaks to OTUs was performed in the statistical computing environment R using the T-RFLP Analyses Matching Program (TRAMP-R) (Fitzjohn and Dickie, 2007). Three out of four of the enzyme / primer combinations within 1.5 bp margin had to be met in a sample for it to be assigned to an OTU.

The sequences obtained were compared with GenBank nucleotide databases using BLAST and considered to belong to a genus or species with similarities of 95 % for an order and 97% for a species. The sequences from this study were deposited to GenBank under accession numbers shown in Table 3.3.

3.3. RESULTS

3.3.1. Fungal biomass

The ergosterol content of the two soils differed significantly ($p<0.001$) before planting (mean 1.41 mg kg-1 dw soil in field BUI and 2.98 mg kg-1 dw soil in field VMD). Ergosterol levels in bulk soils did not change much throughout the growing season (dashed line in Figure 3.1) but did increase in the potato rhizosphere. At the first

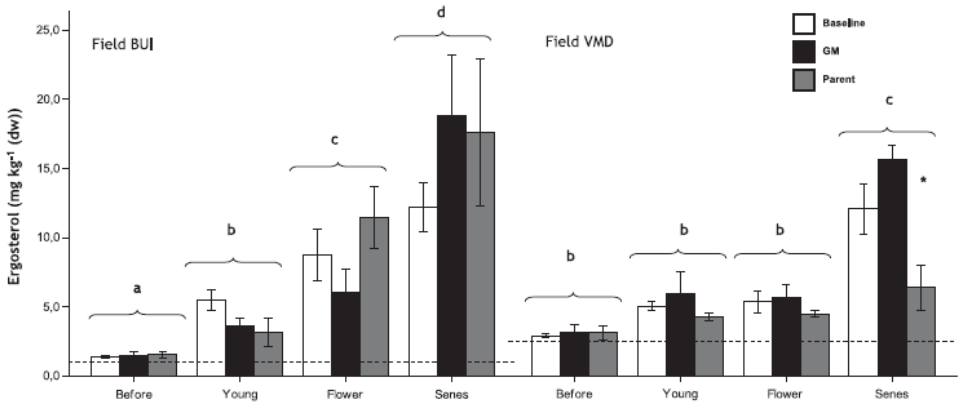

Figure 3.1. Ergosterol content in the soils of both fields at four time points. The dashed line represents the level in the bulk soil throughout the growing season and bars represent the averages of rhizosphere samples (± standard error). The first bar (white) represents the average of four normal cultivars (n=16), the second one (black) represents the GM-variety 'Modena' (n=4) and the third one (grey) represents the parental isoline 'Karnico' (n=4). The letters above growth stages indicate significant differences to other growth stages ($P<0.05$) and * indicate differences between cultivars at any growth stage.

growth stage, the rhizosphere samples of VMD field had a significantly higher ergosterol content ($p=0.002$) than that of BUI field. Thereafter, the amounts of ergosterol in the rhizosphere increased further, and this increase was higher at the BUI site. The highest concentrations of ergosterol were measured at the senescence stage (20 mg kg-1 in field BUI and 15 mg kg-1 in field VMD).
The amount of ergosterol in rhizosphere soil did not differ between GM-variety ('Modena'), its parental variety ('Karnico') and all other cultivars across all growth stages and both field sites. Only at the senescent stage, a significant difference was observed between the parental and GM-cultivars ($p<0.001$), but the ergosterol content of both the GM- and parental cultivar fell within the range of other cultivars.

AMF colonization was significantly different between the two sites at the stage of the young plants, but this may have been affected by the later sampling date of the VMD field. At subsequent later sampling dates, no effect of site on AM-colonization was apparent. Moreover, growth stage did not seem to affect the colonization after EC60 (flowering plants), and no differences between cultivars were found.

3.3.3. Decomposer community function

All ligno-cellulolytic enzyme activities were positively correlated with soil ergosterol content (for all enzymes $n=252$, $R=0.55 – 0.80$, $p< 0.001$), as well as with each other (all correlations significant at the level of $p<0.01$). The bulk soil samples of the VMD site had higher activities of laccases and manganese peroxidases than those of the BUI site (Tukey HSD, $p<0.001$) throughout the growing season (Fig. 3.2). This was not the case for cellulase activities. For rhizosphere samples, significant differences between fields were observed for Mn-peroxidase activity under young and senescent plants, for laccase activity under flowering and senescent plants and for cellulase activity in all growth stages. In general, soil compartment (rhizosphere vs. bulk) and plant growth stage had the most pronounced effects on measured fungal enzyme activities. The enzyme activities generally increased over the course of the growing season in the rhizosphere, especially in the cases of laccase (Fig. 3.2A) and manganese peroxidase (Fig. 3.2B) activities. For cellulases, the plant growth stage had less impact (Fig. 3.2C); with the growth stage effect only being significant in the BUI field. The GM-variety 'Modena' and its parental variety showed the most pronounced trend of increase in fungal enzyme activities in the rhizosphere as compared to the other cultivars. In most cases, they also had the highest activities among the cultivars.

3.3.4. Fungal community structure

The ANOSIM data analysis of T-RFLP profiles indicated that the composition of all fungal communities was significantly different between the two fields (Table 3.1; Fig. 3.3). The most pronounced differences in ascomycete and basidiomycete communities were observed for bulk soils prior to planting (for ascomycetes, HaeIII: $R=0.3509$ [$p< 0.001$] and for basidiomycetes HaeIII: $R= 0.7123$ [$p< 0.001$]) and in the

rhizosphere of young plants. The fungal community composition in the rhizosphere of flowering plants was not different between field sites, and, at the stage of senescence, only small differences between the fields could be detected. AMF community composition differed between fields at senescence (MboI: R=0.2040 and AluI: R=0.1897 for MboI, $p<0.001$) and at flowering (MboI: R=0.2748, $p<0.05$).

The growth stage of the plant had a strong impact on fungal community composition (Table 3.1). The clearest separation was seen between flowering and other stages. Differences between bulk soils and rhizosphere soils were observed for both ascomycetes and basidiomycetes, but not for the AMF. With a single exception, the community structure of all fungal phyla associated with the GM-variety did not differ significantly from any other cultivars (Table 3.1).

All the identified sequences that had a >95 % similarity to a known sequence in the database were grouped into orders. At the level of order, the two fields did not differ from each other (Table 3.2). Moreover, the total numbers of identified OTUs in the rhizosphere or bulk soil did not differ between fields. Only two ascomycetal orders (*Pleurosporales* and *Saccharomycetes*) and two basidiomycetal orders (*Boletales* and *Russulales*) were significantly affected by the field site ($p<0.05$). The growth stage of the plant significantly ($p<0.001$) affected the composition of the basidiomycete community, and this could also be seen at the order level (Table 3.2). In field BUI, the number of OTUs in the rhizosphere at the flowering stage was lower than for the young (Tamhane, $p=0.003$) and senescence ($p=0.012$) stages, whereas in field VMD flowering stage was significantly different from the stage of senescence ($p=0.004$). The number of identified ascomycetal OTUs was not different between growth stages, although differences in the orders *Pleurosporales* and Insertae Sedis were found in the rhizosphere samples. Of the basidiomycete orders, Boletales and *Cantharellales* were most affected by growth stage, being most abundant in the bulk soils in the beginning and in the rhizosphere of young plants. The *Glomeromycota* did not differ in total number of identified OTUs although the BUI field had more ($p=0.035$) *Glomus* species. No cultivar effects could be detected for the distribution of ascomycetal and glomeromycetal OTUs. Only one order of the basidiomycetes, *Agaricales*, was significantly different ($P=0.023$) between cultivars in the BUI field. This was due to one cultivar, 'Karnico', having no *Agaricales* in its rhizosphere at later growth stages.

When looking at individual OTUs, few differences between fields and growth stages could be observed (Table 3.3) and these differences were generally reflected in analyses at the order level. Out of the 63 OTU types observed, seven were present in all the samples (in both fields, rhizosphere and bulk soil and all time points): five ascomycetes (A1-A5), one basidiomycete (B1) and one belonging to the glomerales (G1). When comparing fields with each other, some differences in abundances of the OTU-types were found: four types were present in field VMD, but were not detected in field BUI (A31, A34, G7 and G11), whereas one OTU-type present in field BUI was not detected in field VMD (A36). There was no cultivar specificity in any of the OTUs, meaning that no OTU was consistently present under only one genotype, although at some growth stages unique OTUs were detected for

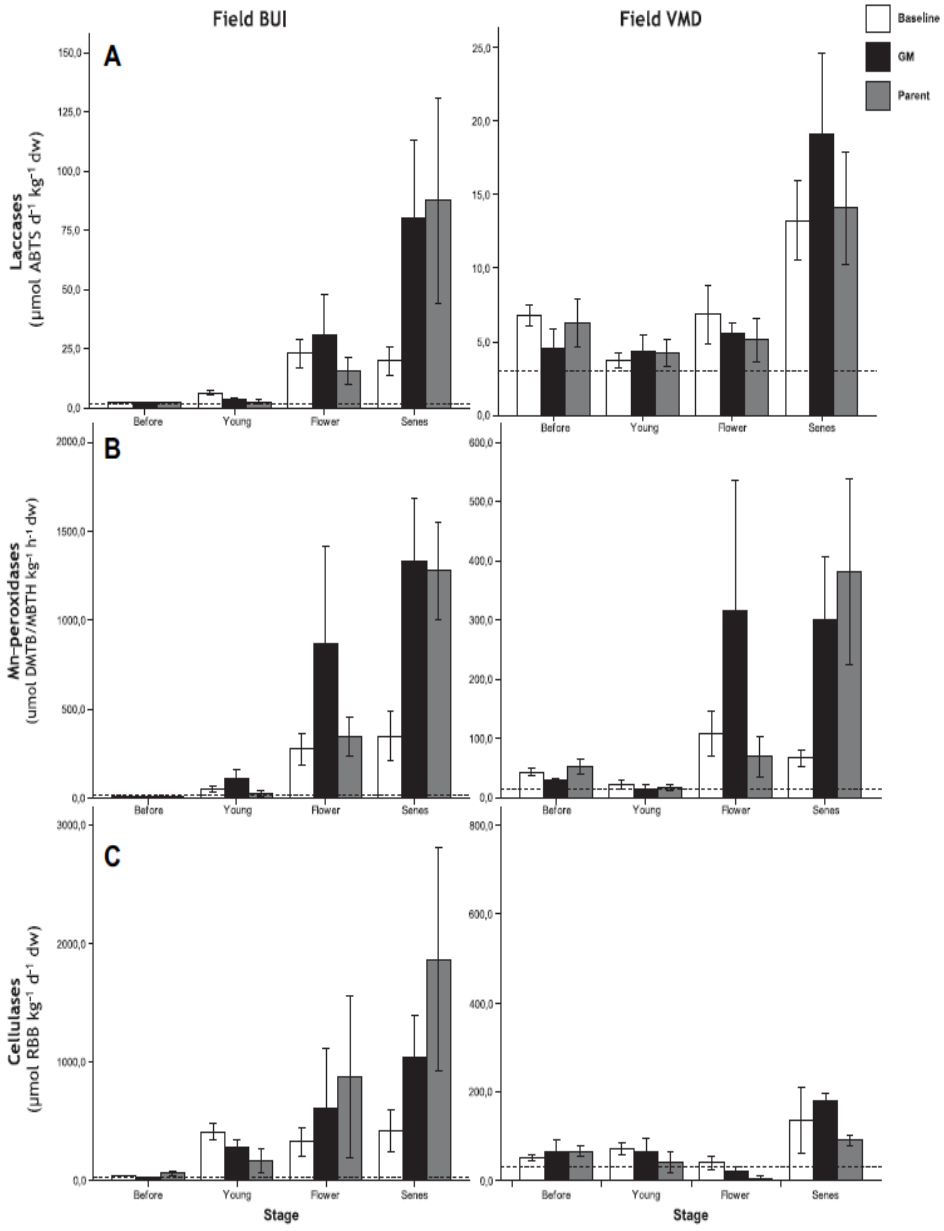

Figure 3.2. Lignolytic extracellular enzymes measured from the soil in both fields at four time points and under different cultivars. Laccases (A), Mn-peroxidases (B) and cellulases (C) were measured from the same samples. Bars (± standard error) present the baseline (four normal cultivars), GM-variety ('Modena') and parental isoline ('Karnico') and the dashed lines present the level in the bulk soil. Note that the axes of different scales are used for different fields.

Table 3.1. Analysis of similarities (ANOSIM) of apparent presence and absence of soil fungal TRFs in different field sites, plant growth stages, soil compartments, cultivars and blocks for each restriction enzyme. Beta diversity of the samples and average TRF richness are presented. Values are the test statistic R for one-way ANOSIM counted in PAST and beta diversity in T-Rex. R equals 1.00 when all replicates within groups are more similar than any replicates from different groups and decreases, as differences between groups weaken. All ANOSIM comparisons were performed using Bray-Curtis index and 10 000 permutations.
Significance of R statistics: ** p<0.005 and * p<0.05. P-values presented are Bonferroni-corrected p-values

	Ascomycetes (HaeIII)	Ascomycetes (HinfI)	Basidiomycetes (HaeIII)	Basidiomycetes (HinfI)	Glomeromycetes (AluI)	Glomeromycetes (MboI)
Site (BUI vs. VMD)	0.17**	0.05**	0.09**	0.10**	0.06**	0.11**
Growth stage[a]						
Field BUI (overall)	0.21**	0.13*	0.39**	0.27**	0.42**	0.30**
Before vs. EC30	0.44**	0.61**	0.37*	0.73**	0.10	0.29*
EC30 vs. EC60	0.22**	0.22	0.45**	0.27*	0.57**	0.37**
EC60 vs. EC90	0.23**	0.31**	0.99**	0.95**	0.31**	0.23*
Field VMD (overall)	0.14*	0.20**	0.21**	0.31**	0.49**	0.38**
Before vs. EC30	0.78**	0.98**	0.26	0.09	0.04	-0.02
EC30 vs. EC60	0.12	0.24**	0.48**	0.38**	0.31**	0.60**
EC60 vs. EC90	0.15	0.13	0.08	0.52**	0.08	0.52**
Soil compartment[b]						
Field BUI	0.63**	0.55**	0.35**	0.77**	-0.05	0.11
Field VMD	0.39**	0.74**	0.05	0.11	0.00	-0.03
Cultivar[c]	0.00	0.00	0.01	-0.02	0.00	-0.02
Field BUI (overall)	0.01	-0.01	0.01	0.01	-0.03	-0.02
EC30	-0.12	0.01	-0.03	-0.04	0.02	-0.10
EC60	0.00	-0.10	-0.11	0.02	0.02	0.16
EC90	0.24*	0.07	0.16	0.11	0.06	0.06
Field VMD (overall)	0.01	0.00	-0.08	-0.06	-0.02	
EC30	-0.08	0.06	-0.11	-0.10	0.00	0.08
EC60	-0.08	-0.12	0.03	-0.25	-0.06	0.17
EC90	0.03	0.08	-0.29	0.06	0.05	0.01
Block[d]						
Field BUI	0.00	-0.03	-0.02	-0.04	0.02	0.02
Field VMD	0.04	0.05	0.12	0.13	-0.04	0.02
Beta diversity	2.52	2.02	2.97	3.13	3.44	2.27

a In the comparison between growth stages, both bulk soil in the beginning and rhizospheric soils during the plant growth were included. Individual differences between growth stages are included and calculated similarly.

b Soil compartment marks comparison between bulk soils and rhizosphere soils during plant growth (excluding before and after crop situations)

c Four replicates of each cultivar were used to compare all the cultivars with each other

d Field blocks (each with all the cultivars present) were compared in order to check the influence of location in the field

single cultivars (Table 3.3).

3.3.5. Fungal richness

The total number of TRFs (sum of the TRFs of the three groups) was positively correlated with the amount of ergosterol in the samples (n=252, R= 0.272, P<0.05). This was also observed for *Ascomycota* TRFs alone (n= 252, R=0.462, P<0.001). Ascomycete and basidiomycete TRF numbers were affected by field site for bulk soils were before planting, and in the rhizosphere of the flowering plants (p<0.05). Glomeromycete TRF richness was not significantly affected by field site (Fig. 3.4). The richness of ascomycete TRFs in the rhizosphere was also significantly different (p=0.002) between the sites at the youngest growth stage. The total number of TRFs was affected by the growth stage for the VMD field (p<0.001), where TRF richness dropped significantly at the stage of flowering. This was not observed for the BUI field. In both fields, ascomycetal and basidiomycetal TRFs were affected by plant growth stage (Fig. 3.4, Table 3.2). At the start of the growing season, the number of basidiomycetal TRFs outnumbered ascomycetes TRFs, with the reverse being the case at the stage of flowering. At the stage of senescence, the numbers of ascomycetal and basidiomycetal TRFs were similar in both fields (36 and 39 in field BUI and 36 and 33 in field VMD for ascomycetes and basidiomycetes, respectively). Both ascomycetes and basidiomycetes generally had lower numbers of TRFs in the bulk soils than in the rhizosphere during the growing season, whereas numbers of glomeromycetal TRFs did not differ between bulk soil and rhizosphere compartments (Table 3.2). The richness of basidiomycetal, ascomycetal and glomeromycetal TRFs was not significantly affected by the cultivar or the GM-variety at any growth stage for either field (TAMHANE, p>0.05).

3.4. DISCUSSION

3.4.1. Soil fungal biomass, functioning and community structure are affected by potato plants

Agricultural management and soil properties like organic matter content, pH, nutrients and water holding capacity have been identified as major abiotic factors affecting soil fungal communities. Similarly, several biotic factors, including plant species identity and plant community diversity have also been shown to influence soilborne fungal communities (Garbeva et al., 2004; Kasel et al., 2008; Berg and Smalla, 2009; Buée et al., 2009). In previous studies, the effect of the soil type was identified as a key factor influencing the bacterial communities in the rhizosphere (Sessitsch et al., 2001). Our data confirm the influence of field site on fungal communities for bulk soil communities. Yet, our data for rhizosphere samples also indicate that the effect of plant growth stage can be larger than the effect of the field site and thus soil type. The role of fungi in organic matter decomposition and nutrient dynamics in intensively managed agricultural soils is often assumed to be less than the impact of

Table 3.2. Effect of soil type, growth stage and cultivar on number of TRFs and identified OTUs at the order level for both of the fields. A total of 63 OTUs was identified and placed into phyla and orders (with >92 % similarity) as shown in table 3. The number of TRFs is counted as an average of the two restriction enzymes.

				All		Ascomycota													
			N	Number of TRFs (stdev)	Total OTUs (66)	Number of TRFs (stdev)	Total asco OTUs (36[a])	Capno-diales (2)	Chaeto-thyriales (1)	Deutero-mycota (3)b	Heloti-ales (2)	Hypo-creales (9a)	Inser-tae sedis (4)	Micro-ascales (1)	Phylla-corales (1)	Pleu-rospo-rales (3)	Not as-signed (1)	Sac-charo (1)	Un-known (10)
Field BUI	Before	Bulk	24	100.35 (33.73)	39	17.66 (6.27)	18	0	1	2	1	5	2	1	1	0	1	0	4
	EC30	Bulk	6	67.01 (5.98)	27	14.81 (4.34)	11	0	0	1	1	2	2	0	0	0	1	0	4
		AV43	4	72.77 (8.96)	48	34.48 (10.95)	27	2	1	3	1	6	3	1	1	2	1	0	6
		Aveka	4	85.33 (31.91)	41	34.64 (8.09)	24	1	1	3	2	5	3	1	1	2	1	0	4
		Aventra	4	76.70 (14.63)	38	32.70 (11.96)	23	0	0	2	2	6	3	1	1	2	1	0	5
		Desiree	4	82.96 (9.39)	44	31.71 (15.54)	26	1	1	2	2	7	3	1	1	2	1	0	5
		Karnico	4	72.14 (2.75)	44	29.41 (9.77)	27	0	0	3	2	5	3	1	1	2	1	1	8
		Premiere	4	76.98 (31.56)	48	34.35 (19.98)	30	2	1	3	2	5	3	1	0	2	1	1	9
		Rhizo	24	77.81 (5.34)	43.8	32.88	26.2	1.0	0.7	2.7	1.8	5.7	3.0	1.0	0.8	2.0	1.0	0.3	6.2
		(stdev)*			(3.9)	(2.06)	(2.5)	(0.9)	(0.5)	(0.5)	(0.4)	(0.8)	(0)	(0)	(0.4)	(0)	(0)	(0.5)	(1.9)
	EC60	Bulk	6	37.89 (7.26)	20	20.05 (3.50)	10	0	0	1	1	2	2	0	0	1	1	0	2
		AV43	4	98.25 (4.60)	37	49.94 (27.30)	26	2	1	3	2	5	3	1	1	2	1	0	5
		Aveka	4	66.75 (9.75)	34	41.56 (10.24)	25	1	1	2	2	6	3	1	1	2	1	0	5
		Aventra	4	85.50 (63.60)	26	59.88 (37.73)	19	0	1	2	1	4	2	1	1	1	1	0	5
		Desiree	4	86.17 (33.70)	29	54.50 (38.63)	19	1	0	2	0	5	2	1	1	2	1	0	4
		Karnico	4	61.38 (15.24)	30	40.75 (19.79)	21	2	1	1	2	6	1	0	1	2	1	0	4
		Premiere	4	95.56 (47.64)	38	49.72 (36.34)	24	2	1	3	1	5	3	1	0	1	1	0	6
		Rhizo	24	82.27 (15.07)	32.3	49.39 (7.38)	22.3	1.3	0.8	2.2	1.3	5.2	2.3	0.8	0.8	1.7	1.0	0	4.8
		(stdev)*			(4.8)		(3.1)	(0.8)	(0.4)	(0.8)	(0.8)	(0.8)	(0.8)	(0.4)	(0.4)	(0.5)	(0)	(0)	(0.8)
	EC90	Bulk	6	33.45 (14.12)	17	11.5 (7.06)	7	0	0	0	0	2	2	0	0	0	1	0	2
		AV43	4	98.19 (17.77)	44	33.74 (20.07)	28	2	1	3	2	7	4	1	1	1	1	0	5
		Aveka	4	99.69 (16.02)	42	41.25 (12.40)	27	1	1	3	2	6	3	1	1	2	1	0	6
		Aventra	4	90.50 (22.63)	44	39.00 (10.76)	26	1	1	3	2	7	3	1	1	1	1	0	5
		Desiree	4	82.75 (16.21)	41	39.38 (8.69)	22	0	1	1	2	7	2	1	1	1	1	0	5
		Karnico	4	74.92 (12.71)	40	33.25 (7.50)	26	1	1	3	2	6	4	1	1	1	1	0	5
		Premiere	4	73.50 (25.46)	37	28.75 (10.80)	23	1	1	3	2	6	2	1	1	1	1	0	4
		Rhizo	24	86.59 (11.35)	41.3	35.90 (4.76)	25.3	1.0	1.0	2.7	2.0	6.5	3.0	1.0	1.0	1.2	1.0	0	5.0
		(stdev)*			(2.7)		(2.3)	(0.6)	(0)	(0.8)	(0)	(0.6)	(0.9)	(0)	(0)	(0.4)	(0)	(0)	(0.6)

Table 3.2. continues

Basidiomycota								Glomeromycota			
Number of TRFs (stdev)	Total basidio OTUs (15c)	Agaricales (1)	Boletales (4)	Cantharellales (3)	Russulales (2)	Tremellomycetes (2c)	Unknown (3)	Number of TRFs (stdev)	Total AMF OTUs (10)	Glomus (6)	Paraglomus (4)
52.92 (16.45)	13	1	4	3	2	1	2	29.77 (11.01)	8	5	3
38.75 (6.54)	8	1	3	0	2	1	1	13.45 (5.21)	8	5	3
26.85 (17.13)	13	1	4	3	2	1	2	11.44 (5.49)	8	5	3
46.19 (24.82)	13	1	4	3	2	1	2	4.50 (2.75)	4	4	0
39.19 (7.37)	12	1	4	2	2	1	2	10.92 (10.25)	3	3	0
44.44 (13.13)	12	1	4	2	2	1	2	6.81 (2.44)	6	5	1
28.17 (4.37)	13	1	4	3	2	1	2	10.33 (4.35)	4	4	0
39.28 (12.30)	12	1	4	3	2	0	2	6.67 (5.78)	6	4	2
37.35 (8.12)	12.5 (0.5)	1.0 (0)	4.0 (0)	2.7 (0.5)	2.0 (0)	0.8 (0.4)	2.0 (0)	8.45 (2.83)	5.2 (1.8)	4.2 (0.8)	1.0 (1.3)
11.98 (2.38)	7	1	3	0	2	0	1	6.79 (5.46)	3	2	1
17.25 (14.40)	8	1	3	0	2	0	2	7.25 (4.61)	3	3	0
14.25 (6.01)	6	1	2	0	1	1	1	4.75 (2.83)	3	3	0
14.17 (11.93)	5	1	2	0	1	0	1	7.81 (5.85)	2	2	0
19.33 (16.43)	8	1	3	0	2	1	1	9.00 (1.67)	2	2	0
13.75 (5.95)	5	0	2	0	1	1	1	6.88 (3.44)	4	3	1
15.75 (9.55)	9	1	4	0	2	1	1	8.08 (3.99)	5	4	1
15.75 (2.18)	6.8 (1.7)	0.8 (0.4)	2.7 (0.8)	0 (0)	1.5 (0.5)	0.7 (0.5)	1.2 (0.4)	7.30 (1.44)	3.2 (1.2)	2.8 (0.8)	0.3 (0.5)
13.97 (9.87)	7	1	3	0	2	0	1	7.92 (4.73)	3	2	1
32.75 (22.27)	13	1	4	3	2	1	2	16.44 (5.70)	3	3	0
48.25 (5.86)	12	1	4	2	2	1	2	10.19 (4.36)	3	3	0
48.50 (9.37)	12	1	4	2	2	1	2	4.50 (3.54)	6	4	2
38.25 (10.73)	13	1	4	3	2	1	2	9.44 (2.48)	6	4	2
31.75 (12.33)	7	0	2	1	2	2	0	6.75 (6.04)	7	5	2
35.88 (7.95)	11	1	4	1	2	1	2	8.38 (4.50)	3	3	0
39.23 (7.45)	11.3 (2.3)	0.8 (0.4)	3.7 (0.8)	2.0 (0.9)	2.0 (0)	1.2 (0.4)	1.7 (0.8)	9.28 (4.05)	4.7 (1.9)	3.7 (0.8)	1.0 (1.1)

Table 3.2. continues

						Ascomycota												
			N	Number of TRFs (stdev)	Total OTUs (66)	Number of TRFs (stdev)	otal asco OTUs (36[a])	Capno-diales (2)	Chaeto-thyriales (1)	Deutero-mycota (3)b	Heloti-ales (2)	Hypo-creales (9a)	Inser-tae sedis (4)	Micro-ascales (1)	Phylla-corales (1)	Pleu-rospo-rales (3)	Not as-signed (1)	Sac-charo (1)	Un-known (10)
Field	Before	Bulk	24	65.50 (12.89)	40	13.61 (4.86)	23	0	0	3	1	6	2	1	1	0	1	1	7
VMD	EC30	Bulk	6	36.94 (17.52)	28	4.25 (1.13)	19	0	0	3	1	5	2	1	1	0	1	1	4
		AV43	4	91.04 (34.80)	50	22.99 (7.29)	33	2	1	3	2	8	4	1	1	2	1	1	7
		Aveka	4	68.70 (24.59)	29	16.65 (7.74)	12	0	0	1	0	5	2	0	0	0	1	1	2
		Aventra	4	79.27 (39.24)	35	37.73 (18.47)	19	0	0	2	2	5	2	0	1	0	1	1	5
		Desiree	4	65.03 (17.91)	38	26.71 (5.98)	23	0	1	3	1	5	4	0	1	1	1	1	5
		Karnico	4	58.05 (25.79)	26	24.68 (9.17)	14	0	0	1	1	3	2	0	1	0	1	1	4
		Premiere	4	60.53 (20.17)	32	20.90 (3.19)	16	0	0	2	1	5	2	0	1	0	1	1	4
		Rhizo	24	70.44 (12.54)	35.0	24.94 (7.15)	19.5	0.3	0.3	2.0	1.2	5.2	2.7	0.2	0.8	0.5	1.0	1.0	4.5
		(stdev)*			(8.5)		(7.7)	(0.8)	(0.5)	(0.9)	(0.8)	(1.6)	(1.0)	(0.4)	(0.4)	(0.8)	(0)	(0)	(1.5)
	EC60	Bulk	6	30.63 (8.47)	16	17.5 (6.7)	10	0	0	1	1	3	2	0	1	0	1	0	1
		AV43	4	43.25 (16.24)	24	35.71 (9.35)	15	1	0	1	2	5	2	0	1	0	1	0	2
		Aveka	4	33.87 (20.66)	33	26.46 (17.30)	23	2	1	2	2	4	3	1	1	1	1	1	4
		Aventra	4	41.27 (8.24)	24	27.45 (4.47)	18	0	0	3	2	4	2	1	1	1	1	0	2
		Desiree	4	33.85 (2.75)	28	20.55 (9.12)	25	2	1	3	2	4	3	1	1	1	1	1	5
		Karnico	4	42.47 (9.41)	31	36.75 (7.33)	27	2	1	2	1	6	3	1	1	1	1	1	7
		Premiere	4	20.10 (5.78)	29	23.37 (9.25)	26	2	1	2	1	6	4	0	1	1	1	1	6
		Rhizo	24	35.80 (8.76)	28.2	28.38 (6.55)	22.3	1.5	0.7	2.2	1.6	4.8	2.8	0.7	1.0	0.8	1.0	0.7	4.3
		(stdev)*			(3.7)		(4.8)	(0.8)	(0.5)	(0.8)	(0.5)	(0.9)	(0.7)	(0.5)	(0)	(0.4)	(0)	(0.5)	(2.1)
	EC90	Bulk	6	25.05 (7.25)	21	5.75 (3.12)	9	0	0	1	1	3	2	0	1	0	1	0	0
		AV43	4	75.63 (11.49)	46	29.58 (5.14)	31	2	1	3	2	7	3	1	1	2	1	1	7
		Aveka	4	86.08 (13.49)	37	37.06 (5.56)	22	1	1	2	1	4	3	1	1	0	1	1	6
		Aventra	4	91.13 (51.98)	42	47.94 (26.68)	28	2	1	3	2	6	3	1	1	1	1	1	6
		Desiree	4	69.58 (28.08)	42	32.81 (24.79)	29	0	1	3	2	7	3	1	1	1	1	1	7
		Karnico	4	93.75 (22.57)	42	33.94 (20.79)	29	0	1	3	2	6	3	1	1	2	1	1	7
		Premiere	4	77.75 (8.84)	31	37.38 (10.64)	18	2	1	0	2	4	2	1	1	0	1	0	4
		Rhizo	24	82.32 (9.49)	40.0	36.45 (6.32)	26.2	1.2	1.0	2.3	1.8	5.7	2.8	1.0	1.0	1.0	1.0	0.8	6.2
		(stdev)*			(5.3)		(5.0)	(1.0)	(0)	(1.2)	(0.4)	(1.4)	(0.4)	(0)	(0)	(0.9)	(0)		(1.2)

Basidiomycota								**Glomeromycota**			
Number of TRFs (stdev)	Total basidio OTUs (15c)	Agari-cales (1)	Bole-tales (4)	Cantharel-lales (3)	Russu-lales (2)	Tremel-lomycetes (2')	Un-known (3)	Number of TRFs (stdev)	Total AMF OTUs (10)	Glomus (6)	Paraglo-mus (4)
33.58 (14.50)	8	1	3	0	2	0	2	18.32 (9.34)	9	6	3
14.29 (3.19)	5	1	2	0	1	0	1	18.40 (6.20)	4	4	0
43.81 (23.84)	12	1	3	3	2	1	2	14.67 (11.04)	5	4	1
44.56 (19.27)	12	1	4	2	2	1	2	7.33 (6.51)	5	4	1
39.75 (31.55)	13	1	4	3	2	1	2	8.25 (5.73)	3	3	0
24.56 (14.71)	10	1	4	0	2	1	2	13.75 (8.77)	5	4	1
27.69 (22.38)	9	1	3	0	2	1	2	5.69 (3.17)	3	3	0
26.94 (20.76)	11	1	4	0	2	2	2	12.69 (3.53)	5	4	1
34.55 (9.14)	11.2	1.0	3.7	1.3	2.0	1.2	2.0	10.40 (3.77)	4.3	3.7	0.7
	(1.5)	(0)	(0.5)	(1.5)	(0)	(0.4)	(0)		(1.0)	(0.5)	(0.5)
7.65 (4.27)	4	1	1	0	1	0	1	7.47 (3.24)	2	2	0
17.00 (5.66)	4	0	2	0	1	0	1	7.25 (1.06)	5	3	2
5.00 (2.00)	4	0	2	0	1	0	1	5.67 (1.61)	6	4	2
8.50 (3.72)	2	1	0	0	1	0	0	4.00 (1.29)	4	4	0
7.00 (3.25)	2	1	0	0	1	0	0	6.75 (5.24)	1	1	0
3.75 (3.18)	2	1	0	0	1	0	0	4.33 (2.08)	2	2	0
4.25 (1.06)	2	1	0	0	1	0	0	7.33 (6.66)	1	1	0
7.58 (4.95)	2.7	0.7	0.7	0	1.0	0	0.3	5.89 (1.46)	3.2	2.5	0.7
	(1.0)	(0.5)	(1.0)	(0)	(0)	(0)	(0.5)		(2.1)	(1.4)	(1.0)
8.58 (4.88)	4	1	1	0	1	0	1	8.55 (1.49)	8	6	2
27.50 (0.71)	8	1	3	0	2	1	1	11.56 (7.42)	7	6	1
38.17 (12.97)	8	1	3	0	2	1	1	11.81 (5.07)	7	5	2
29.81 (20.82)	11	1	4	1	2	1	2	13.38 (9.26)	3	3	0
28.75 (1.89)	10	1	4	0	2	1	2	5.81 (6.79)	3	3	0
34.00 (0.71)	6	1	2	0	1	1	1	8.50 (5.30)	7	4	3
41.17 (15.55)	11	1	4	1	2	1	2	6.50 (5.83)	2	2	0
33.23 (5.53)	9.0	1.0	3.3	0.3	1.8	1.0	1.5	9.59 (3.10)	4.8	3.8	1.0
	(2.0)	(0)	(0.8)	(0.5)	(0.4)	(0)	(0.5)		(2.4)	(1.5)	(1.3)

Table 3.3. The distribution of identified OTUs in the fields during different growth stages. + marks presence in the bulk soil sample and – absence. For rhizosphere samples, the number of cultivars (n = 6) that were positive for an OTU-type is given. The similarity of nearest matches obtained with BLAST is shown for matches with at least 95 % similarity and an overlap of >70 %. Assignment to orders was done if similarity >90 % to known members of an order. Sequences were submitted to GenBank under corresponding accession numbers.

						Field BUI							Field VMD						
Primers	OTU-type	Order	Genbank Acc No.	Closest species match (Accession number)	% identity	Beginning (bulk)	Young (Bulk)	Young (rhizo)	Flower (bulk)	Flower (rhizo)	Senes (bulk)	Senes (rhizo)	Beginning (bulk)	Young (Bulk)	Young (rhizo)	Flower (bulk)	Flower (rhizo)	Senes (bulk)	Senes (rhizo)
ITS1F-4A	A1	Incertae sedis	HM037637	Pseudeurotium bakeri (DQ068995)	100	+	+	6 (6)	+	6 (6)	+	6 (6)	+	+	6 (6)	+	6 (6)	+	6 (6)
	A2	Incertae sedis	HM037638	Pseudeurotium bakeri (DQ529304)	99	+	+	6 (6)	+	6 (6)	+	6 (6)	+	+	6 (6)	+	6 (6)	+	6 (6)
	A3	Not assigned	HM037639	Trichocladium asperum (AM292050)	99	+	+	6 (6)	+	6 (6)	+	6 (6)	+	+	6 (6)	+	6 (6)	+	6 (6)
	A4	Hypocreales	HM037640	Fusarium sp.	96	+	+	6 (6)	+	6 (6)	+	6 (6)	+	+	6 (6)	+	6 (6)	+	6 (6)
	A5	Hypocreales	HM037641	Fusarium sp. 14018 (EU750682)	99	+	+	6 (6)	+	6 (6)	+	6 (6)	+	+	6 (6)	+	6 (6)	+	6 (6)
	A6	Hypocreales	HM037642	Uncultured Verticillium sp. (EU754976)	98	-	-	6 (6)	-	6 (6)	-	6 (6)	+	+	6 (6)	+	6 (6)	+	6 (6)
	A7	Phyllacorales	HM037643	Plectosphaerella sp.	96	+	-	5 (6)	-	5 (6)	-	6 (6)	+	+	5 (6)	+	6 (6)	+	6 (6)
	A8	Deuteromycota*	HM037644	Microsphaeropsis sp. MTFD09 (DQ132840)	99	+	-	6 (6)	+	4 (6)	-	6 (6)	+	+	5 (6)	-	5 (6)	-	5 (6)
	A9	Unknown	HM037645	Unknown1	<90	+	+	6 (6)	-	5 (6)	-	6 (6)	-	+	3 (6)	+	6 (6)	-	5 (6)
	A10	Unknown	HM037646	Unknown5	<90	+	+	6 (6)	-	5 (6)	-	6 (6)	+	-	3 (6)	-	5 (6)	-	6 (6)
	A11	Helotiales	HM037647	Helotiales sp.	95	+	+	6 (6)	+	4 (6)	+	6 (6)	+	+	3 (6)	+	4 (6)	+	5 (6)
	A12	Helotiales	HM037648	Botryotinia fuckeliana isolate (EF207415)	100	-	-	5 (6)	-	4(6)	-	6 (6)	-	-	5 (6)	+	6 (6)	+	6 (6)
	A13	Deuteromycota*	HM037649	Tetracladium sp.	95	+	-	4 (6)	-	5 (6)	-	5 (6)	+	+	4 (6)	+	5 (6)	+	4 (6)
	A14	Hypocreales	HM037650	Clonostachys miodochialis (AF210674)	99	+	-	6 (6)	-	5 (6)	-	6 (6)	+	+	2 (6)	-	4 (6)	-	6 (6)
	A15	Microascales	HM037651	Microascaceae sp. LM278 (EF060607)	98	+	-	6 (6)	-	5 (6)	-	6 (6)	+	+	1 (6)	-	4 (6)	-	6 (6)
	A16	Pleosporales	HM037652	Uncultured Ampelomyces clone (EU516670)	98	-	-	6 (6)	+	6 (6)	-	5 (6)	-	-	2 (6)	-	5 (6)	-	4 (6)
	A17	Unknown	HM037653	Unknown6	<90	-	-	6 (6)	-	3 (6)	-	6 (6)	+	-	3 (6)	-	4 (6)	-	4 (6)
	A18	Deuteromycota*	HM037654	Tetracladium furcatum strain F−11883 (EU883432)	98	-	+	6 (6)	-	4 (6)	-	5 (6)	+	+	3 (6)	-	3 (6)	-	6 (6)
	A19	Chaetothyriales	HM037655	Uncultured Herpotrichiellaceae (FJ554453)	98	+	-	4 (6)	-	5 (6)	-	6 (6)	-	-	2 (6)	-	4 (6)	-	6 (6)
	A20	Hypocreales	HM037656	Nectria sp. ASIN2 (DQ779785)	100	-	-	6 (6)	-	5 (6)	-	6 (6)	+	+	4 (6)	-	1 (6)	-	4 (6)
	A21	Capnodiales	HM037657	Davidiella macrospora (EU167591)	99	-	-	4 (6)	-	5 (6)	-	4 (6)	-	-	1 (6)	-	6 (6)	-	6 (6)
	A22	Unknown	HM037658	Uncultured ascomycota2	98	+	+	5 (6)	+	6 (6)	+	4 (6)	+	+	0 (6)	+	2 (6)	+	2 (6)
	A23	Unknown	HM037659	Unknown3	<90	+	+	6 (6)	+	6 (6)	+	6 (6)	-	-	1 (6)	-	1 (6)	-	2 (6)
	A24	Hypocreales	HM037672	Bionectria cf. ochroleuca (EU552110)	98	+	-	3 (6)	-	3 (6)	-	6 (6)	-	-	1 (6)	-	2 (6)	-	4 (6)
	A25	Unknown	HM037660	Unknown4	<90	-	-	2 (6)	-	0 (6)	-	0 (6)	+	+	6 (6)	-	3 (6)	-	6 (6)
	A26	Unknown	HM037661	Unknown9	<90	-	-	2 (6)	-	0 (6)	-	0 (6)	+	+	6 (6)	-	4 (6)	-	5 (6)
	A27	Saccharomycetales	HM037662	Galactomyces sp. BPY−54 (DQ286062)	99	-	-	2 (6)	-	0 (6)	-	0 (6)	+	+	6 (6)	-	4 (6)	-	5 (6)
	A28	Insertae sedis	HM037663	Leptodontidium sp.	95	-	-	6 (6)	-	1 (6)	-	4 (6)	-	-	2 (6)	-	1 (6)	-	5 (6)

	A29	Capnodiales	HM037664	Cladosporium cladosporioides (AY251074)	99	-	-	2 (6)	-	3 (6)	-	2 (6)	-	-	1 (6)	-	4 (6)	-	3 (6)
	A30	Pleurosporales	HM037665	Drechslera sp. B401 (AY336133)	98	-	-	5 (6)	-	4 (6)	-	1 (6)	-	-	1 (6)	-	0 (6)	-	2 (6)
	A31	Insertae sedis	HM037666	Ascochyta pisi var. pisi (EU167557)	98	-	-	0 (6)	-	2 (6)	-	2 (6)	-	-	2 (6)	-	4 (6)	-	0 (6)
	A32	Unknown	HM037667	Unknown7	<90	-	-	0 (6)	-	0 (6)	-	0 (6)	+	-	4 (6)	-	0 (6)	-	4 (6)
	A33	Hypocreales	HM037668	Fusarium sp. 5/97~45 (AJ279478)	97	-	-	0 (6)	-	0 (6)	-	0 (6)	-	-	3 (6)	-	4 (6)	-	0 (6)
	A34	Pleurosporales	HM037669	Aff. Drechslera MT0008 (AB199583)	99	-	-	1 (6)	-	3 (6)	-	1 (6)	-	-	0 (6)	-	0 (6)	-	1 (6)
	A35	Unknown	HM037670	Unknown2	<90	-	-	1 (6)	-	1 (6)	-	1 (6)	+	-	1 (6)	-	0 (6)	-	0 (6)
	A36	Unknown	HM037671	Unknown8	<90	-	-	2 (6)	-	0 (6)	-	0 (6)	-	-	0 (6)	-	0 (6)	-	0 (6)
ITS1F-4B	B1	Russulales	HM037675	Uncultured fungi (EU645595)	97	+	+	6 (6)	+	6 (6)	+	5 (6)	+	+	6 (6)	+	6 (6)	+	6 (6)
	B2	Boletales	HM037673	Boletales sp.	92	+	+	6 (6)	+	6 (6)	+	5 (6)	+	+	6 (6)	+	2 (6)	+	6 (6)
	B3	Agaricales	HM037674	Agaricales sp.	95	+	+	6 (6)	+	5 (6)	+	5 (6)	+	+	6 (6)	+	5 (6)	+	6 (6)
	B4	Unknown	HM037676	Unknown11	<90	+	+	6 (6)	+	6 (6)	+	5 (6)	+	+	6 (6)	+	2 (6)	+	6 (6)
	B5	Boletales	HM037677	Uncultured Boletus isolate 51/08 (FJ816721)	98	+	+	6 (6)	+	6 (6)	+	5 (6)	+	+	6 (6)	+	2 (6)	-	6 (6)
	B6	Russulales	HM037678	Russulales sp.	96	+	+	6 (6)	+	3 (6)	+	6 (6)	+	-	6 (6)	-	0 (6)	-	5 (6)
	B7	Boletales	HM037679	Boletales sp.	95	+	+	6 (6)	+	3 (6)	+	6 (6)	+	-	6 (6)	-	0 (6)	-	5 (6)
	B8	Tremellales	HM037680	Uncultured Cryptococcus (EU516999)	99	+	+	5 (6)	-	4 (6)	-	6 (6)	-	-	6 (6)	-	0 (6)	-	6 (6)
	B9	Unknown	HM037681	Unknown10	<90	+	-	6 (6)	-	1 (6)	-	5 (6)	+	-	6 (6)	-	0 (6)	-	3 (6)
	B10	Boletales	HM037682	Boletales sp.	96	+	-	6 (6)	-	1 (6)	-	5 (6)	-	-	4 (6)	-	0 (6)	-	3 (6)
	B11	Cantharellales	HM037683	Cantharellales sp.2	96	+	-	6 (6)	-	0 (6)	-	4 (6)	-	-	3 (6)	-	0 (6)	-	2 (6)
	B12	Cantharellales	HM037684	Ceratobasidium sp. aurim1217 (DQ093646)	98	+	-	6 (6)	-	0 (6)	-	5 (6)	-	-	3 (6)	-	0 (6)	-	2 (6)
	B13	Hypocreales	HM037685	Volutella ciliata (AJ301966)	97	+	-	1 (6)	-	0 (6)	-	3 (6)	+	-	4 (6)	-	0 (6)	-	2 (6)
	B14	Cantharellales	HM037686	Canthrellales sp.	95	+	-	4 (6)	-	0 (6)	-	3 (6)	-	-	2 (6)	-	0 (6)	-	0 (6)
	B15	Unknown	HM037687	Unknown12	<90	-	-	0 (6)	-	0 (6)	-	0 (6)	-	-	0 (6)	-	0 (6)	-	0 (6)
FLR3-FLR4	G1	Glomerales	HM037688	Unknown Glomus	<95	+	+	6 (6)	+	6 (6)	+	6 (6)	+	+	6 (6)	+	6 (6)	+	6 (6)
	G2	Glomerales	HM037689	Uncultured glomeromycete (FJ820912)	98	+	+	6 (6)	+	6 (6)	+	6 (6)	+	+	6 (6)	-	1 (6)	+	6 (6)
	G3	Glomerales	HM037690	Unknown13	<95	+	+	6 (6)	-	4 (6)	-	6 (6)	+	+	6 (6)	-	0 (6)	+	3 (6)
	G4	Glomerales	HM037691	Glomus mosseae (AY639156)	97	+	+	4 (6)	-	0 (6)	-	1 (6)	+	-	4 (6)	-	2 (6)	+	1 (6)
	G5	Paraglomerales	HM037692	Uncultured paraglomus	99	+	+	3 (6)	-	1 (6)	-	3 (6)	+	-	0 (6)	-	2 (6)	+	3 (6)
	G6	Paraglomerales	HM037693	Uncultured paraglomus3	<95	+	+	2 (6)	+	1 (6)	+	3 (6)	+	-	0 (6)	-	2 (6)	-	2 (6)
	G7	Glomerales	HM037694	Glomus eburneum (AM713413)	97	-	-	0 (6)	-	0 (6)	-	0 (6)	+	+	0 (6)	+	4 (6)	+	4 (6)
	G8	Paraglomerales	HM037695	Uncultured paraglomus sp. 2	<95	+	+	1 (6)	-	0 (6)	-	0 (6)	+	-	4 (6)	-	0 (6)	+	1 (6)
	G9	Tremellales	HM037696	Trichosporon dulcitum strain HB940 (AJ507663)	98	-	-	0 (6)	-	0 (6)	-	1 (6)	-	-	1 (6)	-	0 (6)	-	1 (6)
	G10	Glomerales	HM037697	Uncultured Glomus (DQ677409)	97	+	+	3 (6)	-	1 (6)	-	3 (6)	+	-	0 (6)	-	0 (6)	+	3 (6)
	G11	Paraglomerales	HM037698	Unknown Paraglomus	<95	-	-	0 (6)	-	0 (6)	-	0 (6)	-	-	0 (6)	-	2 (6)	-	0 (6)
	G12	Glomerales	HM037699	Uncultured soil fungus (EU690958)	92	+	-	0 (6)	-	0 (6)	-	0 (6)	-	-	0 (6)	-	0 (6)	-	0 (6)

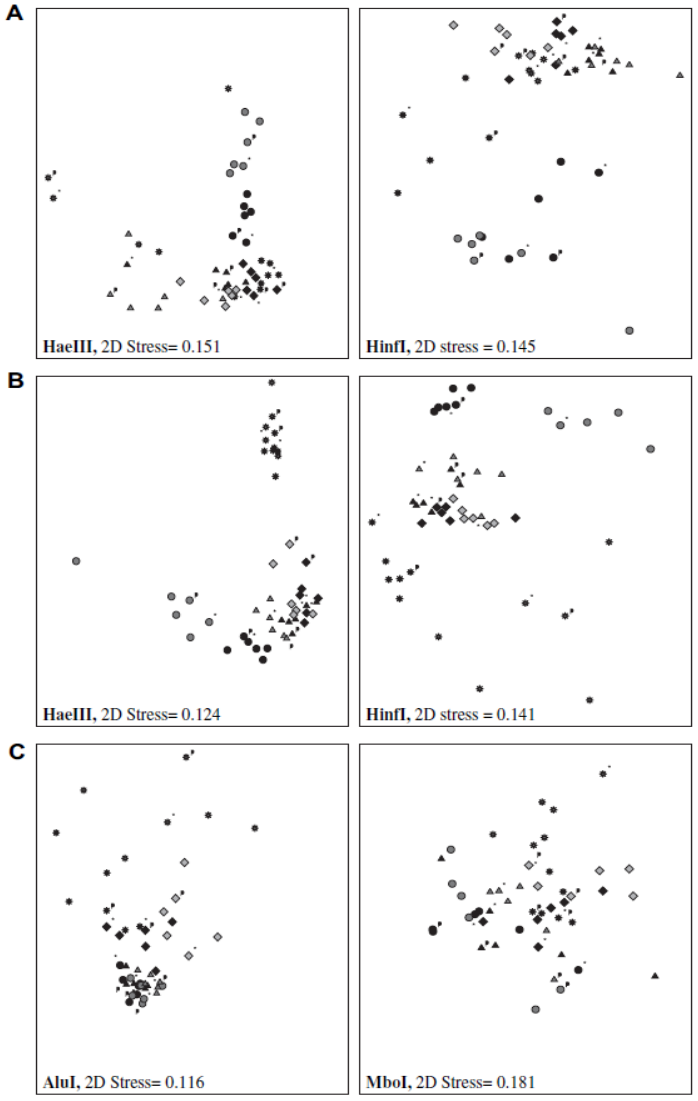

Figure 3.3. NMDS ordination plots of Ascomycota (A), Basidiomycota (B) and Glomeromycota (C) communities in the soil based on TRFs for each restriction enzyme and the 2D stress values of each ordination. The black symbols present samples from the field V and gray symbols samples from field B. Circles mark situation before planting (bulk soil), triangles rhizosphere of young plants, stars flowering plants and diamonds senescent plants. The GM-variety 'Modena' is indicated with an asterisk and parental isoline 'Karnico' with a small p. Symbols presented here are counted averages within treatments (n=4) for sake of simplicity. See Table 3.2 for significance of differences between the treatments

bacteria. This is because fungal biomass in such soils is usually low and appears to increase when agricultural activities are stopped (van der Wal et al., 2006). The ergosterol concentrations in the bulk soil of the two soils under study were in the range of those found for other intensively managed agricultural soils. The highest concentrations were found in the organic-rich soil, which is in line with the role of fungi in decomposition of recalcitrant organic matter (de Boer et al., 2006). However, the current study revealed a strong stimulating effect on fungal abundance and activity by potato roots, in particular in the flowering stage and senescent stage. Hence, fungi may be more important in the functioning of these intensively managed agricultural ecosystems than presumed beforehand. The strong increase of fungal biomass and ligno-cellulolytic enzyme activities during the later growth stages of potato suggest that fungi play a major role in the decomposition of rhizodeposits derived from older roots.

In a recent study on the fungal community composition under a GM-crop, no seasonal effects were found (Hart et al., 2009). We could not detect strong seasonal effects when looking at the level of the total fungal community, however phylum-specific community profiling showed that all communities responded separately to the growth stage of the plant and (Fig. 3.4). The separation of the communities between stages was clearest at the stage of flowering and was seen both in community function (Fig. 3.2) and structure (Fig. 3.3; table 3.2). Only the glomeromycetes were not affected by plant growth stage. Apparently, after establishing a certain level of colonization, they seem to remain relatively evenly distributed (Cesaro et al., 2008).

In the absence of plants, the phylum *Basidiomycota* showed greatest diversity in the number of TRFs (Fig. 3.4), probably due to the basidiomycete ability to degrade the more complex C derived from remainders of the previous crops. Members of the phylum *Ascomycota* became more diverse during flowering, and, at the stage of senescence, the *Basidiomycota* again showed greater diversity, suggesting that both phyla play important, yet temporally distinct roles in the rhizosphere. The dynamic change between these two phyla was seen clearly at the BUI field while the trend was less clear in the VMD field, where the high organic matter content may override plant growth stage effects (Fig. 3.2). Especially at the stage of flowering, a clear change in the community fingerprints of the different phyla was observed (Fig. 3.4).

Other studies have concluded that fungi could play an important role in the decomposition of more complex organic compounds derived from older root cells (Mougel et al., 2006; Broeckling et al., 2008). However, these conclusions have been based on observed increases in fungal diversity of fungi in the rhizosphere during reproductive growth stages and not on biomass and activity measurements like presented in this study. As the seasonal effect on the fungal biomass and enzyme activity may be due to quantitative changes in rhizodeposition, the observed changes in the community structure may also be due to changes in the proportion of phytosynthates released in the rhizosphere and their composition which is known to vary during plant's life cycle according to changes in plant health status and physiology (Sessitsch et al., 2004; Mougel et al., 2006; Artz et al., 2007; Singh et al., 2007; Hart et al., 2009). As we detected no differences in the moisture content of the soils during

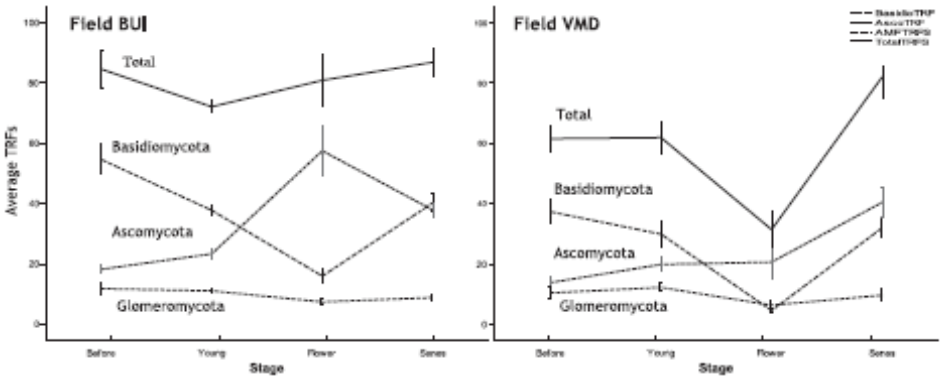

Figure 3.4. Dynamics of the fungal phyla in the rhizosphere of the plants during growth and in the bulk soil before planting. Changes in the TRF abundance in both fields during season. The error bars represent 1 times standard error.

the growing season, and the effect of growth stage was not extended from the rhizosphere to the bulk soil, we conclude that changes in root exudation (priming effect) and possibly alterations in root morphology are the most likely factors affecting the fungal community.

3.4.2. The effects of plant genotype are minor compared to other factors

We did not detect consistent differences between the different cultivars. Previous studies have reported distinct compositions of rhizosphere bacterial communities under different cultivars of various crops (Di Giovanni et al., 1999; Dunfield and Germida, 2001; Kowalchuk et al., 2002; LeBlanc et al., 2007). However, fungal communities have not been observed to be as responsive to different cultivars (Milling et al., 2004; Turrini et al., 2004; Götz et al., 2006; Hart et al., 2009; Wang et al., 2009), and only one study (Donegan et al., 1995) reported differences between genotypes using community fingerprinting methods. In our study, we observed differences between cultivars at certain growth stages, but these effects were transient and specific for only that particular growth stage. The approach of looking at many different cultivars and not just comparing the modified variety and its parental isoline, allowed us to examine the range of variation among cultivars. The fungal communities under the GM crop variety did not differ beyond this variation range. Similar observations of variation among cultivars extending that introduced by a GM-trait have been made earlier for bacterial communities (van Overbeek and van Elsas, 2008; Weinert et al., 2009). The only major difference observed was a difference in laccase and Mn-peroxidase activities during senescence between the rhizospheres of groups of three cultivars. 'Aventra', 'Premiere' and 'Désirée' seemed to have lower activities of these enzymes in their rhizosphere than the other three varieties. This might be due to differences in timing of the carbon allocation and root morphology, as at flowering stage these differences were not obvious.

None of the fungal groups examined showed significant differences between the plant genotypes. Moreover, the data on the lower levels of organization of the fungal communities confirmed the lack of cultivar specificity for any fungal OTUs or orders. T-RFLP as an analysis method will not show one or two OTUs differences in the samples (Edwards et al., 2004), especially when richness of the OTUs is as large as we see here. However, as we saw a clear difference between growth stages, soil types and compartments, we conclude that the potato cultivar genotype is not an important factor in shaping soil fungal communities, when compared to the effects of these other variables.

We acknowledge that potential effects of GM-crops on soil fungal communities may vary between types of modifications, and, although we found no effect of GM-trait on soil fungal communities, we cannot state for sure that this will hold for other modified traits, making a case-by–case evaluation strategy advisable. We did not expect amylose or amylopectin to leak out of the roots, and this research was designed to study undesirable side-effects of GM-crops on soil fungal communities. As mentioned, these side-effects can arise from unintended effects of the genetic modification on the plant physiology including production of different exudates. Importantly, here we offer valid tools and a baseline approach to study the potential risks of GM-crops on soil communities, which should be applicable to evaluating the effects of any crop.

3.4.3. Final remarks

From the results presented here, we conclude that the fungal communities in intensively managed agricultural soil strongly respond to the presence of plants and to changes in plant growth stages during the growing season, while the fungal community in the bulk soil is mainly driven by environmental factors and soil characteristics. However, plant genotype, including the GM trait under study, did not have a lasting effect on soil fungal communities and was the least explanatory factor driving the fungal communities in the soil, even though changes in root exudate composition due to this particular modification might have been expected. Measuring root exudation might explain much of the variation in soil fungal communities and should be an important part of the future studies evaluating possible effects of GM crops. Moreover, the combination of phylogenetic analyses with functional assays proved highly useful, providing a more complete picture of fungal community dynamics. However, also, we acknowledge that insight in the community structure of soil fungi is not always sufficient to determine the functionality of the fungal community (Hanson et al., 2008; Höppener-Ogawa et al., 2009) as functions are not conserved among the different phyla and orders of fungi (James et al., 2006). Thus, it is important to consider the results on community structure in the light of the functional data.

Chapter 3

4

A 3-year study reveals that **plant growth stage**, **season** and **fieldsite** affect soil fungal communities while cultivar and GM-trait have minor effects

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Chapter 4

In this three year field study the impact of different potato (Solanum tuberosum L.) cultivars including a genetically modified (GM) amylopectin-accumulating potato line on rhizosphere fungal communities is investigated using molecular microbiological methods. The effects of growth stage of a plant, soil type and year on the rhizosphere fungi were included in this study. The GM cvariety, its parental isoline and four non-related cultivars were planted in the fields and analyzed using T-RFLP on the basis of fungal phylum specific primers combined with multivariate statistical methods. Additionally, fungal biomass and some extracellular fungal enzymes (laccases, Mn-peroxidases and cellulases) were quantified in order to gain insight into the function of the fungal communities. Plant growth stage and year (and agricultural management) had the strongest effect on both diversity and function of the fungal communities while the GM-trait was the least explanatory factor. The impact of cultivar and soil type was intermediate. Occasional differences between cultivars, the amylopectin-accumulating potato line, and its parental variety were detected, but these differences were mostly transient in nature and detected either only in one soil, one growth stage or one year.

4.1. Introduction

Genetic engineering of plants has been used to improve the quality and quantity of crop production in a cost-effective way (e.g. by enhancing resistance to pests and diseases or introducing tolerance to herbicides) (Wolfenbarger and Phifer, 2000). Despite the great potential of this technology to advance agricultural yields, there are major concerns about the ecological impacts of genetically modified (GM) crops on soil ecosystem functioning. The impacts can be (1) direct (e.g. toxicity of an expressed introduced gene on key non-target species of important functional groups), (2) indirect (e.g. effects via unintended changes in the metabolism of the plant thereby affecting root exudates composition and fluxes) or (3) caused by changes in management regime used with GM crops (Birch et al., 2007).

The rhizosphere, is a hot-spot of microbial abundance and metabolic activity due to the resources released by plants (Lynch and Whipps, 1990; Raaijmakers et al., 2009). Hence, possible side-effects of GM plants on functioning of soil microbes should be first considered for the rhizosphere. Together with bacteria, fungi in the rhizosphere are very important to functioning of the soil-plant system and their functions range from symbiotic arbuscular mycorrhizal fungi (AMF) and plant pathogens to decomposers (Carlile et al., 2001; Buée et al., 2009).

The structure and functioning of soil microbial communities is affected by soil type (Garbeva et al., 2004; Berg and Smalla, 2009; Wang et al., 2009), plant growth stage (Gomes et al., 2001; Buyer et al., 2002; Gomes et al., 2003; Wang et al., 2009; Gschwendtner et al., 2010), and other abiotic and biotic factors such as agricultural management (Griffiths et al., 2007; Verbruggen et al., 2010). The magnitude of the effects exerted by these factors compared to possible effects of cultivar and GM-crops is still largely unknown although knowledge of these sources of natural variation is critical for the assessment of the relative effects of specific potential perturbations such as introduced GM-traits.

Most of the studies on soil fungal communities have shown that GM-crops affect soil fungi in a similar way as its isoline (Donegan et al., 1996; Donegan et al., 1999; Milling et al., 2004; Hart et al., 2009; Wang et al., 2009; Weinert et al., 2009; Gschwendtner et al., 2010; Lee et al., 2011), and only three studies (Donegan et al., 1995; Götz et al., 2006; Wei et al., 2006) observed significant differences between the GM-variety and its parental isoline which could, however, be explained by factors other than GM-trait. Common to these studies was that the normal variability between cultivars under field conditions was usually very high and that other factors than cultivar-type affected the soil fungal communities more than the cultivar-type did. The aforementioned studies usually focused on one growth stage or one season/year without investigating variability over seasons. Thus, the question remains if different cultivars of potato, including a GM variety, have different effects on diversity or functioning of the soil microbes over multiple years.

Identifying the normal variation in fungal community structure and function in the soil is very important when aiming to evaluate the possible effects of GM-crops on soil communities (van Overbeek and van Elsas, 2008). In this study

we followed the fungal community structure and function in two fields located in the Netherlands during 3 years of growing potatoes (*Solanum tuberosum* L.). Three growth stages of six cultivars (including a GM-variety with modified starch quality and its parental isoline) were included in the study allowing us to determine the long-term (years) and short term (within growth season) effects of the potato cultivars on fungal community dynamics and fungal decomposing activities. This approach facilitated an evaluation of the normal variation in fungal communities between years, growth stages, soils and under different cultivars, thereby providing a necessary baseline for assessing the potential impact of this GM potato variety. Further, we sampled the fields also after the growing seasons as well as in the rhizosphere of the succeeding crop (barley) to learn about possible long term effects of the starch-modified GM-potatoes.

4.2. Materials and methods

4.2.1. Field set-up and sampling

Two agricultural sites VMD and BUI were selected for this experiment (chapters 1 and 3). They are both located in the northern part of the Netherlands and are 10 km apart. Details on soil type, soil parameters and fertilizer treatments are presented in table S4.1. Cropping in these sites consists of potato-barley rotation (1 crop per year). Plots with six cultivars of potato were sampled in years 2008, 2009, and 2010 and barley fields were sampled after cultivation with potato in 2009. The exact sampling dates are presented in table S4.1. The fields were fertilized with 180 – 220 kg ha -1 nitrogen (N) in the form of calcium ammonium nitrate, 56 – 81 kg ha -1 phosphorous (P) as P2O5 and 145 – 200 kg ha -1 potassium (K) as K2O or K2SO4 in 2008 and 2009. In 2010 organic fertilizer in form of pig manure (14 ton ha-1in field VMD and 25 ton ha-1 in field BUI, respectively) was added together with inorganic fertilizers (table S4.1). Six cultivars of potato; 'Aveka', 'Aventra', 'Désirée', 'Premiere', 'Karnico' and 'Modena' (the modified variety of 'Karnico') were grown each in four replicates on these fields in randomized block design and locations were varied between years. The variety 'Modena' was genetically modified for its starch composition by complete inhibition of the production of amylose via introduction of a RNAi construct of the granule-bound starch synthase gene inhibiting GBSS and amylose formation, which yields pure amylopectin (de Vetten et al., 2003). Cultivars 'Aventra', 'Aveka', 'Karnico' and 'Modena" produced tubers with a relatively high starch content and had a low to medium growth rate, whereas cultivars 'Désirée' and 'Premiere' had lower starch content in the tubers and higher growth rates.

Soil samples were collected from bulk soil before and after harvest whereas both rhizosphere and bulk soil were collected at the growth stages EC30 (seedling/young), EC60 (flowering) and EC90 (senescence) (Hack et al., 2001). Bulk soil was collected using 0-15 cm soil corers (diameter 10 cm) and 5 cores per plot were randomly sampled and used to form a composite sample per plot that was further homogenized and sieved (4 mm mesh) to remove possible root fragments and stones.

Rhizosphere soil was collected from a combination of 4 plants per plot by brushing roots. Part of the soil sample was subsequently frozen at -80 °C for molecular analyses, another part was kept at -20 °C prior to enzymatic analyses and ergosterol measurements and another part was used for immediate analyses of soil water content and pH (table S4.1). Soil water content was determined from fresh material as weight loss after overnight drying at 105 °C.

4.2.2. Enzymatic analyses

Quantification of ergosterol, via the alkaline extraction method, was used as an estimate of fungal biomass (de Ridder-Duine et al., 2006). Analyses of activities of enzymes involved in decomposition of lignocellulose-rich organic matter, i.e. laccase, cellulase and Mn-peroxidase were performed according to van der Wal et al. (2006).

4.2.3. Molecular analyses

DNA was extracted from soil (0.5 g wet weight) with a Power Soil DNA isolation kit (MOBIO Laboratories, Inc. Carlsbad, CA, USA) using a bead beating system. Yields of genomic DNA were checked on 1 % agarose gel and visualized under UV after ethidium bromide staining.

Terminal restriction fragment length polymorphism (T-RFLP) combined with the construction of a small library of the most dominant operational taxonomical units (OTUs) was used to determine the fungal community compositions over years. The structures of the three fungal phyla studied, ascomycetes, basidiomycetes and glomeromycetes, were assessed separately. For the analysis of ascomycete and basidiomycete communities, internal transcribed spacer (ITS) regions were used as target regions and the large subunit of ribosomal genes (LSU) was used as a target region for AMF (*Glomeromycota*). PCR conditions, primers and restriction enzymes are given in chapter 3. Appropriate dilutions based on test runs of terminal restriction fragments (TRFs) were analyzed with an ABI 3130 sequencer using GeneScan™ -500 LIZ (Applied Biosystems) and used as a size standard.

Clone libraries were constructed as described in chapter 3 and partially the same clone libraries were used. The sequenced clones were assigned to OTUs based on comparisons with GenBank using BLAST and considered to belong to a genus or species with similarities of 95 % for an order and 97% for a species. These OTUs were related to the orginal peaks and their presence and absence in field samples were evaluated in T-RFLP Analyses Matching Program (TRAMP-R) (Fitzjohn and Dickie, 2007) in the statistical computing environment R. Three out of four of the enzyme / primer combinations within 1.5 bp margin had to be met in a sample for it to be assigned to an OTU.

4.2.4. Data analyses

Analyses of variance (ANOVA) with a linear mixed effect model was used to compare the ergosterol and enzymatic data as well as number of TRFs using SPSS for windows (Release 17.0.). The assumption of normality was tested with Shapiro-Wilk statistics and homogeneity of variances was assessed with Levene's test. The field

site, growth stage, year of sampling, cultivar and GM-variety were used as fixed factors and block was set as the random factor. Differences between treatments were compared by a post hoc Tukey's honestly significant difference (HSD) test. Log transformation was used when data were not normally distributed. To estimate the possible effects of GM variety 'Modena' to its parental variety over years, a mixed model with repeated measure (growth stage) and block as a random factor was built separately for both fields.

The quality of T-RFLP data was first visually inspected in GeneMapper Software v4.1 (Applied Biosystems) and then transferred to T-Rex (Culman et al., 2008). True peaks were identified for both labels as those of which the height exceeded the standard deviation (assuming zero mean) computed over all peaks and multiplied by two (Abdo et al., 2006). Non-Metric Multidimensional Scaling (NMDS) with Jaccard as distance measure were used to assess the similarity of the fungal communities after the harvest and in the rhizosphere of next crop, barley. Principal component analyses (PCA) were used to analyse the communities between years, fields, growth stages and cultivar. The community fingerprints were compared using ANOSIM in PAST (Hammer et al., 2001). In short, ANOSIM is a non-parametric test of significant differences between groups by comparing distances between groups to distances within groups (resulting to R-values between 1 and -1). We used Jaccard as a distance index and 10000 permutations. Pairwise ANOSIMs between field sites, growth stages, years and cultivars are provided.

The diversity was calculated from the matched samples with both Shannon-H' and Simpson diversity indexes and compared with ANOVA as explained above.

4.3. Results

4.3.1. Soil enzymatic analyses, fungal biomass and fungal richness

Fungal-related parameters in plots cropped with the GM-variety seemed to fall within the normal variation among potato cultivars observed in time (table 4.1). The

Table 4.1. ANOVA comparisons of several fungal-related parameters between fields, years, growth stages, cultivars and GM-trait and the interaction effects of the cultivar. Significant P-values are marked with bold. Only samples from rhizosphere were included in analyses of growth stage, cultivar and GM-parent comparison. # indicates richness of the fungi.

	Field			Year			Growth stage			Cultivar			GM-parent		
	df.	*F*	*P*	*df.*	*F*	*P*	*df.*	*F*	*P*	*df.*	*F*	*P*	*df.*	*F*	*P*
Ergosterol (mg / g)	1	0.13	0.72	2	48.17	**<0.001**	3	19.38	<0.001	5	1.47	0.20	1	0.12	0.73
Laccases (µmol / g)	1	0.63	0.43	2	14.39	**<0.001**	3	21.19	<0.001	5	1.05	0.39	1	0.36	0.55
Mn-Peroxidases (µmol / g)	1	1.06	0.10	2	1.96	0.14	3	9.81	<0.001	5	3.31	0.06	1	0.67	0.42
Cellulases (µmol / g)	1	17.74	**<0.001**	2	23.94	<0.001	3	19.01	<0.001	5	1.08	0.37	1	0.04	0.83
# of Ascomycetes	1	0.41	0.52	2	6.28	<0.001	3	25.15	<0.001	5	1.51	0.19	1	2.73	0.11
# of Basidiomycetes	1	1.65	0.20	2	51.60	<0.001	3	20.14	<0.001	5	0.72	0.61	1	0.16	0.69
# of AMF	1	0.61	0.44	2	15.29	<0.001	3	6.09	<0.001	5	0.66	0.65	1	0.35	0.55

largest explaining factor for most of the measured parameters was the plant phenological growth stage, followed by year and the soil type (table 4.1).

Ergosterol analyses indicated that soil fungal biomass was strongly dependent on plant growth stage and year (table 4.1, Fig. 4.1). Although growth stage was affecting the fungal biomass, there were no significant differences between pre- and post-cropping situations or in bulk soils (N=288, F=1.31, p=0.25). Hence, no long term effects of cultivation were detected. Cultivar did not affect the fungal biomass in the rhizosphere in general, however, differences between some cultivars were detected in pairwise comparisons: cultivar 'Premiere' had a significantly lower fungal biomass as assayed by the ergosterol method in its rhizosphere than cultivars "Aveka' and 'Désirée' (N=432, F=4.131 and 4.181, p<0.05) over the entire period. In field BUI significant effects of cultivar on fungal biomass were detected at the stage of flowering in 2008 and the stage of young plant in 2010 (table 4.2) while in field VMD there were no effects of cultivar at any stage. Furthermore, there was no consistency in cultivars having the lowest or highest amount of ergosterol in their rhizosphere (Fig.4.1). The GM cultivar 'Modena' was not significantly different from the other cultivars or the parental variety (table 4.2) but rather in the middle range of the cultivars in the field BUI. The only significant difference between the GM-variety and its parental variety was the amount of ergosterol in the rhizosphere in the senescent stage (table 4.2).

Correlations revealed that all the extracellular enzymes measured in this study (laccases, cellulases and Mn-peroxidases) were positively correlated with the fungal biomass indicator ergosterol (n = 702, R2 between 0.23 - 0.29 and p<0.001). Further, there were strong positive correlations among all enzyme activities measured. The richness of both ascomycetes and basidiomycetes was positively correlated with the amount of ergosterol (n=702, for basidiomycetes $R^2 = 0.27$ and P<0.001 and ascomycetes $R^2 = 0.08$ and P<0.05). AMF richness was negatively correlated with the amount of ergosterol ($R^2 = 0.11$ and P<0.05). Furthermore, the amount of Mn-Peroxidases in the soil was positively correlated with the ascomycete diversity ($R^2 = 0.16$, P<0.001) while the AMF richness was negatively correlated with production of cellulases ($R^2 = 0.11$ and P<0.005).

Table 4.1. continues

Year x cultivar			Field x cultivar			Growth stage x cultivar			Field x year x growth stage x cultivar		
df.	*F*	*P*	*df.*	*F*	*P*	*df.*	*F*	*P*	*df.*	*F*	*P*
10	1.40	0.18	5	1.00	0.42	14	0.97	0.49	14	1.72	0.071
10	1.84	0.052	5	1.27	0.28	14	2.39	0.004	14	1.72	0.052
10	1.02	0.43	5	1.07	0.38	14	1.86	0.031	14	1.69	0.043
10	4.03	<0.001	5	3.96	0.002	14	3.12	<0.001	14	1.29	0.35
10	0.72	0.69	5	0.48	0.79	14	2.67	0.001	14	1.38	0.16
10	0.52	0.88	5	0.08	1.00	14	1.12	0.34	14	0.39	0.97
10	0.49	0.88	5	0.89	0.49	14	0.34	0.98	14	0.50	0.91

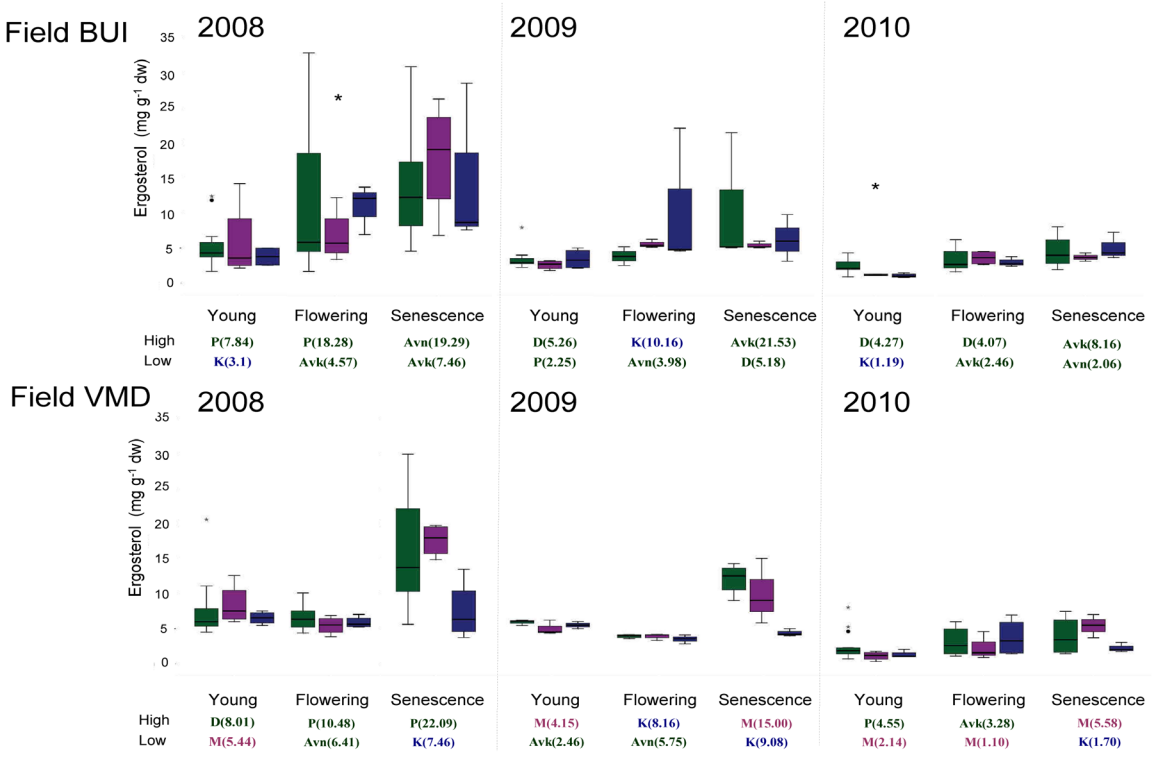


Figure 4.1. Change in fungal biomass. Boxplots of fungal biomass in the rhizosphere as measured by ergosterol concentrations during 3 years in different growth stages and in both field locations. The baseline (all other cultivars combined, n=16) is marked with green boxplots, the GM-variety (n=4) with purple and the parental variety 'Karnico' (n=4) with blue markers. The star indicates a significant cultivar effect at the indicated time point. The values under the graphs are the cultivars with highest and lowest values (on average) colored the same as in the boxplots where 'D' = 'Désirée', 'Avk'='Aveka', 'Avn'='Aventra', 'P'= 'Premiere', 'K' = 'Karnico' (parental cultivar) and 'M'='Modena' (modified cultivar).

The measured extracellular enzymes (laccases, Mn-peroxidases and cellulases) were all affected by plant growth stage; highest activities were measured during senescence (table 4.1). The amount of laccases and cellulases in the rhizosphere was significantly affected by year and the highest activity of these enzymes was found in 2009. On average the BUI location had higher laccase and cellulase activity than field VMD. The amount of Mn-peroxidases was associated with cultivar, but other enzymes were not. The cultivar 'Modena' had similar amounts of Mn-peroxidase in its rhizosphere as the parental cultivar 'Karnico', but more Mn-peroxidases in its rhizosphere than was found in the rhizospheres of Premiere and Aveka.

When looking at individual time points and fields the ascomycete, basidi-

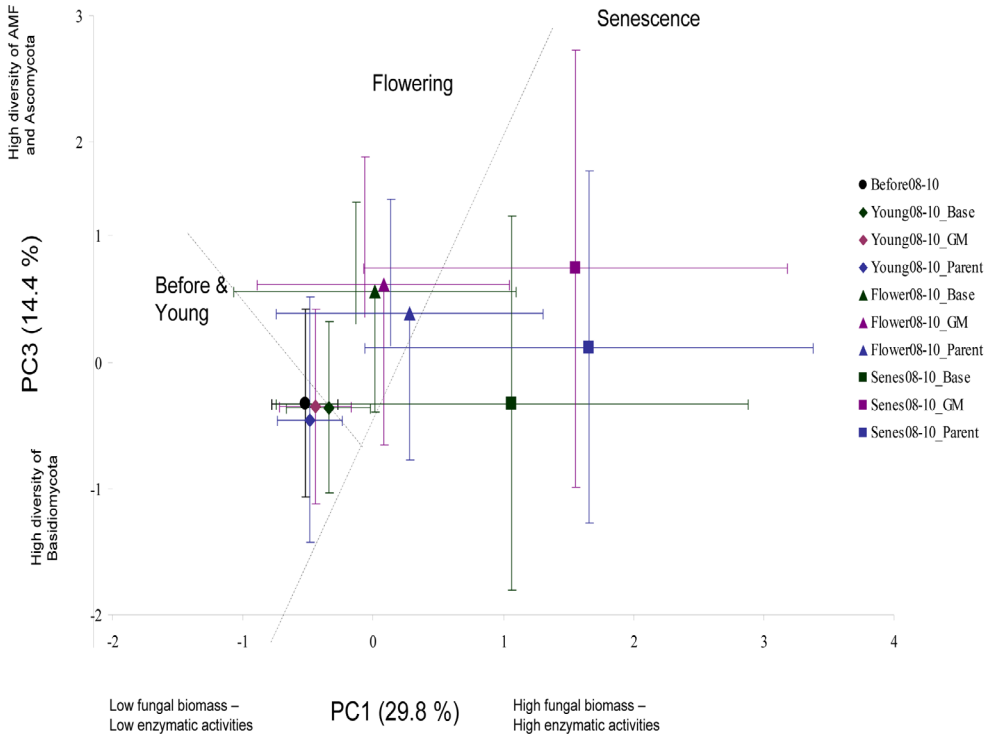


Figure 4.2. Principal component analysis of functioning and diversity of fungal communities in plots cropped with different potato cultivars. For clarity, the years and field sites are combined. Pre-cropping samples are represented by black circles, young plant stage samples with diamonds, flowering plants stage samples with triangles and senescence stage samples with squares. Green markers and error bars represent baseline cultivars (n=96), purple markers the GM-variety (n=24) and blue markers the parental variety 'Karnico' (n=24). The explanatory parameters are mentioned next to the axis. The enzymes measured as functional parameters were laccases, Mn-peroxidases and cellulases.

Table 4.2. ANOVA analysis of effects of cultivar (including all cultivars) and GM-cultivar 'Modena' versus parental cultivar 'Karnico'on fungal biomass, enzymatic activities and fun-

				Ergosterol (mg /g)		Laccases (µmol / g)		Mn-Peroxidases (µmol / g)	
				Cultivar	GM-Parent	Cultivar	GM-Parent	Cultivar	GM-Parent
			df.	5	1	5	1	5	1
Field BUI	2008	Young	F	0.86	0.24	0.89	2.99	1.02	1.32
			P	0.53	0.65	0.51	0.15	0.44	0.30
		Flowering	F	**12.64**	2.13	1.00	0.59	0.76	0.42
			P	**<0.001**	0.20	0.44	0.47	0.59	0.84
		Senescence	F	2.07	0.15	2.61	0.20	**9.19**	0.85
			P	0.12	0.72	0.06	0.67	**<0.001**	0.40
	2009	Young	F	1.22	1.13	1.24	0.50	0.73	0.61
			P	0.36	0.33	0.35	0.51	0.59	0.46
		Flowering	F	1.04	0.72	0.45	2.63	1.00	0.34
			P	0.41	0.45	0.77	0.16	0.45	0.58
		Senescence	F	1.32	0.20	1.68	1.32	1.42	1.36
			P	0.32	0.68	0.26	0.33	0.33	0.33
	2010	Young	F	**9.49**	0.01	1.09	0.11	0.11	0.26
			P	**0.001**	0.94	0.41	0.75	0.99	0.63
		Flowering	F	0.85	0.77	1.06	0.64	0.84	0.69
			P	0.54	0.42	0.41	0.46	0.54	0.80
		Senescence	F	4.73	1.42	0.65	0.22	1.89	0.30
			P	0.11	0.30	0.63	0.66	0.17	0.61
Field VMD	2008	Young	F	1.96	1.49	1.20	1.21	0.76	0.09
			P	0.13	0.27	0.35	0.31	0.59	0.78
		Flowering	F	0.67	0.33	1.43	1.19	0.73	0.65
			P	0.65	0.59	0.27	0.32	0.61	0.46
		Senescence	F	1.43	**17.50**	1.24	0.51	2.43	0.18
			P	0.26	**0.006**	0.33	0.50	0.08	0.69
	2009	Young	F	2.91	0.54	0.66	0.22	0.27	0.06
			P	0.17	0.50	0.59	0.66	0.85	0.82
		Flowering	F	7.03	0.62	0.98	0.18	**4.24**	1.18
			P	0.23	0.47	0.44	0.69	**0.03**	0.32
		Senescence	F	0.77	**14.16**	1.57	1.18	1.35	1.24
			P	0.68	**0.011**	0.23	0.32	0.30	0.31
	2010	Young	F	0.86	0.30	0.83	1.05	0.65	2.97
			P	0.53	0.61	0.55	0.34	0.66	0.14
		Flowering	F	0.75	0.99	1.76	1.00	2.13	1.00
			P	0.60	0.36	0.17	0.36	0.11	3.56
		Senescence	F	0.27	**13.59**	1.54	1.17	0.18	0.13
			P	0.84	**0.014**	0.30	0.54	0.90	0.74

gal richness in the rhizosphere in different fields, years and growth stages. Significant P-values are marked with bold.

Cellulases (µmol / g)		# of Ascomycetes		# of Basidiomycetes		# of AMF	
Cultivar	GM-Parent	Cultivar	GM-Parent	Cultivar	GM-Parent	Cultivar	GM-Parent
5	1	5	1	5	1	5	1
0.48	0.92	0.22	0.06	0.90	0.05	1.09	0.10
0.79	0.38	0.95	0.82	0.50	0.83	0.42	0.78
0.79	0.00	0.38	0.25	0.48	0.02	1.89	0.47
0.57	0.96	0.86	0.63	0.79	0.89	0.15	0.53
1.25	0.51	**3.67**	**39.06**	0.66	0.38	1.93	0.28
0.33	0.50	**0.018**	**0.025**	0.66	0.57	0.15	0.62
0.94	0.61	0.13	0.07	0.47	0.19	1.12	2.42
0.47	0.46	0.97	0.80	0.75	0.69	0.40	0.17
0.69	5.53	0.17	3.00	0.87	0.00	0.04	5.44
0.62	0.06	0.85	0.33	0.49	0.96	0.96	0.26
1.80	0.71	0.32	1.93	1.60	2.10	1.24	1.15
0.23	0.46	0.81	0.21	0.27	0.24	0.37	0.40
1.32	0.49	0.92	1.94	0.84	1.26	0.34	0.35
0.31	0.52	0.49	0.21	0.54	0.31	0.88	0.58
0.47	0.13	0.19	0.02	2.52	0.06	0.34	1.08
0.79	0.73	0.96	0.89	0.31	0.81	0.88	0.49
1.55	1.51	0.33	1.04	0.68	0.00	1.53	**6.42**
0.24	0.27	0.86	0.38	0.62	0.95	0.24	**0.044**
1.03	0.34	2.13	2.50	0.34	0.10	0.55	1.54
0.43	0.58	0.11	0.17	0.89	0.77	0.74	0.26
1.23	1.93	0.56	0.33	0.46	1.47	2.10	1.03
0.35	0.21	0.73	0.59	0.72	0.44	0.12	0.35
0.81	0.10	0.66	1.93	0.56	0.26	0.66	0.93
0.56	0.76	0.66	0.21	0.73	0.65	0.66	0.38
0.32	0.15	0.57	0.24	0.58	0.36	1.06	0.62
0.81	0.72	0.65	0.64	0.64	0.57	0.40	0.46
1.22	2.83	0.67	2.00	0.25	0.85	1.58	0.29
0.35	0.14	0.54	0.22	0.90	0.41	0.29	0.63
0.36	0.01	1.35	1.04	**6.00**	4.09	2.38	5.89
0.84	0.92	0.36	0.38	**0.03**	0.11	0.13	0.06
0.68	0.93	0.53	0.18	0.83	1.67	**4.01**	4.17
0.65	0.37	0.75	0.68	0.55	0.24	**0.03**	0.11
0.78	0.86	1.08	0.74	0.25	0.09	2.02	8.17
0.58	0.39	0.41	0.42	0.90	0.78	0.18	0.07
0.30	0.06	0.95	0.46	0.47	1.02	0.12	0.52
0.82	0.82	0.45	0.53	0.71	0.35	0.95	0.51

omycete and glomeromycete richness was only once significantly different between cultivars (table 4.2). The richness of ascomycetes and glomeromycetes in the rhizosphere of GM-cultivar was only once different from the parental cultivar, namely at senescence 2008 and senescence 2010 in field BUI. The basidiomycete richness was at no occasion different between GM- and parental cultivar (table 4.2).

Data on community function, as based on activities of enzymes involved in decomposition of lignocellulose-rich organic matter, and richness were analysed by principal component analyses (PCA). The PCA analyses revealed that growth stage was the strongest explanatory factor of differences in the community function (Fig. 4.2). The stage senescence clearly separated from the other stages along PC1 (ANOVA; $F = 9.57\text{-}13.74$, $p<0.001$) which was explained with higher ergosterol and enzymatic activities during senescence. The PC2 was explained by same factors as PC1 and is thus not used here. The flowering stage separated along PC3 ($F = 4.22 – 8.28$, $p<0.05$) which is explained by more AMF and ascomycetes and less basidiomycetes during that stage compared to the other stages. Further, the years separated along both axes (PC1: $F=8.5$, $p<0.001$ and PC3: $F=124.6$, $p<0.001$) and fields along PC3 ($F=33.9$, $p<0.001$) (Fig. S4.1). Cultivar had no significant contribution to explanation of PC1 ($F=1.83$, $P=0.15$), PC2 ($F=1.92$, $P=0.12$) nor PC3 ($F=0.88$, $P=0.47$) and the GM-variety was not significantly different from its parental isoline 'Karnico' (Fig. 4.2).

4.3.2. Fungal diversity and community structure

According to the ANOSIM, the community fingerprints of all TRF peaks as well as identified OTUs of *Ascomycota*, *Basidiomycota* and *Glomeromycota*, were affected by the growth stage of the plant, field site and year (Fig. 4.3, Table 4.3). The fungal community structure was most strongly influenced by year-to-year variation ($R>0.22$) and difference in growth stage ($R>0.09$). The R values for the field site were close to 0, but due to the size of the data-set a significant difference between fields were found. Plant cultivar did not predict fungal community structure when all growth stages, years and both fields were considered together (Table 4.3). There were no significant differences in the community structure of ascomycetes, basidiomycetes, glomeromycetes or total fungi between GM-cultivar 'Modena' and its parental variety 'Karnico' in any pairwise comparisons (Fig. 4.3)

The diversity of all fungal phyla was expressed both by the Shannon-Wiener index (H') and Simpson diversity index. The ascomycete diversity was significantly correlated with ascomycete richness ($R^2 = 0.55$ for total diversity, $R^2 = 0.45$ for orders and $R^2 = 0.36$ for classes, $P<0.001$ for all) and basidiomycete diversity with basidiomycete richness ($R^2 = 0.51$ for total diversity and $R^2 = 0.41$ for orders, $P<0.001$ for both). Further, the ascomycete diversity was negatively correlated with basidiomycete diversity ($R^2 = 0.15$, $P<0.005$). Ascomycete richness was correlated with the amount of Mn-peroxidases in the soil ($R^2 = 0.15$, $P<0.05$) and basidiomycete richness with ergosterol ($R^2 = 0.18$, $P<0.001$). The AMF diversity was positively correlated with soil moisture content ($R^2 = 0.15$, $P<0.001$), AMF richness ($R^2 = 0.58$, $P<0.001$) and ascomycete diversity ($R^2 = 0.10$, $P<0.05$).

Table 4.3. ANOSIM comparisons between the fields, years, growth stages, cultivars and GM-trait for *Ascomycota*, *Basidiomycota* and *Glomeromycota*. Significant P-values are marked with bold.

	Field		Year		Growth stage		Cultivar*		GM-parent*	
	R	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*
Ascomycota	0.07	**<0.001**	0.29	**<0.001**	0.10	**<0.001**	0.013	0.131	-0.006	1
Basidiomycota	0.04	**<0.001**	0.25	**<0.001**	0.19	**<0.001**	0.008	0.188	0.015	0.915
Glomeromycota	0.11	**<0.001**	0.22	**<0.001**	0.09	**<0.001**	-0.005	0.689	-0.011	0.863

*Only samples where plant was present are included in the analyses

Table 4.4. The effect of field site, year, growth stage and cultivar on soil ascomycete, basidiomycete and glomeromycete diversity for different taxonomic levels. All diversities were calculated using both Shannon H' and Simpson diversity indexes and presented in the table as Shannon H' / Simpson diversity. If both P-values are the same, only one value is presented. Diversity index for classes was not calculated for basidiomycetes and glomeromycetes due to low numbers or unevenness of classes. Significant P-values are marked with bold.

		Field		Year		Growth stage		Cultivar*		GM-parent*	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Ascomycota	OTUs	0.005 / 0.004	0.94 / 0.94	7.80 / 3.89	**<0.001 / 0.02**	12.76 / 9.16	**<0.001**	0.65 / 0.32	0.66 / 0.91	2.67 / 0.49	0.11 / 0.49
	Orders	0.33 / 0.009	0.57 / 0.92	7.44 / 3.56	**<0.005 / 0.03**	10.8 / 13.22	**<0.001**	0.59 / 0.52	0.64 / 0.76	2.74 / 1.58	0.10 / 0.21
	Classes	9.30 / 9.50	**0.03 / 0.02**	10.80 / 9.64	**<0.001**	6.78 / 5.76	**<0.001**	15.58 / 34.61	**<0.001**	2.97 / 2.31	0.09 / 0.31
Basidiomycota	OTUs	1.803 / 0.523	0.18 / 0.47	9.49 / 6.64	**<0.001 / 0.002**	13.84 / 9.37	**<0.001**	1.24 / 1.41	0.29 / 0.23	0.03 / 0.02	0.87 / 0.90
	Orders	0.04 / 0.002	0.85 / 0.97	21.85 / 17.86	**<0.001**	8.99 / 6.48	**<0.001**	1.85 / 2.08	0.13 / 0.09	0.19 / 0.37	0.67 / 0.54
Glomeromycota	OTUs	14.67 / 15.04	**<0.001**	24.48 / 20.72	**<0.001**	3.01 / 2.76	**0.03 / 0.04**	1.91 / 1.63	0.09 / 0.15	1.91 / 1.40	0.17 / 0.24
	Orders	38.22 / 35.98	**<0.001**	12.50 / 9.99	**<0.001**	2.29 / 2.13	0.08 / 0.09	1.91 / 1.89	0.09 / 0.10	1.17 / 1.59	0.19 / 0.21

*Only samples where plant was present are included in the analyses

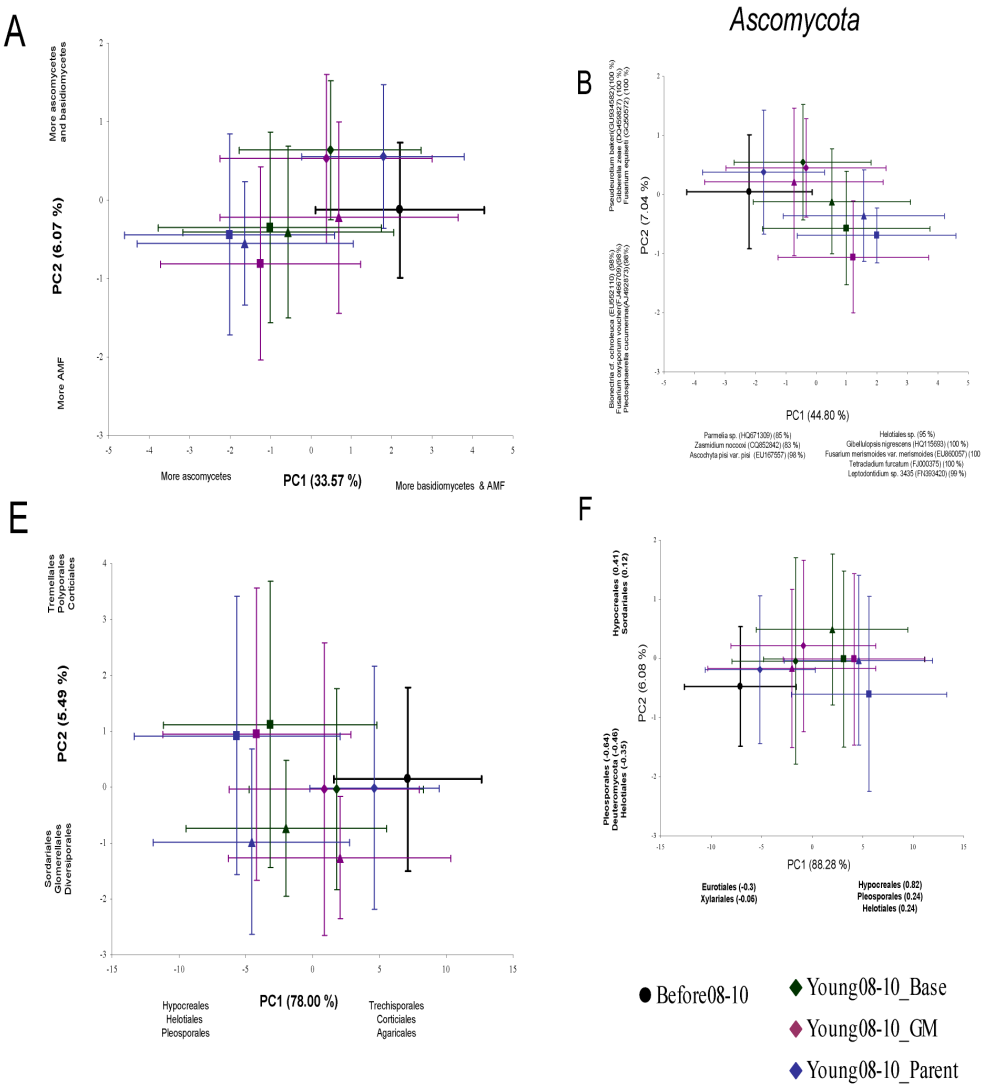
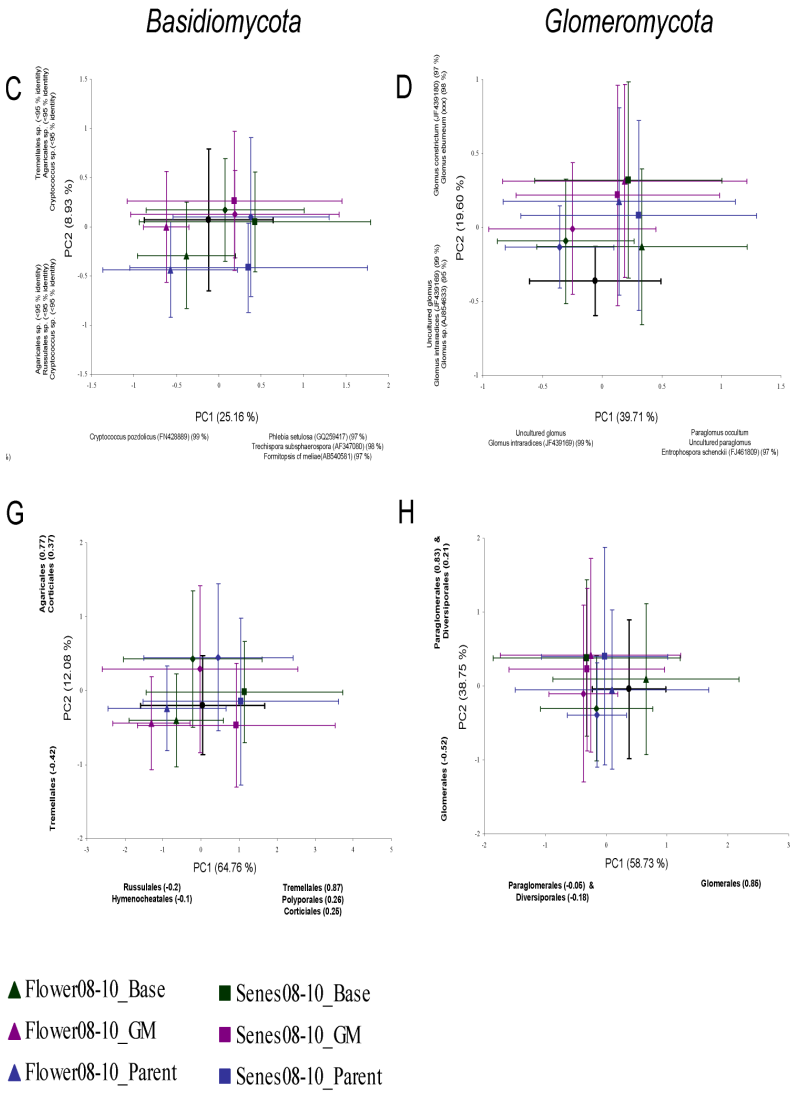


Figure 4.3. Principal component analysis of community structure of identified fungi. The PCA analysis was done both at the level of individual OTUs and of orders for total fungi (A & E), *Ascomycota* (B & F), *Basidiomycota* (C & G) and *Glomeromycota* (D & H). Figures A-D depict the identified fungal OTUs whereas figures E-H indicate the levels of orders. For clarity, the years and field sites are combined. Pre-cropping soil samples are marked with black circles,



young plants stage with diamonds, flowering plant stage with triangles and senescence stage with squares. Green markers and error bars represent baseline cultivars (n=96), purple markers the GM-variety (n=24) and blue markers the parental variety 'Karnico' (n=24). The OTUs (figures A-D) and orders (E-H) that do significantly explain the components are mentioned next to the axis.

4.3.2. Fungal diversity and community structure

According to the ANOSIM, the community fingerprints of all TRF peaks as well as identified OTUs of *Ascomycota*, *Basidiomycota* and *Glomeromycota*, were affected by the growth stage of the plant, field site and year (Fig. 4.3, Table 4.3). The fungal community structure was most strongly influenced by year-to-year variation (R>0.22) and difference in growth stage (R>0.09). The R values for the field site were close to 0, but due to the size of the data-set a significant difference between fields were found. Plant cultivar did not predict fungal community structure when all growth stages, years and both fields were considered together (Table 4.3). There were no significant differences in the community structure of ascomycetes, basidiomycetes, glomeromycetes or total fungi between GM-cultivar 'Modena' and its parental variety 'Karnico' in any pairwise comparisons (Fig. 4.3)

The diversity of all fungal phyla was expressed both by the Shannon-Wiener index (H') and Simpson diversity index. The ascomycete diversity was significantly correlated with ascomycete richness ($R^2 = 0.55$ for total diversity, $R^2 = 0.45$ for orders and $R^2 = 0.36$ for classes, $P<0.001$ for all) and basidiomycete diversity with basidiomycete richness ($R^2 = 0.51$ for total diversity and $R^2 = 0.41$ for orders, $P<0.001$ for both). Further, the ascomycete diversity was negatively correlated with basidiomycete diversity ($R^2 = 0.15$, $P<0.005$). Ascomycete richness was correlated with the amount of Mn-peroxidases in the soil ($R^2 = 0.15$, $P<0.05$) and basidiomycete richness with ergosterol ($R^2 = 0.18$, $P<0.001$). The AMF diversity was positively correlated with soil moisture content ($R^2 = 0.15$, $P<0.001$), AMF richness ($R^2 = 0.58$, $P<0.001$) and ascomycete diversity ($R^2 = 0.10$, $P<0.05$).

The diversity of ascomycetes or basidiomycetes at the level of OTUs or orders was not significantly affected by field site. However, AMF diversity was. There was no significant difference in diversity of ascomycetes at the level of OTUs and orders from year to year, although diversity between years 2009 and 2010 was significantly different. However, at the level of classes also 2008 and 2009 were different and year was a more pronounced factor explaining the diversity. For basidiomycetes and AMF, year had a strong influence on diversity both at the level of OTUs and orders (table 4.4). Growth stage, had a strong significant effect on ascomycete and basidiomycete diversities (Fig. 4.4, table 4.4) but less effect on the AMF diversity in the rhizosphere.

Cultivar-type had no overall effect on basidiomycete, ascomycete and AMF diversity at the level of OTUs or orders. However, at the level of classes cultivar 'Désirée' had a significantly less diverse community of ascomycetes in its rhizosphere than all the other cultivars causing a general cultivar effect (table 4.4). When the field sites, growth stages and years were considered separately, cultivar was a weak explanatory factor for the diversity of ascomycetes, basidiomycetes and AMF (Fig. 4.4, table 4.5). Both cultivar and GM-variety had an effect on diversity of ascomycetes in the rhizosphere in field BUI 2010 in the young-plant stage where 'Karnico' had a low diversity. The GM-variety had a significantly less diverse community of ascomycetes compared to its parental variety in field VMD 2010 at the stage of flowering plants (table 4.5). Basidiomycete diversity was different in rhizospheres between cultivars

Table 4.5. ANOVAs of the effect of cultivar (including all cultivars) and GM-cultivar 'Modena' versus parental cultivar 'Karnico' on diversity of ascomycetes, basidiomycetes and glomeromycetes in the rhizosphere in both fields, all years and growth stages. The diversities were estimated using Shannon-H'. The first two columns of each fungal group are performed at the level of OTUs and the third column indicates significance at the level of orders. Significant P-values are marked with bold.

				Diversity of *Ascomycota* (Shannon H)			Diversity of *Basidiomycota* (Shannon H)			Diversity of *Glomeromycota* (Shannon H)		
				Cultivar	GM-Parent	Order level	Cultivar	GM-Parent	Order level	Cultivar	GM-Parent	Order level
				df. 5	*df. 1*		*df. 5*	*df. 1*		*df. 5*	*df. 1*	
Field BUI	2008	Young	F	0.12	0.12	n.s.	0.21	0.60	n.s.	0.38	0.38	n.s.
			P	0.89	0.75		0.95	0.48		0.86	0.85	
		Flowering	F	0.92	0.00	n.s.	0.79	0.56	n.s.	0.62	2.34	n.s.
			P	0.91	1.00		0.58	0.50		0.69	0.22	
		Senescence	F	2.93	0.05	n.s.	0.99	0.30	n.s.	0.93	0.60	n.s.
			P	0.11	0.83		0.47	0.61		0.50	0.50	
	2009	Young	F	0.88	1.22	n.s.	0.23	0.40	n.s.	0.99	0.88	n.s.
			P	0.51	0.52		0.92	0.55		0.46	0.38	
		Flowering	F	2.86	1.14	n.s.	3.28	0.21	n.s.	0.64	0.71	n.s.
			P	0.14	0.35		0.10	0.63		0.56	0.44	
		Senescence	F	1.35	3.29	n.s.	4.88	1.19	n.s.	nd	nd	n.d.
			P	0.35	0.21		0.77	0.29		nd	nd	
	2010	Young	F	**6.25**	**13.80**	**3.83 / 11.27**	0.79	5.98	n.s.	0.54	0.60	n.s.
			P	**0.03**	**0.01**	**0.02 / 0.001**	0.58	0.07		0.74	0.50	
		Flowering	F	0.46	0.00	n.s.	2.86	2.24	n.s.	0.43	0.13	n.s.
			P	0.80	0.98		0.14	0.38		0.82	0.74	
		Senescence	F	1.37	1.14	n.s.	0.31	0.29	n.s.	4.41	0.82	n.s.
			P	0.30	0.35		0.86	0.82		0.99	0.40	
Field VMD	2008	Young	F	1.73	1.89	n.s.	0.45	0.19	n.s.	4.77	38.37	**2.38 / 40.38**
			P	0.19	0.49		0.80	0.69		0.01	0.00	**0.02 / 0.001**
		Flowering	F	0.85	4.35	n.s.	0.43	0.37	n.s.	2.23	0.41	n.s.
			P	0.52	0.17		0.79	0.58		0.15	0.59	
		Senescence	F	0.99	0.04	n.s.	0.57	0.34	n.s.	0.13	0.32	n.s.
			P	0.48	0.86		0.73	0.59		0.93	0.62	
	2009	Young	F	0.64	1.79	n.s.	0.45	0.26	n.s.	0.51	0.20	n.s.
			P	0.62	0.41		0.73	0.64		0.69	0.67	
		Flowering	F	0.64	1.08	n.s.	**4.95**	0.90	n.s.	0.47	0.71	n.s.
			P	0.61	0.41		**0.04**	0.39		0.76	0.44	
		Senescence	F	0.35	1.21	n.s.	**5.54**	0.93	**4.37 / 1.61**	2.31	23.78	n.s.
			P	0.84	0.44		**0.02**	0.38	**0.04 / 0.26**	0.15	0.16	
	2010	Young	F	0.49	0.42	n.s.	0.62	2.92	n.s.	1.12	1.89	n.s.
			P	0.78	0.84		0.66	0.15		0.56	0.49	
		Flowering	F	2.66	**7.84**	**1.43 /1.17**	2.72	2.34	n.s.	0.46	0.33	n.s.
			P	0.08	**0.05**	**0.28 / 0.03**	0.13	0.22		0.77	0.67	
		Senescence	F	0.84	0.91	n.s.	0.54	2.87	n.s.	0.49	0.42	n.s.
			P	0.50	0.38		0.67	0.17		0.75	0.86	

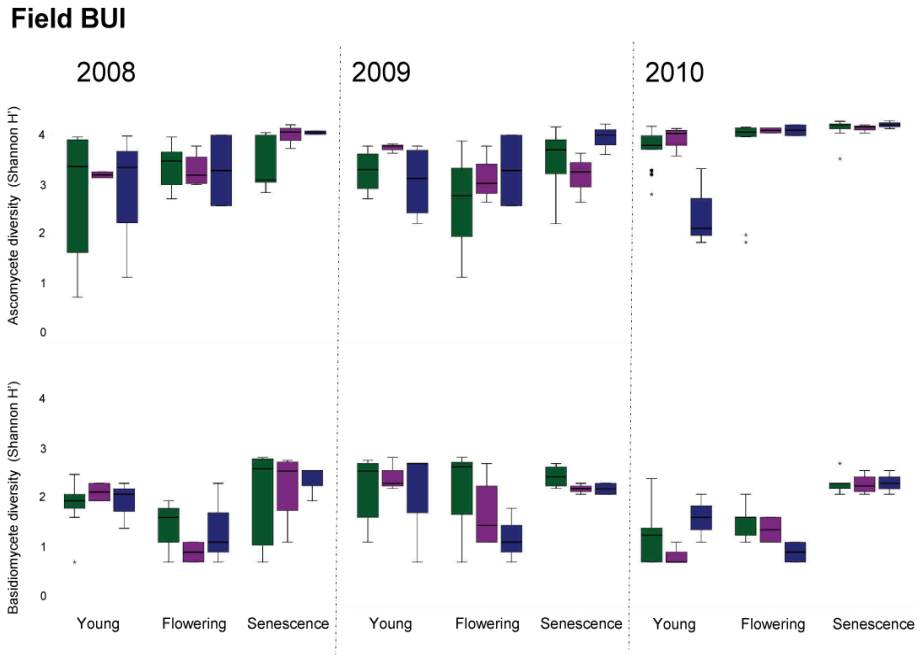


Figure 4.4. Effect of cultivar, year, growth stage and field on fungal diversity. Boxplots of changes in diversity of *Ascomycota* and *Basidiomycota* between years, growth stages, fields and between baseline ,GM and its parental variety. The baseline (all other cultivars combined, n= 16) is marked with green boxplots, the GM-variety (n=4) with purple and the parental variety 'Karnico' (n=4) with blue markers. Diversity was calculated using Shannon-Wiener index (H') and statistical comparisons are presented in table 4.5.

both during flowering and senescence 2009 in field VMD but never between GM and its parental cultivar. For AMF effects of cultivar and GM-variety were observed only at the first sampling moment of rhizosphere field in VMD (young 2008).

4.4. Discussion

The composition and function of fungal communities in the rhizosphere was shown to be highly dynamic and influenced by plant growth stage, soil type, year and, to a smaller extent, also cultivar-type. The largest explaining factor for most of the measured parameters was plant phenological growth stage, followed by year and soil type. In addition, these results confirmed our previous observations that fungal composition and abundance is strongly influenced by the presence of potato roots i.e. a strong rhizosphere effect (chapter 3).

The succession of microbial communities during plant growing season can be explained by two possible mechanisms (Wang et al., 2009). The first one is related

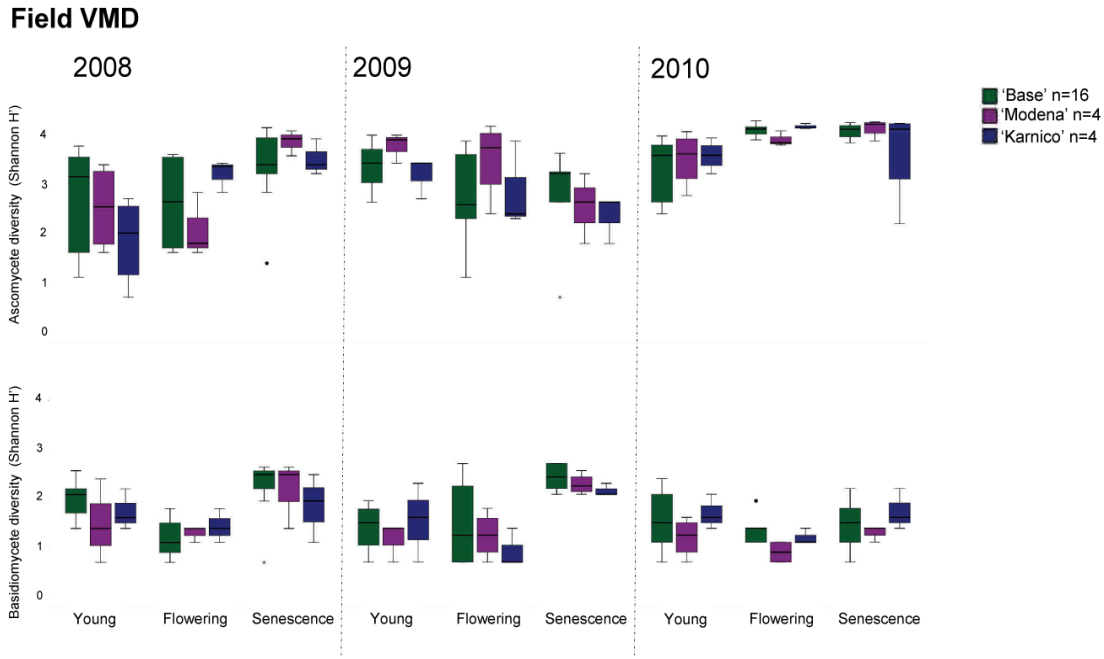


Figure 4.4. continued

to temporal changes in abiotic conditions such as soil moisture and temperature. However this is not a likely option to explain the fungal community dynamics observed in this study as the three years of study were very contrasting in the temperatures and moisture. The second, more likely, mechanism is the changes in quality and quantity of root exudates and rhizodeposits with growth stage (Marschner et al., 2002; Jones et al., 2004) and or changes in root morphology. Although root exudates were not measured in this study, there is evidence of the effect of plant growth stage on root exudate fluxes which in turn affect soil microbial communities (Duineveld et al., 2001; Garbeva et al., 2004). Earlier studies indicated that the size of bacterial and fungal communities in the rhizosphere would either decrease (Milling et al., 2004; Acosta-Martínez et al., 2008; Wang et al., 2009; Gschwendtner et al., 2010) or increase (Lottmann et al., 2000; Gomes et al., 2001; Smalla et al., 2001; Gomes et al., 2003; Sessitsch et al., 2004) during plant maturation. Our results clearly indicate that the plants at the senescence stage (EC90) harbor the most diverse, active and abundant fungal communities. The presence of the highest fungal biomass and diversity at the stage of senescence was expected as at that stage decomposable material (dead roots and leaves) is already available while root exudation still continues thereby broadening the spectrum of substrate availability (Broeckling et al., 2008). Yet, the increase and the magnitude of the fungal biomass and its activity in the rhizosphere at that stage is remarkable as until now the general idea is that the fungal biomass is low in soils under intensive agricultural management. Earlier results with the same

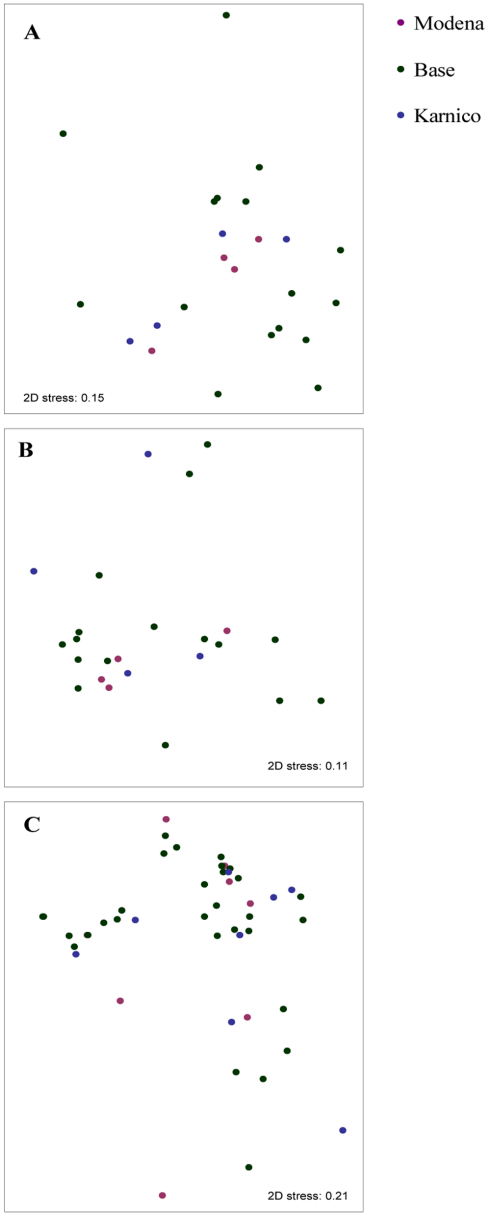


Figure 4.5. Long term effect of GM-trait on fungal community. NMDS of effects of GM-variety in the next crop (barley) rhizosphere in field BUI on ascomycetes (A), basidiomycetes (B) and in fields BUI and VMD on glomeromycetes (C). Both fields are presented for glomeromycetes due to the significant difference between fields. The GM-variety 'Modena' is marked with purple markers, the parental cultivar 'Karnico' with blue markers, and baseline (all other cultivars combined) green markers. Details on statistical analysis are given in table 4.6.

		Field BUI				Field VMD				Barley rhizosphere			
		Cultivar		GM-parent		Cultivar		GM-parent		Cultivar		GM-parent	
		F / R	P	F / R	P	F / R	P	F / R	P	F / R	P	F / R	P
ANOVA	Ergosterol	0.14	0.89	0.00	0.95	0.81	0.47	1.04	0.35	0.03	0.98	0.00	0.98
	Laccases	0.70	0.63	0.28	0.62	3.63	0.02	0.06	0.82	0.56	0.58	1.03	0.36
	Mn-Peroxidases	1.77	0.19	2.76	0.15	1.30	0.31	0.00	0.98	0.00	0.99	0.00	0.99
	Cellulases	1.00	0.43	0.35	0.58	1.56	0.22	5.53	0.06	0.06	0.95	0.05	0.84
	Diversity of basidiomycetes									1.37	0.29	0.06	0.82
	Diversityof ascomycetes	0.87	0.72	0.33	0.64	0.34	0.72	0.58	0.48	0.72	0.51	0.60	0.48
	Diversity of AMF	0.61	0.50	0.02	0.89	0.53	0.68	1.00	0.36	0.55	0.60	1.14	0.35
ANOSIM	Community of ascomycetes	-0.22	0.88	-0.32	1.00	-0.05	0.72	0.07	0.24	0.01	0.42	0.56	0.33
	Community of basidiomycetes	0.03	0.65	-0.12	0.95	-0.13	0.73	-0.38	0.97	-0.24	0.91	-0.58	1.00
	Community of AMF	-0.04	0.67	-0.16	0.80	0.02	0.37	-0.13	0.81	-0.28	1.00	-0.38	1.00

Table 4.6. Statistical analysis of the effects of cultivar and GM-trait on fungal-related parameters in post-harvest soil samples as well as in the rhizosphere of next plant barley. ANOVA was used as a similarity measure for fungal biomass, enzymatic measurements and diversity and F values are presented in the table. ANOSIM was used for the community data derived from T-REX and R-values are presented in the table. Significant P-values for both ANOVA and ANOSIM are marked with bold.

cultivars under controlled conditions confirm these observations (chapter 3).

Surprisingly, despite the strong differences in soil organic matter content, the field location did not affect the community function or diversity of the higher fungi much and results from the two fields could be even combined for baseline purposes. Earlier studies have found soil type as one of the most explanatory factor (Buyer et al., 2002; Garbeva et al., 2004; Berg and Smalla, 2009; Wang et al., 2009; Weinert et al., 2009) affecting the soil microbial communities. Bacterial communities appeared to differ strongly between the two fields used in this study, both for bulk soil and rhizosphere (Inceoglu et al., 2010). In our study, however, only total fungal community structure and diversity of AMF were strongly affected by the field site while fungal biomass and functional parameters such as enzymatic activities seemed to respond to the field type only slightly. The difference in AMF between fields could be probably explained by the higher organic matter content and thus higher AMF diversity in field VMD (Verbruggen et al., 2010).

We detected interesting differences between the years. In the first years, mineral fertilizer was used and only from the beginning of 2010 pig manure was used as a fertilizer. This might explain differences in fungal communities observed between 2008 and 2010. Previously, it has been shown that different types of fertilizer treatments contribute to different microbial communities (Marschner et al., 2003). Notably, in our study we detected more ascomycetes and less basidiomycetes and fungi in general in 2010 compared to 2008 in both fields (Fig. 4.1) which might be an indication of changed community structure due to changed fertilizer treatment. Also the diversity and richness of AMF was higher in 2010.

Community structure and diversity of the soil fungi are important determinants of key soil ecosystems functions such as decomposition of organic matter. Indeed, we could detect a correlation between community structure of fungi and decomposition-related enzyme activities. Moreover, the combination of phylogenetic analyses with functional assays proved highly useful, providing a more complete picture of fungal community dynamics. We found a correlation between Mn-peroxidases produced and the ascomycete diversity (and richness). Mn-peroxidases can be produced by some ascomycetal groups although most notable producers are basidiomycetes (Bödeker et al., 2009) while not much is known of the ecology of ascomycetal Mn-peroxidases. AM fungi are strongly affected by agricultural practices and changes in soil characteristics (Helgason et al., 1998; Turrini et al., 2004; Giovannetti et al., 2005) such as moisture and manure addition. Indeed, we saw an increase of AMF diversity in 2010 when the fertilizer was changed from mineral to pig manure which is in correspondence with results from Verbruggen et al. (2010) who found organic fertilizers having a positive effect on AMF diversity.

Only few studies have evaluated the potential impacts of GM-plants in the context of impacts of multiple cultivars on fungal rhizosphere communities. Most of them have found some degree of cultivar dependence of soil fungal community composition (Turrini et al., 2004; Weinert et al., 2009; Gschwendtner et al., 2010) while another one (Milling et al., 2004) found no cultivar dependent alterations in the fungal communities. We found some indications of cultivar dependence, for in-

stance the cultivar 'Premiere' had a lower amount of fungi, as measured by ergosterol, in its rhizosphere than two other cultivars 'Aveka' and 'Désirée' Despite some differences in enzymatic activities, total fungal diversity was not affected by the cultivar-type at the level of OTUs and orders. Ascomycetal diversity was affected at the level of classes as one cultivar, 'Désirée', had a less diverse community in its rhizosphere. To conclude, we found some degree of cultivar dependence in measured parameters at some time points, but these differences were mostly not persisting over time and not observed in both fields, similarly as found by Weinert et al. (2009).

Furthermore, in this study the GM-variety 'Modena' was not significantly different from its parental variety 'Karnico' in any measured parameter and it seemed that these cultivars had a very similar effect on both the structure and function of soil fungal communities. The only more lasting effect was the difference in the amount of fungi in the rhizosphere of these cultivars in the field VMD in the stage of senescence in 2008, 2009 and 2010. This was, however, seen only in one of the two soils studied and can, thus, be ruled out as a cultivar-soil interaction effect. There was no overall trend of multiple parameters being consistently changed by any of the cultivars while the other factors (i.e. growth stage and season) had consistent effect on multiple parameters measured.

The selection of the growth stage can also affect the outcome of the comparison between the cultivars. Other authors have found differences in microbial communities associated with GM-potatoes mostly at the senescent growth stage (Lottmann et al., 1999; Lottmann et al., 2000; Lukow et al., 2000; Abdo et al., 2006). As soil micro-organisms have an important role in soil ecosystem functioning it is possible that the differences at the stage of senescence as found in this study could lead to changes in function and might, thus, have long lasting effects. In this study, all analyses indicated that when the fungal communities were assessed after removal of the plant or in the rhizosphere of the next crop in rotation, there were no differences between fungal communities from field plots that contained harvested modified potato plants. So, we did not detect any significant connection between the previous cultivar of potato on the fungi in the rhizosphere of the next crop barley. Hence, the changes in the fungal biomass associated with starch modified potato plants detected at certain time points and fields in this study were temporary and did not persist into the next field season. A similar observation was made for bacteria after cropping of transgenic canola (Dunfield and Germida, 2003).

In conclusion, plant growth stage, year and field site were the factors contributing most to variation in the potato-associated fungal communities. Despite some differences in fungal-related parameters between individual cultivars, there were no directional effects and most of the differences observed were not consistent between fields and years. Even at the level of individual OTUs, there were no consistent significant differences between cultivars in community structure and no differences in community function were found during and after the growth of the plant. However, as was seen from conflicting evidence between different studies, we acknowledge that potential effects of GM-crops on soil fungal communities vary

between crop species and types of modifications done to the plant making a case-by–case evaluation strategy advisable. Data presented in this study allowed us to conclude that the modification studied here has no long-lasting effects on soil fungal communities and that the potato plant growth stage, season and field location affect the soil fungal community structure and function more than the cultivar-type or starch modification of tubers.

Table S4.1. Soil characteristics and fertilizers added to the fields and sampling dates (average temperature). In the fertilizer treatments CAN = Calcium Ammonium Nitrate, NP = nitrogen as ammonium sulphate and phosphorous as P2O5 and ORG = organic fertilizer = pig manure.

	Soil	BUI	VMD
Soil charasteristics	Soil texture	Loamy sand	Sandy peat
	pH (H20)	5.0	5.0
	Organic mat. (%)	5	19 - 25
	Sand (%)	90	94
	Silt (%)	5.7	2.8
	Clay (%)	4.3	3.2
	Water retention (%)	25 - 29	40 - 46
	K (mg/kg)	90	164
	P (mg/kg)	9.1	6.9
Fertilizer treatment	2008 (kg / ha)	N: 104 (NP) + 81(CAN) = 185 P: 56 (P2O5) K: 200 (K2O)	N: 150 (CAN) + 25 (N) = 175 P: 25 (P2O5) + 45 (P) = 67 K:150 (K2O)
	2009 (kg / ha)	N: 189 (CAN) P: - K: 175 (K2O)	N: 137 (CAN) + 33 (N) = 150 P: 33 (P2O5) + 45 (P) = 78 K: 200 (K2O)
	2010 (kg/ha)	N: 90 (org) + 122 (CAN) = 212 P:45 (org) K: 75 (org) + 100 (K2SO4) = 175	N: 25 (org) + 25 (CAN) + 104 (NP) = 179 P: 25 (org) + 56 (NP) = 81 K: 15 (org) + 150 (K2O) = 165
Sampling dates (daily average temperature in C)	Before 2008	7.4.2008 (3.7)	22.4.2008 (11.1)
	Young 2008	21.5.2008 (11.3)	21.5.2008 (11.3)
	Flowering 2008*	sampled many times	sampled many times
	Senescence 2008	3.9.2008 (13.8)	5.9.2008(15.8)
	After 2008	3.12.2008 (1.4)	3.12.2008 (1.4)
	Before 2009	3.4.2009 (12.9)	3.4.2009 (12.9)
	Young2009	14.5.2009 (13.4)	14.5.2009 (13.4)
	Flowering 2009*	sampled many times	sampled many times
	Senescence 2009	17.9.2009 (13.1)	18.9.2009 (12.5)
	After 2009	12.11.2009 (5.6)	12.11.2009 (5.6)
	Before 2010	3.5.2010 (7.2)	3.5.2010 (7.2)
	Young 2010	3.6.2010 (14.1)	4.6.2010 (12.6)
	Flowering 2010*	sampled many times	sampled many times
	Senescence 2010	8.9.2010 (14.6)	8.9.2010 (14.6)
	After 2010	11.9.2010 (4.2)	11.9.2010 (4.2)

5

^{13}C **pulse-labeling** assessment of the community structure of **active fungi** in the rhizosphere of a genetically starch-modified potato cultivar and its parental isoline

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Chapter 5

Summary

1. The aim of this stugy was to gain understanding of the carbon flow from the roots of a genetically modified amylopectin-accumulating potato cultivar and its parental isoline to the soil fungal community using stable isotope probing (SIP).

2. The microbes receiving ^{13}C from the plant were assessed through RNA/PLFA-SIP at three time points (1, 5 and 12 days after the start of labeling). The communities of *Ascomycota*, *Basidiomycota* and *Glomeromycota* were analysed separately with RT-qPCR and T-RFLP.

3. Ascomycetes and glomeromycetes received carbon from the plant already 1 and 5 days after labeling while basidiomycetes were slower in accumulating the labeled carbon. The rate of carbon allocation by the GM-variety differed from its parental variety thereby affecting the soil fungal communities.

4. We conclude that both saprotrophic and mycorrhizal fungi are rapidly metabolizing organic substrates flowing from the root into the rhizosphere, that there are large differences in utilization of root-derived compounds at a lower phylogenetic level within investigated fungal phyla and that active communities in the rhizosphere differ between GM-plant and its parental cultivar through effects of differential carbon flow from the plant.

5.1. Introduction

It has been estimated that 20 to 50 % of the carbon obtained by the plants via photosynthetic assimilation is transferred to the roots and about half of this is further released into the soil (Kuzyakov and Domanski, 2000). This release of exudates is strongly affecting the soil microbial composition and activity close to the roots giving rise to so called rhizosphere effect (Lynch and Whipps, 1990; Jones et al., 2009). Whereas the rhizosphere effect has mostly been studied for bacteria, an increasing number of studies point at the importance of fungi in metabolizing root-derived organic compounds (Buée et al., 2009). In a previous study, we described the community dynamics of saprotrophic fungi in the rhizosphere of potato cultivars in intensively managed agricultural soils (chapter 3). Contrary to the expected low abundance and activity of saprotrophic fungi in intensively managed soils (Van der Wal et al., 2006) , we found that fungi made up a significant part of the rhizosphere microbial biomass, especially during the flowering and senescent stages.

Many approaches have been used to monitor the response of the rhizosphere microbial communities to root exudates (Kuzyakov and Domanski, 2000). One method that has proven very useful is the application of different carbon isotopes in tracking 13C in cellular components (e.g. lipids and nucleic acids) to determine which functional groups actively assimilate ^{13}C labeled substrates (Boschker et al., 1998 ; Radajewski et al., 2000; Manefield et al., 2002).

Use of phospholipid fatty acid analyses in combination with stable isotopes (PLFA-SIP) has indicated that fungi are a very important group of organotrophic organisms in the rhizosphere and even inside roots receiving considerable amount of plant derived carbon (Butler et al., 2003; Lu et al., 2004; Wu et al., 2009; Gschwendtner et al., 2011). In addition, fungi are known to respond rapidly to addition of easily degradable substrates such as root exudates (Broeckling et al., 2008; De Graaff et al., 2010). Unfortunately, the use of PLFA-SIP does not give information on the identity of the active fungi. It is known that the diversity of fungi in soils is enormous and the functions range from obligate mutualists (*Glomeromycota*), to saprobes and pathogens (*Ascomycota* and *Basidiomycota*), all being very important in rhizosphere (Carlile et al., 2001; Buée et al., 2009). All three fungal phyla are influenced by the plant in one way or another but the relationships of individual taxa or even species is not known (Christensen, 1989; Broeckling et al., 2008; Buée et al., 2009).

Due to high variation in rhizodeposition patterns between different plant species, it can be assumed that genetic modification in plants, especially if the modification is targeting carbon related compounds, could result in a change in carbon allocation patterns and thus may give rise to shifts in abundance of fungal species. It has been reported that carbon allocation within plants is strongly regulated by genotype and stage of development. Several manuscripts (Milling et al., 2004; Götz et al., 2006; Griffiths et al., 2007; O'Callaghan et al., 2008; Weinert et al., 2009) have provided information on the effects of transgenic crops on soil bacterial and fungal communities but only few have addressed the question from the carbon partitioning

perspective (Wu et al., 2009; Gschwendtner et al., 2011).

The aim of this study was to identify and compare fungal communities actively assimilating root exudates of a GM-potato (Solanum tuberosum L.) 'Modena' with modified starch metabolism and its parental variety 'Karnico' cultivated in the same soil by applying both RNA-SIP and PLFA-SIP to the ^{13}C labeled plants. As this particular modification is targeting a biosynthetic pathway, it was hypothesized that this could also result in changes in composition of rhizodeposition and of rhizosphere microbial communities . The main focus of the study was to improve our understanding of the relationship between plants and different fungal phyla, namely *Ascomycota*, *Basidiomycota* and *Glomeromycota*, in the rhizosphere and to assess how the GM-trait would affect these relationships.

5.2. Materials and methods

5.2.1. Greenhouse experiment and ^{13}C labeling

A greenhouse experiment was performed comparing a genetically modified potato line ('Modena') with altered starch quality used for industrial purposes with its parental isoline ('Karnico'). The altered starch composition was created by complete inhibition of the production of amylose via introduction of a RNAi construct of the granule-bound starch synthase gene inhibiting amylose formation, which yields pure amylopectin (de Vetten et al., 2003). The soil used for the experiments was collected from a Dutch agricultural field (field VMD in chapters 3 and 4) after the growing season of 2009. The soil was a sandy peat soil with the following characteristics: silt fraction 2.8 %, sand fraction 94.3 %, organic matter content 25 g 100 g-1 dry soil, pH 5.0. The soil was homogenized and sieved (< 2mm) and transferred to pots (volume 10 liters). One tuber of either cultivar was planted per pot and the plants were grown in the greenhouse until they reached the phenological stage of senescence (EC90) (Hack et al., 2001). This stage was selected because in an earlier field experiment it was shown that at this stage the highest abundance of fungal biomass in the rhizosphere occurred and the differences between the modified cultivar and its parental variety were most pronounced (chapter 3). The day- night period was set at 16/8 and maximum daily temperature was around 22 ^{o}C. Triplicate pots with soil but without plants (bulk soil) were incubated under the same conditions and used as controls of possible accumulation of labeled carbon by fungi without presence of a plant.

Twelve plants of each cultivar and two bulk soil pots were labeled with 99.99 atom-% $^{13}CO_2$ (Cambridge Isotope Laboratories, Andover, MA, USA) in an artificially lit air-tight growth chamber for a total of 30 hours. The same number of plants was placed in a similar chamber and kept under identical conditions but with a $^{12}CO_2$ atmosphere, representing the control treatment. The CO_2 concentrations in the chambers were monitored through-out the experiment. Prior to the start of the labeling the plants were allowed to assimilate carbon until the CO_2 concentration fell to 200 µl l^{-1}. During this period the photosynthetic rate was determined. When the CO_2 concentration of 200 µl l^{-1} was reached, $^{13}CO_2$was injected into the chamber

using a gas tight pumping system until the CO_2 concentration reached 380 ppm. During the labeling period additional $^{13}CO_2$ was injected when the concentration fell below 350 ppm. The plants were labeled during two intervals of 12 hours in the light, interrupted by 6 hours of non-labelling in the darkness during which no $^{13}CO_2$ was added and excess CO_2 was removed. Thus, in total, the plants were labeled for 24 hours in the light. The total amount of $^{13}CO_2$ added to the chamber was 25 liters.

5.2.2. Harvest

After the labeling period all pots were removed from the chambers and rhizosphere soil of 3 replicate plants per cultivar was immediately harvested from both the $^{13}CO_2$ and $^{12}CO_2$ treatment. The rhizosphere soil was collected by brushing the roots and immediately frozen in liquid nitrogen and kept in -80 ^{o}C prior to nucleic acid extractions. Bulk soil samples (soil not adhering to roots) were also taken and treated similarly. Part of the soil samples (both rhizosphere and bulk soil) were kept separate, frozen and freeze dried before lipid fatty acid analyses.

Shoot, leaves, roots and tubers were collected, weighted and tuber production was estimated. Representative samples of plant parts were frozen, freeze dried and kept in -80 ^{o}C until further analyses. The same harvesting procedure was repeated 5 and 12 days after the end of the labeling period to monitor the carbon flow in time. These sampling dates were selected based on previous studies (Drigo et al., 2010).

5.2.3. ^{13}C content in different parts of the plant

Freeze-dried plant parts were grinded to mesh size 0.1 µm. The $\delta^{13}C$ value of these samples was analyzed using an elemental analyzer coupled to an isotope ratio mass spectrometer (Thermo Finnigan) to determine the amount of photosynthates allocated to above- and belowground parts.

The incorporation of ^{13}C into plants was expressed as the increase of $\delta^{13}C$ value relative to the $\delta^{13}C$ values of unlabeled control plants (δ ^{13}C values). Isotope ratios and atom% of ^{13}C were calculated using the equations described earlier (Werner and Brand, 2001). Vienna PeeDee Belamnite (V-PDB) was used as reference material.

5.2.4. PLFA analyses of the soil

PLFAs were extracted, and concentrations and $\delta^{13}C$ values were measured on a Finnigan Delta-S gas chromatograph - isotope ratio monitoring mass spectrometer (GC-IRMS) as described in (Boschker, 2004). The internal standard methyl nonadecanoate fatty acid (19:0) was used for calculating concentrations. The following fatty acids were used as biomarkers for bacterial biomass: i14:0, i15:0, a15:0, i16:0, 16:1ω7t, 17:1ω7, a17:1ω7, i17:0, cy17:0, 18:1ω7c and cy19:0 (Mauclaire et al., 2003). PLFA10Me16:0 was used as specific indicator for actinomycetes (Frostegård et al., 1993). PLFA 18:2ω6.9 was considered as an indicator for fungal biomass (Bååth, 2003; Bååth and Anderson, 2003). . Unfortunately, the NLFA extractions were not successful and we could not relate the NLFA marker with the PLFA marker. Thus, the PLFA 16:1ω5 which is found mainly in AMF fungi and that often correlates with the

corresponding NLFA, was used as an indicator of AMF (Olsson et al., 1995; Drigo et al., 2010). PLFA 20:4ω6 was used to assess the amount of ^{13}C incorporated to protozoan biomass (Mauclaire et al., 2003). The percentage of ^{13}C allocated to a certain PLFA was calculated from the amount of ^{13}C in each PLFA and total ^{13}C accumulation (excess ^{13}C pmol g-1) in all PLFAs used as biomarkers for different microbial groups and these values were used in data analyses.

5.2.5. RNA extraction and gradient fractionation

Total nucleic acids were co-extracted from 400 mg of frozen rhizosphere and bulk soils following the protocol given by Griffiths et al. (2000). RNA was retrieved by treating the total nucleic acids with DNAse (Turbo DNAse, Ambion), inspected for its integrity using the Experion RNA StdSens Analysis System (ExperionTM, Bio-Rad Laboratories Inc., the Netherlands) and stored at -80 ^{o}C. Total RNA was quantified using a NanoDrop ND-1000 Spectrofotometer (Bio-Rad Laboratories Inc., the Netherlands). ^{13}C-enriched RNA was separated from non-labeled RNA by density-gradient centrifugation and analysed as described in Manefield et al. (2002). 500 ng of RNA was used per sample and 20 fractions (of 100 µl) of the developed density gradient were collected after centrifugation. The fractionated RNA was combined into samples called 'heavy' (densities ≥ 1.82 g ml^{-1}) and 'light' (densities ≤1.78 g ml^{-1}) based on the presence of nucleic acids (measured with NanoDrop) in desired densities, the first one containing fractions with ^{13}C-enriched RNA and latter fractions containing unlabelled ^{12}C RNA. The ^{12}C labeled plants were used as controls and analysed as the ^{13}C labeled plants.

5.2.6. RT-qPCR and T-RFLP

The 'light' and 'heavy' fractions were separately reverse transcribed using random hexamers (0.2 µg μl^{-1}) according to the manufacturer's protocol (RevertAidTM First Strand cDNA synthesis Kit, Fermentas). The cDNA produced was further used to quantify the ITS region of basidiomycetes and ascomycetes by real-time PCR using ABsolute QPCR SYBR green mix (AbGene, Epsom, UK) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) with primers presented in table 1. All samples were analyzed in at least two different runs to confirm the reproducibility of the quantification. Standard curves were prepared from ITS DNA isolated from purified plasmids and exhibited a linear relationship between the log of the ITS copy number and the calculated threshold (Ct) value ($R^2 > 0.98$). The plasmid DNAs were run as triplicates per dilution in each run and further used to calculate the number of ITS copies in the samples.

Terminal restriction fragment length polymorphism (T-RFLP) was used as a fingerprinting method to assess the diversity and community composition of *Ascomycota*, *Basidiomycota* and *Glomeromycota* (AMF) also from the same cDNA. T-RFLP was done using primers and conditions presented in table 5.1 and restriction was done like in chapter 2.

In order to identify specific OTUs which cause the differences between the samples, clone libraries were created for all three fungal groups. PCR products of

'heavy' and In order to identify specific OTUs which cause the differences between the samples, 'light' fragments were purified with Qiaqen PCR purification kit and pooled per treatment after purification. The pooled fragments were cloned into Escherichia coli JM109 using the pGem-T Easy System II cloning kit (Promega, UK) with a vector : insert ratio of 3:1. Approximately 50 successful transformants per time and fragment i.e. 'heavy' and 'light' were selected for amplification, restriction digest and identification with labeled primers as described in table 1. The clones producing unique fragments with both restriction enzymes were amplified using vector-based M13 primers and sequenced. Selected plasmids were isolated using a plasmid mini kit (Qiaqen, Valencia, CA) according to manufacturer's instructions and further used for qPCR analyses.

Table5.1. Used primers , PCR conditions and enzymes used for restriction analyses

Target	Primers	PCR conditions	Restriction enzymes used	Reference
Ascomycota	ITS1F: CTT GGT CAT TTA GAG GAA GTA A ITS4a: CGC CGT TAC TGG GGC AAT CCC TG	95°C 5 min, 35 cycles of (95 °C 15s, 62°C 30s, 72°C 90s), 72°C for 10 min	HaeIII, HinfI	Gardes & Bruns, 1993 Larena et al., 1999
Basidiomycota	ITS1F: CTT GGT CAT TTA GAG GAA GTA A ITS4b:CAG GAG ACT TGT ACA CGG TCC AG	95°C 5 min, 35 cycles of (95 °C 15s, 55°C 30s, 72°C 90s), 72°C for 10 min	HaeIII, HinfI	Gardes & Bruns, 1993
Glomeromycota	1st LR1: GCATATCAATAAGCGGAGGA FLR2: GTCGTTTAAAGCCATTACGT	95°C 5 min, 35 cycles of (95 °C 30s, 58°C 30s, 72°C 70s), 72°C for 10 min		
			AluI, MboI	Gollotte et al., 2004
	2nd FLR3: GTT GAA AGG GAA ACG RTT RAA G FLR4: ATTACGTCAACATCCTTA	95°C 5 min, 27 cycles of (95 °C 30s, 56°C 40s, 72°C 60s), 72°C for 16 min		

5.2.7. Data analyses

Data on ^{13}C enrichment in plant parts, PLFA data, diversity and richness of fungi and copy numbers of ascomycetes and basidiomycetes were analyzed using univariate regression within the general linear mode (GLM) procedure in statistical program PAST (Hammer et al., 2001). The assumption of normality was tested with Shapiro-Wilk statistics and homogeneity of variances was assessed with Levene's test. Differences between time points and cultivars were tested for significance with Tukey's HSD test, or, when variances were unequal, with Tamhane's T2 test. All the statistics were done with original non-transformed values.

The quality of T-RFLP data was first visually inspected in GeneMapper Software v4.1 (Applied Biosystems, Carlsbad, CA) and then transferred to T-Rex (Culman et al., 2008). True peaks were identified as those of which the height exceeded the standard deviation (assuming zero mean) computed over all peaks and multiplied by two (Abdo et al., 2006).

Although the number of TRFs obtained with different restriction enzymes and labels correlated (spearman 2-tailed <0.01), the lowest value of the four restriction enzyme – primer combinations was used for further analyses to exclude false positives and diversity was calculated from that. Moreover, any peak occurring only once (not found in replicates or different fraction) was deleted from further analyses. Non-Metric Multidimensional Scaling (NMDS) with Jaccard as distance measure was used to assess the similarity of the fungal communities in the different fractions and between the cultivars. The effect of the treatments was tested using one- or two-way ANOSIM with Jaccard as a distance measure. Only presence-absence data were used.

The assignment of peaks (TRFs) to OTUs was performed in the statistical computing environment R using the T-RFLP Analyses Matching Program (TRAMP-R) (Fitzjohn and Dickie, 2007). Three out of four of the enzyme / primer combinations within 1.5 bp margin had to be met in a sample for it to be assigned to an OTU. The diversities of OTUs, assigned to classes and orders and the TRF data were compared with Shannon-Weaver H' diversity index and diversity t-test was used to compare diversities. All statistics were done in statistical program PAST (Hammer et al., 2001).

The PLFA ^{13}C-labeling data was evaluated with Principal Component Analyses (PCA) and multivariate analysis of variance (MANOVA) was used to determine the overall effects of time and cultivar on mole percentages and ^{13}C values of PLFAs compared to the controls.

5.3. Results

5.3.1. ^{13}C enrichment in potato plants and rhizosphere microbes

During the incubation in a $^{13}CO_2$ atmosphere a steady consumption of CO_2 was measured by the automatic monitoring system which coincided with a detectable amount of ^{13}C in the plant parts and in the rhizosphere microbes (Figure 5.1). The ^{13}C values in the control plants were in a normal range (in average $\delta^{13}C$ -28 ‰). The amount of labeled carbon in the roots was highest at the first sampling (Fig. 5.1). This indicates a rapid flux of labeled carbon into the rhizosphere in very early stages of the experiment. After the first sampling time, the amount of labeled carbon got diluted by ongoing photosynthesis and 12 days after labeling only 35 % (significantly less after 12 days than right after labeling, $F=4.24$, $P<0.05$) of carbon (16 % in leaves and 37 % in roots) was left in the plant tissues. At the last sampling point (12 days after labeling) most of the carbon allocated below-ground was detected in the potatoes and this amount was significantly ($F=7.37$, $p<0.05$) higher after 12 days than right after labeling. After 5 days of labeling there was a difference between cultivars, but the ^{13}C data of Karnico did not fit in the pattern of other harvests and might, thus not be reliable (data not shown).

Directly after labeling the ^{13}C content of the GM and its parental cultivar did not differ significantly neither in their total plant biomass nor for any of the plant

parts. Analysis of 13C enrichment in PLFAs in the rhizosphere showed that most of the label accumulated in 18:2ω6.9, which is commonly used as a fungal biomarker (Fig. 5.2). Total ^{13}C in below ground parts of the plant was positively correlated with amount of label in the AMF marker 16:1ω5 ($r = 0.64$, $p<0.001$) and the amount of label in the fungal marker 18:2ω6.9 was positively correlated with amount of label in root samples ($r=0.68$, $p<0.001$) and in 16:1ω5 marker ($r=0.70$, $p<0.001$). Further,

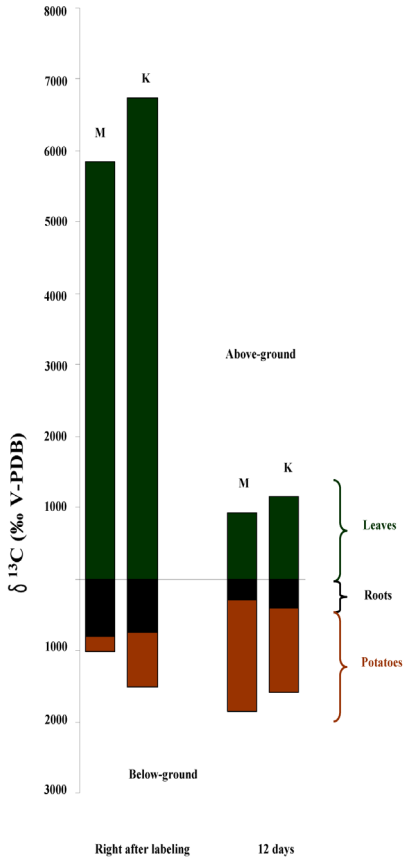

Figure 5.1. Distribution of ^{13}C in potato plants and rhizosphere microbes. The ^{13}C content in different parts of labeled potato plants is expressed as excess compared to non-labeled control plants harvested at the same time and separated in above-ground parts (leaves and stem combined) and below-ground parts (roots and potatoes). The first columns represent the GM-variety 'Modena' and second columns its parental isoline 'Karnico'. The natural isotopic signatures of the control plants were the same for both cultivars (average δ13C -28 ‰).

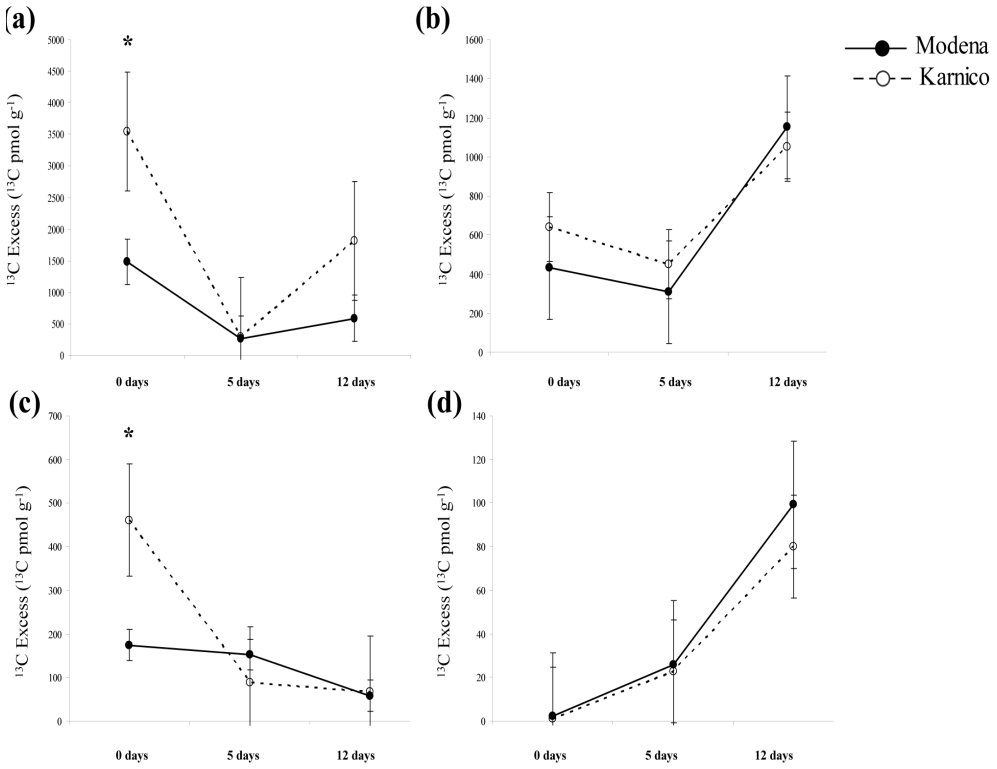

Figure 5.2. The amount of excess ^{13}C in different microbial groups as measured by PLFA analyses. The incorporation of ^{13}C into the markers was calculated for (a) fungi, (b) bacteria, (c) AMF and (d) protozoa based on markers specific to these groups mentioned in text in three time points. The closed symbols represent the GM-variety 'Modena' and open symbols its parental cultivar 'Karnico'. PLFAs used as indicator for the different microbial groups are given in the material & methods section. Note that all axis are different and ordered from highest to lowest.

the amount of labeled carbon in PLFA markers 18:2ω6.9 and 18:1ω9 positively correlated ($r=0.98$, $p<0.005$) with each other but not with any other markers (Fig. 5.3b). No excess ^{13}C was detected in the PLFAs from plants treated with ^{12}C or in the pots with only bulk soil subjected to ^{13}C labeling.

Five days after labeling, total bacterial PLFAs contained more or less the same amount of ^{13}C as fungal PLFAs. At the last sampling point (12 days), fungal PLFAs contained again more ^{13}C than bacterial PLFAs in the rhizosphere of 'Modena' but not in that of 'Karnico'. The total enrichment of ^{13}C at the first sampling was higher in rhizosphere PLFAs of cultivar 'Karnico' than of 'Modena' (Fig. 5.2). However, this difference appeared to be caused by a higher accumulation of ^{13}C in

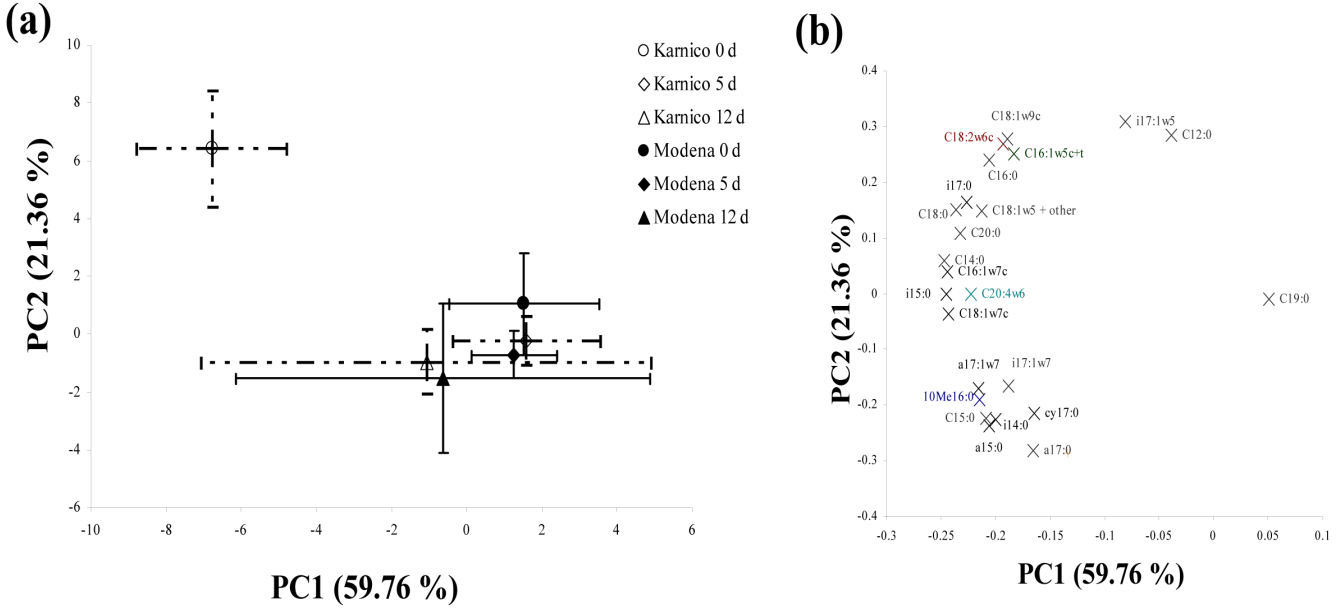

Figure 5.3. PCA analyses of the labeled PLFA (excess ^{13}C pmol g^{-1}) patterns of the rhizosphere of both cultivars at all time points (a) and PLFAs explaining this pattern (b). The closed symbols and solid variances represent the GM-variety 'Modena' and open symbols and dotted line parental cultivar 'Karnico'. The variance is based on triplicates of each treatment. The bacterial PLFAs explaining the patterns are marked with black, fungal marker with red, AMF with green, actinomycetes with blue and non-identified with grey markers. For grouping of the PLFAs, see text.

fungal PLFAs (F=7.098, p=0.04) but not for any other group. In the rhizosphere of both cultivars the amount of ^{13}C in bacterial PLFAs was similar for the first two sampling periods but increased 12 days after labeling (Fig 5.2). The heaviest labeling of bacterial PLFAs was observed for two Gram-negative markers (16:1ω7t and 18:1ω7c) (data not shown). Protozoan and actinomycetal PLFAs had the highest labeling at later stages (data not shown.). There were no differences in the ^{13}C in protozoan or actinomycete PLFA markers in the rhizosphere soil of 'Karnico' compared to 'Modena'.
Similarly, the PCA of labeled PLFAs of rhizosphere microbes revealed a difference between growth stages and at the first time point also between the GM- and parental variety (Fig. 5.3). Based on MANOVA of the eigenvalues, there were no significant temporal effects on the overall PLFA labeling profiles for both cultivars (Wilks' lambda = 0.629, p>0.05), and there were no overall differences between the cultivars (Wilks' lambda = 0.93, p>0.05). The only significant effect of cultivar on PLFAs was directly after labeling (Wilks' lambda = 0.053, p<0.05) which could be explained by different labeling of the fungal specific marker (18:2ω6,9) and AMF marker (16:1ω5) (Fig. 5.3b).

5.3.2. Ascomycota and Basidiomycota receiving carbon from the plant

The total number of ITS copies in the ^{13}C-enriched RNA fractions was positively correlated with the labeling of PLFA 18:2ω6.9 (R=0.82, p<0.05). The number of ITS copy numbers in ^{13}C-enriched RNA fractions extracted from the rhizosphere 1 day after labeling were, ten times higher for *Ascomycota* than for *Basidiomycota*. Furthermore, for Modena copy numbers of *Ascomycota* and *Basidiomycota* showed opposite temporal patterns (Fig. 5.4). There were no significant differences in total ('heavy'

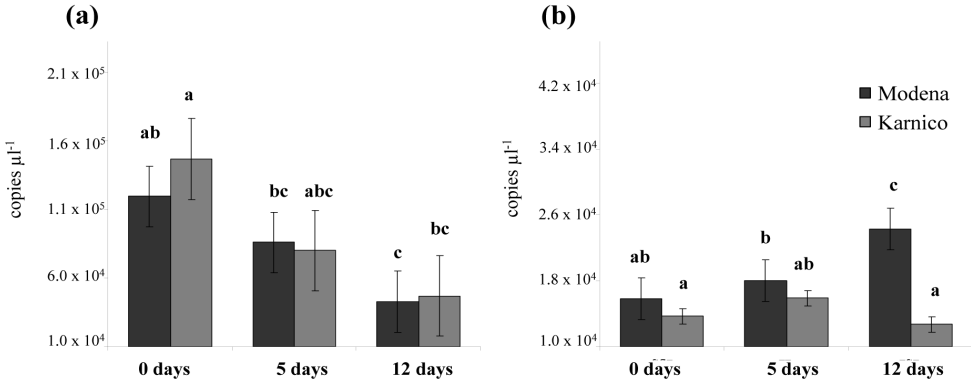

Figure 5.4. ITS copy numbers of (A) *Ascomycota* and (B) *Basidiomycota* in the heavy fraction at different time points after labeling. The first bars represent the genetically modified cultivar 'Modena' and second bar its parental cultivar 'Karnico'. Letters above bars indicate significant differences at the level of P<0.05. Note that axis of A and B are not the same.

and 'light' fraction combined) numbers of fungal ITS copies between measuring times or cultivars (data not shown). Furthermore, there were no differences in the total ITS copy numbers between the ^{13}C labeled and control plants and no ITS copies were detected in the 'heavy' fraction of control plants thus confirming that the ^{13}C enrichment of fungi was real.

There were no significant differences between cultivars in ^{13}C-enriched ascomycetal ITS copy numbers at any time point (Fig. 5.4a). The decrease in the labeled ITS copy numbers of ascomycetes with prolonged sampling time correlated with the amount of labeled carbon in the roots ($r=0.77$, $p<0.05$). The percentage of total ascomycete copies in the 'heavy' fraction was 70 % and 81 % right after labeling, 56 % and 49 % after 5 days and 28 % and 27 % after 12 days for 'Modena' and 'Karnico, respectively.

The ^{13}C-enriched ITS copy numbers of basidiomycetes did neither reveal significant differences between cultivars for the first two sampling time points or if all time points were combined (Fig. 4b). There was, however, a difference at the last time point (12 days) when the GM cultivar 'Modena' had more labeled basidiomycetal ITS copy numbers in its rhizosphere than 'Karnico' ($F=18.7$, $p<0.05$). The percentage of ^{13}C-enriched copies of basidiomycetes compared to total number of copies ranged from 35 % to 51 %.

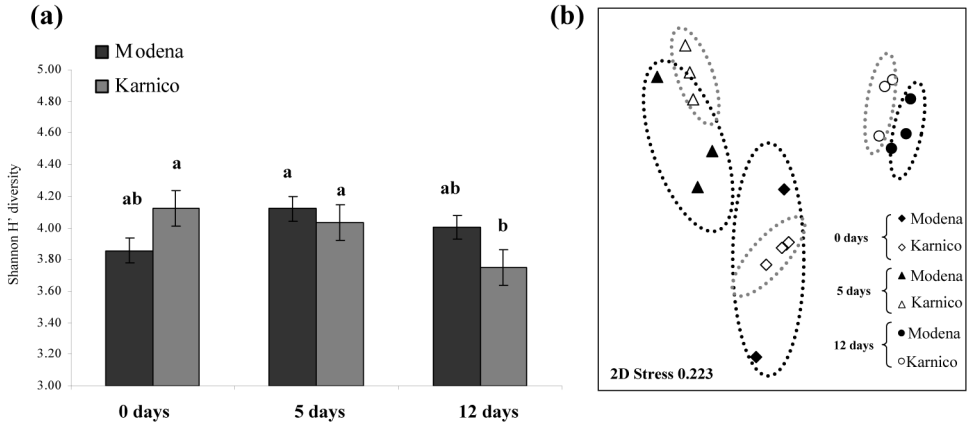

Figure 5.5. Diversity (a) and community structure (b) of all active (labeled RNA pool) fungal groups combined. (a) Black bars represent average diversity (n=3) (±SD) of fungi in the rhizosphere of 'Modena' and gray bars average diversity (±SD) in the rhizosphere of 'Karnico' at three different time points after 13CO2 pulse-labeling. Letters above bars indicate significant differences in diversity (diversity t-test) at the level of $P<0.05$. (b) In the NMDS plot the open symbols represent the parental variety and closed symbols the GM-variety. Circles around samples are distinct cultivar and time combinations.

Table 5.2. Diversity of TRFs and identified fungal OTUs (at different taxonomic levels) in the heavy RNA fraction right after labeling and after 5 days1, 5, and 12 days after labeling and in the light fraction (combined). Total fungi is calculated by combining the three phyla. The letters behind numbers in level of TRFs and OTUs indicate significance at level $p<0.05$. The OTUs are assigned into orders as presented in table 5.3.

		Right after labeling		5 days after labeling		12 days after labeling		Light Fractions	
		Karnico	Modena	Karnico	Modena	Karnico	Modena	Karnico	Modena
Total	Shannon - H' TRFs	3.86ab	4.12a	4.12a	4.03a	4.00ab	3.75a	4.64	4.65
	Number of TRFs	55	63	74	58	60	49	103	106
	Shannon - H' OTUs	3.33a	3.33a	3.78b	3.56ab	3.37a	3.18a	3.85	3.95
	Number of OTUs	28	28	44	35	29	24	47	52
	Shannon - H' orders	2.35	2.32	2.66	2.70	2.49	2.38	2.59	2.69
	Number of orders	14	15	19	18	14	13	19	20
	Shannon - H' classes	1.94	1.96	1.99	1.72	1.42	1.29	1.86	1.92
	Number of classes	9	10	11	10	7	6	11	11
Ascomycota	Shannon - H' TRFs	3.05ac	3.37ab	3.55b	3.37ab	2.71c	2.71c	3.53	3.65
	Number of TRFs	21	29	35	29	15	15	34	40
	Shannon - H' OTUs	2.77ab	2.30a	3.14a	2.94b	2.08c	2.30c	2.83	2.94
	Number of OTUs	16	10	23	19	8	10	17	19
	Shannon - H' orders	1.98	1.90	2.19	2.28	1.61	1.81	1.95	2.12
	Number of orders	10	9	12	12	6	8	10	11
	Shannon - H' classes	1.40	1.17	1.11	1.25	0.64	0.84	1.00	1.21
	Number of classes	6	5	5	6	3	4	5	5
Basidiomycota	Shannon - H' TRFs	2.83a	2.83a	2.77a	3.09ab	3.18b	3.14b	3.56	3.58
	Number of TRFs	17	17	16	22	24	23	35	36
	Shannon - H' OTUs	1.95a	2.08a	2.34ab	2.64b	2.77b	2.63b	2.94	3.00
	Number of OTUs	7	8	11	14	16	14	19	20
	Shannon - H' orders	0.87	0.80	1.15	1.47	1.71	1.57	1.49	1.54
	Number of orders	3	3	4	5	6	5	6	6
	Shannon - H' classes	0.64	0.60	0.69	0.66	0.60	0.62	0.69	0.68
	Number of classes	2	2	2	2	2	2	2	2

Table 5.2. continues

		Right after labeling		5 days after labeling		12 days after labeling		Light Fractions	
		Karnico	Modena	Karnico	Modena	Karnico	Modena	Karnico	Modena
Glomeromycota	Shannon - H' TRFs	2.34a	2.75a	2.55a	1.75b	2.78a	1.77b	3.53	3.40
	Number of TRFs	17	17	23	7	21	11	34	30
	Shannon - H' OTUs	1.61ab	2.30b	2.30b	0.69a	1.61ab	n.d.	2.34	2.57
	Number of OTUs	5	10	10	2	5	0	11	13
	Shannon - H' orders	0.00	0.64	0.85	0.00	0.50	n.d.	0.76	0.72
	Number of orders	1	3	3	1	2	0	3	3
	Shannon - H' classes	0.00	0.64	0.85	0.00	0.50	n.d.	0.71	0.68
	Number of classes	1	3	3	1	2	0	3	3

The classes investigated were (orders included): *Deuteromycota* (unassigned), *Dothideomycetes* (*Capnodiales* and *Pleosporales*), *Eurotiales (Eurotiomycetes*), *Leotiomycetes (Helotiales* and *Thelebolales*), *Sordariomycetes (Chaetothyriales, Hypocreales, Magnaporthales, Microascales, Phyllacorales, Sordariales* and *Xylariales), Ascomycota* incertae sedis, *Agaricomycetes (Agaricales, Cantharellales, Corticiales, Hymenomycetales, Polyporales* and *Trechisporales), Mitosporic Agaricomycotina, Tremellomycetes (Tremellales), Diversiporales (Acaulosporales* and *Diversiporales), Glomerales (Glomerales*) and *Paraglomerales (Paraglomerales).*

5.3.3. Diversity and community structure of Ascomycota, Basidiomycota and Glomeromycota active in the rhizosphere

The number of fungal OTUs in the heavy RNA fraction ranged from 49 to 74 and Shannon H' diversity ranged from 3.75 to 4.12 (Fig. 5.5a, table 5.2). There were no significant differences between the cultivars although the diversity in the ^{13}C fraction was lower in the rhizosphere of 'Modena' 12 days after labeling compared to 'Karnico' ($t=1.68$, $p=0.09$). This was mainly due to decrease in the diversity of *Basidiomycota* and *Glomeromycota*. The diversity of identified OTUs at all levels corresponded well to the diversity of TRFs. Significant differences in fungal community structure between the cultivars were also detected after 12 days but not at the earlier sampling dates compared to sampling dates (Fig. 5.5b). The fungal community structure in the heavy fractions differed significantly (ANOSIM: $R= 0.977$, $p<0.001$) in time (Fig. 5.5b) and between all time points ($R>0.92$ and $p<0.005$).

There were no significant differences between cultivars or harvest times in total number of ascomycetal TRFs, i.e. when light and heavy fractions were combined. The diversity of ascomycetes ranged from 2.71 in fraction labeled with ^{13}C sampled after 12 days to 3.55 on day 5 (Fig. 5.6a). There were no differences in diversity between cultivars at any time point. Directly after labeling 20 TRF types under 'Modena' and 29 under Karnico' had already received labeled carbon and incorporated it into their RNA corresponding to diversity levels of 3.05 and 3.17 (table 5.3). Five days later 'Modena' and 'Karnico had 29 and 35 TRFs active in their rhizosphere, respectively, of which 11 (for Modena) and 13 (for Karnico) were the same as at day 0. The community structure of active ascomycetal OTUs was significantly

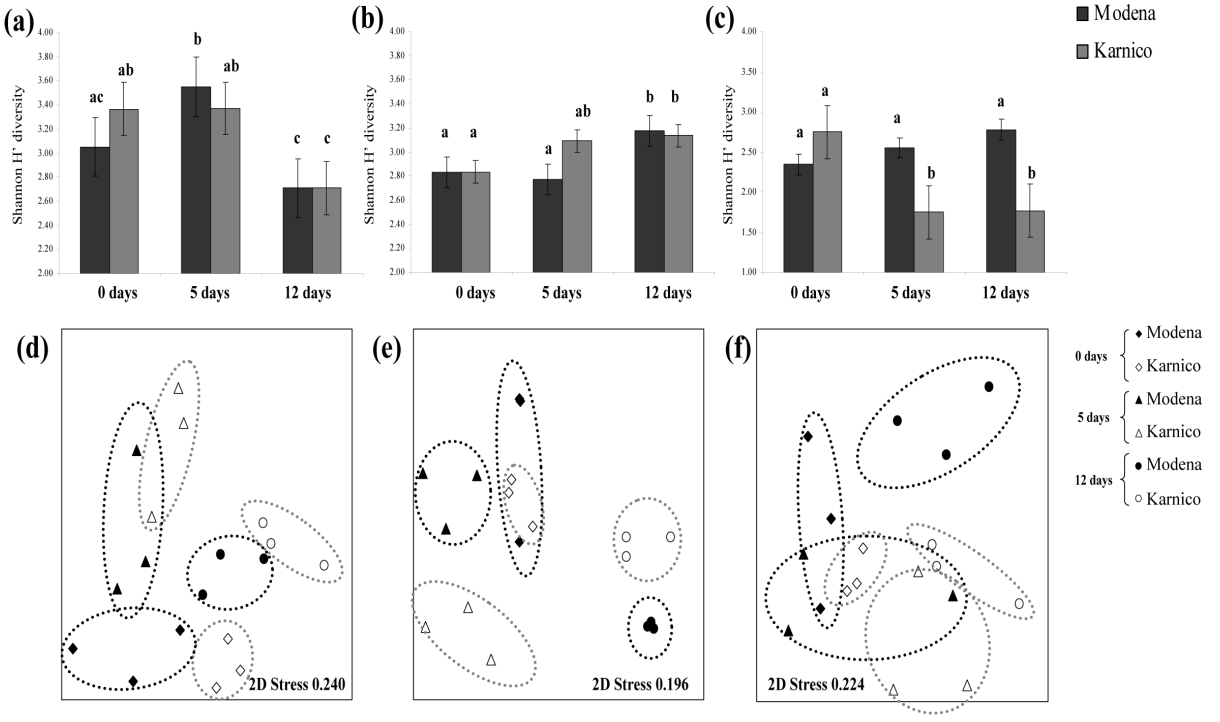

Figure 5.6. Diversity (a-c) and community structure (d-e) of *Ascomycota* (a & d), *Basidiomycota* (b & e) and *Glomeromycota* (c & f) in the ^{13}C-enriched fraction of RNA extracted from potato rhizosphere soil. Bars represent average diversity (n=3) (±SD) of (a) ascomycetes, (b) basidiomycetes and (c) glomeromycetes in the rhizosphere of 'Modena' (black bars) and 'Karnico' (gray bars) at three measuring points. Letters above bars indicate significant differences at the level of $P<0.05$. In the NMDS plots of (d) ascomycetes, (e) basidiomycetes and (f) glomeromycetes different symbols represent different time points and colors different cultivars. Circles around samples represent distinct cultivar and time combinations

different between the time points (ANOSIM: R=0.5, p<0.001) (Fig. 5.6d). Although number of active ascomycetal OTUs did not differ significantly between cultivars, the community structure did, (ANOSIM, R>0.5, p<0.05) at time points 0 and 12 days after labeling (Fig. 5.6d).

Not only were there less copies of *Basidiomycota* than *Ascomycota*, the diversity of basidiomycetes in the ^{13}C fraction was also lower with around 20 TRFs of which about half could be identified (table 5.2). The basidiomycete diversity increased with sampling time (Fig. 5.6b). The diversity of active basidiomycetes between cultivars was not significantly different overall or at any time point. Further, the community structure of active basidiomycetal OTUs was significantly affected by the sampling time (ANOSIM: R=0.98, p<0.001) (Fig. 5.6e) and cultivar. In addition, the cultivar affected the community structure in the last two sampling points s (R=0.97, p<0.001).

The glomeromycetes showed the clearest differences in diversity between the two cultivars: in the rhizosphere of the cultivar 'Karnico' the diversity of labeled AMF was higher at the last two sampling time points (t=2.99, p<0.01 and F=3.92, p<0.001) than under the genetically modified cultivar 'Modena' which had the highest diversity right after labeling (Fig. 5.6c). The AMF community in the ^{13}C fraction was less diverse in the rhizosphere of 'Modena' than in that of 'Karnico' 5 and 12 days after labeling. The community fingerprints were, however, not, significantly different between the cultivars (Fig. 5.6f) probably due to the low amount of TRFs and thus, lack of statistical power.

5.3.4. Species composition of active community

The observed differences in community fingerprints and diversities can be partially explained by differences in species identified (table 5.3). A total of 72 different OTUs could be identified from the fractions. Of these, the majority (37) were ascomycetes. Differences observed in community fingerprints between 'Karnico' and 'Modena' could be explained by labeled OTUs (i.e. 'Cap2', 'Hel1' and 'Deu3') receiving heavy carbon already after 24 hours from 'Karnico' and later also from 'Modena' and some OTUs showing the opposite ('Phy1' and 'US3'). Furthermore, some OTU types in the heavy fraction were only apparent under one of the cultivars. There were 9 OTUs found to receive labeled carbon only in the rhizosphere of 'Karnico' and four OTUs) that were found only in fraction with ^{13}C in the rhizosphere of 'Modena' (table 5.3). This might explain the observed differences in the community structure when the diversity was similar. The differences in observed OTUs composition had only minor impact at the level of orders (table 5.2).

In total 29 basidiomycetal sequences were identified from the ^{13}C fractions. In general, directly after labeling there were mostly Cryptococcus yeasts found in the heavy fractions while in later measuring times Agaricales, Cantharellales and Corticiales were dominating the community. Three OTU types ('Cor2', 'Pol2' and 'Trem1') were detected in the heavy fraction at all time points. Of these only one OTU, 'Pol1' was closely related to a known plant pathogenic species *Limonomyces roseipellis* (EU622845) while the others were closer to yeasts ('Trem1') and even to jelly rot fungi ('Cor2'). Differences detected in diversity and community structure in

the heavy fraction five days after labeling (Fig. 5.4) can be explained with delayed labeling of few OTUs ('Cor1', 'Cor3' and 'Can1') in the rhizosphere of Karnico. All of these OTUs were already detected after 5 days in the rhizosphere of 'Modena' but only after 12 days in the rhizosphere of 'Karnico'.

There were in total 13 OTUs of *Glomeromycota* identified to be active in the rhizosphere during this experiment (table 5.3). The differences seen in the diversity between 'Modena' and 'Karnico' could be explained by some Glomus OTUs ('Glo4', 'Glo7', 'Glo8' and 'Glo9', closely related to *Glomus eburneum*, *Glomus caledonium*, *Glomus geosporum* and *Glomus verruculosum*, respectively) receiving carbon from both cultivars right after the labeling but not from cultivar 'Modena' at the later stages.

5.4. Discussion

5.4.1. ^{13}C distribution in the plants

Immediately after labeling, a substantial amount of ^{13}C was already transferred to the roots (Fig. 5.1). This is in accordance with earlier findings of quick allocation of carbon into the roots by grass land species (Vandenkoornhuyse et al., 2007). No significant differences were detected in the initial amounts of labeled carbon in roots between the GM- and its parental cultivar. Similar observations were made for earlier phenological stages of the same modification (Gschwendtner et al., 2011).

5.4.2. Active microbial communities in the rhizosphere

There is evidence from stable isotope experiments that fungi are a very important group of organotrophic organisms in the rhizosphere receiving considerable amount of plant derived carbon (Butler et al., 2003; Lu et al., 2004; Wu et al., 2009). Furthermore, it has been shown that fungi can respond rapidly to addition of easily degradable substrates to soil (Broeckling et al., 2008; De Graaff et al., 2010). It was indeed confirmed by our PLFA analysis that fungi were the dominant organisms incorporating ^{13}C from the plant immediately after labeling (Fig 5.2a). There, is however a possibility that some fast growing bacteria could have already metabolized the carbon before the first sampling point of this study and thus no trace of them would be left in the PLFA fingerprints. Vanderkoornhuyse et al. (2007) showed a rapid (within 5 hours) incorporation of carbon into the RNA of endophytic bacteria but studies based on PLFAs have detected slower incorporation of the carbon into lipids (Rinnan and Bååth, 2009). We could detect that immediately after labeling the major part (>70 %) of the ^{13}C found in microbial phospholipids was found in the PLFA marker 18:2ω6.9 which is commonly used as indicator for fungi. The use of this marker as indicator of fungal biomass is often debated, but, as we saw a highly significant positive correlation between PLFA 18:2ω6.9 and active fungal ITS copy number, we conclude that this markers is a useful indicator of fungal biomass in the rhizosphere, despite the presence of living roots (Frostegård et al., 2011). Earlier studies have also shown that fungi are quickly incorporating carbon from the plants into their phospholipids (Lu et al., 2007; Wu et al., 2009; Drigo et al., 2010; Gschwendtner et al., 2011). Another large part (around 9 % of the total in the first sampling) of the total

^{13}C was detected in PLFA 16:1ω5 mainly representing AMF (Olsson and Johnson, 2005; Denef et al., 2007). This is interesting, as it has been thought that despite the importance of mycorrhiza in nutrient uptake, their importance would be minor in a high nutrient environment like intensively managed agricultural soils (Cesaro et al.,2008; Cheeke et al., 2011). Yet, results obtained from earlier developmental stages of potato showed similar results with 6.3 % of the ^{13}C allocated to the AMF specific PLFA marker (Gschwendtner et al., 2011).

Further, by using RNA based techniques we could confirm these findings as we detected ^{13}C incorporation in several fungal species immediately after the period of pulse-labeling (Fig 5.4, table 5.3). We conclude that these rapidly responding fungal species in the rhizosphere are truly plant-dependent organisms. It should be noted that we did not differentiate between rhizosphere fungal species with and without access to ^{13}C inside roots. Penetration of living roots by saprotrophic rhizosphere fungi has been reported (Harman et al., 2004). Hence, part of the allocation of ^{13}C to saprotrophic rhizosphere fungi may be independent from the rhizodepositions. In addition to the fast accumulators, we could detect another group of fungi benefiting from plant-derived carbon at later time-points after labeling and probably being able to use more recalcitrant compounds. Some (mostly Gram-negative) bacteria were also labeled immediately after the end of the aboveground labeling procedure which is in accordance with earlier studies (Wu et al., 2009; Gschwendtner et al., 2011). In this study, however, the majority of bacteria, received the labeled carbon later than fungi, possibly through fungal related exudation processes (Vandenkoornhuyse et al., 2007; Drigo et al., 2010) or due to their inability to have access to the interior of the root. The PLFA marker for protozoa (20:4ω6), not known to be able to use plant derived carbon readily, revealed delayed response to the ^{13}C addition possibly because they were feeding on labeled bacteria or fungi.

5.4.3. Active fungal communities in the rhizosphere

When root derived products enter the soil, they are rapidly metabolized and the microbial community is likely to shift in favor of those species that are able to compete for these resources (Dennis et al., 2010). The copy number calculations revealed that mostly ascomycetes, glomeromycetes and some basidiomycetal yeasts received carbon immediately released by the plant while later fungal community changed in favor of (basidiomycetal and ascomycetal) species probably better adapted to different carbon source or secondary carbon from dead plant parts or from other organisms (Lu et al., 2004; Rangel-Castro et al., 2005; Lu et al., 2007; Vandenkoornhuyse et al., 2007; Dennis et al., 2010). The carbon sources at these later stages may consist of more complex substrates e.g. sloughed-off root cells.

We could detect certain fungal orders and species that were labeled at the first sampling point but not at later stages (table 5.3). These OTUs are expected to be good competitors for simple root exudates but not for more complicated carbon sources and thus active only right after labeling. Orders typically recieving carbon right away from the plant were the basidiomycetal Tremellales and the ascomycetal Capnodiales while basidiomycetal orders Agaricales, Cantharellales, Sordariales,

Table 5.3. Distribution of identified OTUs in the rhizosphere of the GM-variety and its parental isoline at different time points (right after labeling and, 5 and 12 days after) in the heavy fraction and the commonly occurring (all time points) OTUs in the light fraction indicated as presence-absence. Closest species match (% identity) was obtained by comparison to known species GenBank using BlastN and assignment to orders is based on this similarity.

				Right after labeling		5 days after labeling		12 days after labeling		Light Fractions	
Phyla	**Name**	**Order**	**Closest species (% identity)**	**K**	**M**	**K**	**M**	**K**	**M**	**K**	**M**
Ascomycota	Cap1	Capnodiales	*Davidiella macrospora* (EU167591) (99)	X	X					X	X
	Cap2	Capnodiales	*Cladosporium cladosporioides* (AY251074) (99)	X			X				X
	Cap3	Capnodiales	*Cladosporium herbarum* (AF177734) (80)			X	X				X
	Cap4	Capnodiales	*Zasmidium nocoxi* (CQ852842) (83)	X	X	X	X			X	
	Cap5	Capnodiales	*Devriesia* sp. NG_p52 (HQ115717) (100)				X		X		
	Chae1	Chaetothyriales	*Cladophialophora chaetospira* (EU035405) (100)			X	X	X		X	X
	Chae2	Chaetothyriales	*Exophiala* sp. Ppf18 (GQ302685) (97)			X	X	X	X		
	Chae3	Chaetothyriales	Uncultured Herpotrichiellaceae (FJ554453) (98)	X	X						
	Deu1	Deuteromycota	*Tetracladium furcatum* (FJ000375) (98)			X	X			X	
	Deu2	Deuteromycota	*Stilbella fimetaria* strain MH178 (96)	X	X						X
	Deu3	Deuteromycota	*Microsphaeropsis* sp. MTFD09 (DQ132840) (99)	X		X	X		X		
	Eur1	Eurotiales	*Capronia* sp. 94003b (EU129158) (81)	X	X	X	X	X	X		
	Hel1	Helotiales	*Botryotinia fuckeliana* (EF207415) (99)	X		X	X	X			
	Hel2	Helotiales	*Meliniomyces variabilis* (EF093178) (95)			X					X
	Hyp1	Hypocreales	*Clonostachys miodochialis* (AF210674) (99)	X		X				X	X
	Hyp2	Hypocreales	*Bionectria cf. ochroleuca* (EU552110) (98)	X	X	X		X	X		
	Hyp3	Hypocreales	*Fusarium* sp. 5/97–45 (AJ279478) (97)							X	X
	Hyp4	Hypocreales	*Gibberella fujikuroi* 3 (HM165488) (100)			X	X		X		
	Hyp5	Hypocreales	*Gibellulopsis nigrescens* (HQ115693) (100)			X				X	
	Hyp6	Hypocreales	*Fusarium equiseti* (GQ50572) (100)			X					
	Hyp7	Hypocreales	*Fusarium merismoides var. merismoides* (EU860057) (100)	X	X	X	X	X	X	X	X

	Hyp8	Hypocreales	*Eucasphaeria capensis* (EU272516) (89)	x	x	x	x	x	x	x	x	x	x
	Hyp9	Hypocreales	*Nectria* sp. ASIN2 (DQ779785) (100)	x	x	x	x	x				x	x
	Hyp10	Hypocreales	*Fusarium* sp. HMA-16 (GU480953) (100)	x	x	x	x					x	x
	Hyp11	Hypocreales	*Volutella ciliata* (AJ301966) (98)	x	x				x	x		x	x
	Hyp12	Hypocreales	*Fusarium* sp. (96)	x								x	x
	IS1	Incertae sedis	*Pseudeurotium bakeri* (DQ068995) (100)									x	x
	IS2	Incertae sedis	*Pseudeurotium bakeri* strain MCJAxII (DQ529304) (99)	x	x								x
	IS3	Incertae sedis	*Leptodontidium* sp. (95)					x					
	Ma1	Mitosporic ascomycota	*Zalerion varium* (AJ608987) (98)	x							x	x	
	Mag1	Magnaporthales	*Phialophora* sp. DF36 (EU314710) (99)			x	x	x	x	x		x	
	Micr1	Microascales	*Microascaceae* sp. LM278 (EF060607) (98)	x		x					x		
	Phy1	Phyllacorales	*Plectosphaerella* sp. (96)		x	x	x				x	x	
	Pleo1	Pleosporales	Uncultured Ampelomyces clone IIP2–29 (EU516670) (98)	x									x
	Pleo2	Pleosporales	*Aff. Drechslera* MT0008 (AB199583) (99)	x		x					x	x	
	Pleo3	Pleosporales	*Dendryphion nanum* (AY387657) (98)			x	x						
	Pleo4	Pleosporales	*Coniothyrium* sp. 229 (FJ228186) (93)	x									x
	Pleo5	Pleosporales	*Pyrenochaeta* sp. ZLY-2010b (HM5955516) (90)		x								x
	Sor1	Sordariales	*Podospora minigluttinans* (FJ946483) (94)			x	x	x	x	x	x	x	x
	Sor2	Sordariales	*Podospora glutinans* (AY615208) (96)			x	x				x	x	
	Sor3	Sordariales	*Podospora* sp. (80)			x	x						
	Sor4	Sordariales	*Chaetomium* sp. 15003 (EU750691) (98)	x	x							x	
	Thel1	Thelebolales	*Thelebolus* sp. (FJ613125) (99)			x						x	
	Xyl1	Xylariales	*Sarcostroma bisetulatum* (EU552155) (80)				x			x			x
Basidiomycota	Aga1	Agaricales	*Rhodocybe mundula* (DQ089017) (98)						x	x	x	x	x
	Can1	Cantharellales	*Rhizoctonia solani* (EU730860) (97)	x		x	x		x	x	x	x	x
	Can2	Cantharellales	*Utlhatobasidium fusisporum* (AF518593) (96)							x		x	x
	Can3	Cantharellales	*Thanatephorus cucumeris* (HM625913) (98)				x		x			x	x
	Can4	Cantharellales	*Ceratobasidium* sp. FO 38200 (DQ520098) (93)			x	x		x	x	x	x	x
	Cor1	Corticiales	*Hyphodontia hastata* (DQ340311) (100)				x		x	x			
	Cor2	Corticiales	*Phlebia tremellosa* (DQ384584) (96)	x	x	x	x		x	x	x	x	x

Table 5.3. Continues

				Right after labeling		5 days after labeling		12 days after labeling		Light fractions	
Phyla	**Name**	**Order**	**Closest species (% identity)**	**K**	**M**	**K**	**M**				
Basidiomycota	Cor3	Corticiales	Hyphoderma praetermissum (AY707094) (95)				X	X		X	X
	Hym1	Hymenomycetales	Hymenochaetales sp. (FN907922) (100)				X	X		X	X
	MB3	Mitosporic agaricomycotina	*Mycotribulus mirabilis* (EF589734) (97)			X	X			X	X
	Pol1	Polyporales	*Limonomyces roseipellis* (EU622845) (96)	X	X	X	X	X	X	X	X
	Trem1	Tremellales	*Cryptococcus festucosus* (FR717832) (99)	X	X	X	X	X	X	X	X
	Trem2	Tremellales	*Cryptococcus podzolicus* (FN428889) (100)	X	X	X	X		X	X	X
	Trem3	Tremellales	*Cryptococcus* sp. (92)	X	X	X		X		X	X
	Trem4	Tremellales	*Cryptococcus terreus* (AB032649) (99)		X	X	X	X	X	X	X
	Trem5	Tremellales	*Holtermannia corniformis* (GU937753) (96)							X	X
	Trem6	Tremellales	*Trichosporon dulcitum* strain HB940 (AJ507663) (98)		X					X	X
	Trem7	Tremellales	*Cryptococcus podzolicus* (FN428938) (97)	X		X	X	X	X	X	X
	Trec1	Trechisporales	*Trechispora farinacea* (EU909231) (100)		X	X	X	X	X		X
Glomeromycota	Glo1	Glomerales	*Glomus aurantium* (FN547663) (97)		X					X	X
	Glo2	Glomerales	*Glomus* cf. *claroideum* (AY639343) (96)		X						X
	Glo3	Glomerales	*Glomus mosseae* (AY639156) (97)		X	X		X		X	X
	Glo4	Glomerales	*Glomus eburneum* (AM713413) (96)	X	X	X		X		X	X
	Glo5	Glomerales	*Glomus* sp. (93)		X	X				X	X
	Glo6	Glomerales	*Glomus versiforme* (95)							X	X
	Glo7	Glomerales	*Glomus caledonium* (Y17653) (99)	X	X	X				X	X
	Glo8	Glomerales	*Glomus geosporum* (AJ245637) (97)	X	X	X		X		X	X
	Glo9	Glomerales	*Glomus verruculosum* (AJ301858) (97)	X	X	X		X		X	X
	Para1	Paraglomerales	*Paraglomus* sp. (93)			X	X			X	X
	Para2	Paraglomerales	*Paraglomus* sp. (89)		X	X				X	X
	Acau1	Acaulosporales	*Acaulospora* sp. W4699 (FN825900) (95)			X		X		X	X
	Div1	Diversiporales	*Diversispora* sp. (95)		X						
Mycoromycotina	Muc1	Mucorales	*Rhizopus oryzae* strain CAF276 (EU399919) (95)	X		X	X				X

Magnaporthales and Chaetothyriales seemed to receive carbon only later. The presence of basidiomycetal yeasts in the rhizosphere that are able to use simple root-exudate compounds has been observed in earlier studies (Botha, 2011; Mestre et al., 2011). Although we could see this pattern at the level of orders, some of the OTUs within orders had very different functions. For instance, one OTU assigned to Cantharellales ('Can1') received heavy carbon already right after labeling while the other OTUs assigned to the same order only 5 or 12 days later. These observed differences between individual OTUs within orders points at differences between closely related species with respect to carbon resource utilization. The high amount of OTUs closely related to known decomposer species can partially be attributed to the late phenological stage at which the labeling was performed. Although no senescent leaves were allowed to drop on the soil, we could detect sequences from orders with many known decomposer species receiving labeled carbon especially 5 and 12 days after labeling (table 5.3) indicating that besides root exudates, there might be another pathway for the fungi to receive carbon, probably decomposing dead root material. (Dennis et al., 2010).

5.4.4. Effect of GM-trait on active soil microbial communities

PLFA analyses showed no overall effect of cultivar (GM versus parental cultivar) on the total amount of carbon allocated to fungi. However, differences between cultivars in ^{13}C allocation to both fungi and AMF were found at different sampling times and this was related to the amount of carbon allocated to the roots (Fig. 5.2). Furthermore, differences in basidiomycete diversity and copy numbers and AMF diversity could be detected which can be explained by the difference in the amount of carbon released from the plant and thus a difference in the speed of succession. A recent study done for the same genetic modification (although in a different soil) using PLFA markers revealed no significant differences between the GM-trait and its parental isoline in fungal biomass or the plant exudation patterns (Gschwendtner et al., 2011). However, that study was done in the earlier growth stages EC30 and EC60 while our study focused on the senescent stage EC90. This can explain the differences in results as it has been shown that amount of carbon allocated to the roots would increase with increased age of the plant and initiation of carbon storage structures (i.e. tubers in potato) (Timlin et al., 2006) making the possible differences more obvious in later growth stages. These age-dependent exudation patterns might explain the differences in outcome of earlier studies conducted on GM-plants as they have been done on different growth stages (Rossi et al., 2007; Wu et al., 2009; Gschwendtner et al., 2011) and thus confirming the importance of considering the plant phenological state when designing experiments (van Overbeek and van Elsas, 2008; Weinert et al., 2010). Indeed, it was shown that differences between this GM- and its parental variety in carbon allocation belowground and microbial communities in the field could be seen at the stage of senescence (chapter 3).

While some studies reported effects of modified crops on soil bacterial numbers (Siciliano and Germida, 1999; Dunfield and Germida, 2001), others have documented only minor or transient effects reviewed by Kowalchuk et al. (2003). A

few studies have addressed the effects of GM-crops on general fungal community structures but none have detected cultivar dependent significant differences (Milling et al., 2004; Götz et al., 2006; Hart et al., 2009). The approach of using RNA-SIP on fungal communities as tool to investigate side-effects of GM-plants is very promising as differences between GM- and parental variety could be detected. Earlier Rasche et al. (2009) investigated differences in shoot endophytic bacteria between two cultivars of potato using DNA-SIP and found cultivar related shift in bacterial communities after 4 days of labeling very similar to the differences that we observed here for soil fungal communities. In this study we could show that potato modified for differential tuber starch quality and its parental isoline differed in their carbon allocation patterns and this in turn coincided with differences in soil fungal communities. Contrastingly, by using PLFA-SIP as an indicator of microbial communities under Bt-rice and its parental isoline, Wu et al. (2009) did not find differences in ^{13}C distribution in roots or rhizosphere indicating that observed differences might be modification-dependent.

The largest differences for the three fungal phyla were seen for the diversity of active AMF especially at later sampling times (Fig. 5.6). Earlier, Vanderkoornhuyse et al. (2007) observed differences in active glomeromycete communities between plant species and explained it as a consequence of competition among colonizers occupying the same ecological niche. We took this one step further and could, indeed, detect differences in active communities between the two cultivars. Earlier, some studies done on Bt-maize isolines expressing Cry1Ab reported reduced AMF colonization (Turrini et al., 2004; Girlanda et al., 2008; Cheeke et al., 2011). In the current study the observed differences in AMF communities in the rhizosphere of the two cultivars could be explained by presence and absence of certain OTUs in the heavy fraction in the rhizosphere of only one cultivar, Karnico. Most of the OTUs were present, though, in the light fraction of both cultivars indicating differences in carbon uptake abilities of the AM species.

Although we could detect these differences in the speed of carbon flow to fungal communities under greenhouse conditions between the GM-crop and its parental isoline, caution in extrapolating these results field scale is warranted. Earlier field observations did not reveal significant differences in bacterial or fungal communities between this GM and its parental cultivar (Inceoglu et al., 2010) although differences between the two were the largest at the stage of senescence, probably due to differences in rhizodeposition (Weinert et al., 2009). Moreover, comparing the genetically modified cultivar only to its parental variety and neglecting intraspecific variation in carbon distribution can cause false significant results, especially when evaluating potential risks of GM-crops (chapter 2). Differences between variety of cultivars in their carbon allocation patterns should be investigated to strengthen the results presented here.

5.4.5. Conclusions

We conclude that both saprotrophic and mycorrhizal fungi are rapidly metabolizing organic substrates flowing from the root into the rhizosphere and that there are large

differences in utilization of root-derived compounds. Furthermore, we showed that there are differences in active fungal communities in the rhizosphere between a starch modified GM-plant and its parental isoline which are probably due to different composition of rhizodeposits. The differences in carbon allocation and microbial communities assimilating carbon between GM and its parental variety, although convincing, might not reflect long term effects in natural systems. However, the current study was especially done to show that measurements of active fungal communities may enhance the sensitivity of detection of effects exerted by GM crops which may be helpful for the evaluation of possible risks of GM-crops.

Chapter 5

6

Effect of genetic modification of potato starch on **decomposition of leaves and tubers** and on fungal **decomposer communities**

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manuscript

As part of a risk evaluation of growing genetically modified crops, we investigated the effects of a genetic modification of starch quality (increased level of amylopectin) in potato tubers (*Solanum tuberosum* L.) on the decomposition of tissues (tubers and leaves) as well as on the associated fungal functional and phylogenetic diversity. The weight loss of both leaves and tubers in litterbags were analysed after 1, 3 and 6 months of incubation in soils and combined with measurements of fungal extracellular enzyme activities (laccases, Mn-perixidases and cellulases) as well as molecular analyses of the fungal community (ITS regions and cellobiohydrolase I (cbhI) genes). The study revealed that initial decomposition of both tubers and leaves of the parental isoline was significantly faster than that of the GM-variety. This coincided with differences in fungal community composition. After this initial difference, no significant differences in any of the parameters measured could be detected after 3 and 6 months of decomposition illustrating the transient nature of the initial difference between the cultivars. Thus, it can be concluded that the starch modified tubers do not bear any risk to fungal decomposer community and despite initial differences in decomposition, the total decomposition rate of the GM-variety is similar to its parental variety. Furthermore, interesting dynamics of fungal phyla and species during decomposition were observed; the basidiomycetal yeasts and ascomycetes were primary colonizers of the potato litter detected with both functional and phylogenetic markers while basidiomycetes were dominant in the more decomposed and lignin-rich litter.

6.1. Introduction

Large-scale cultivation of GM crops is still a matter of debate because of contrasting ideas about the risks of harmful side-effects such as effects on non-target soil organisms and microbe-mediated soil processes and functions (Icoz and Stotzky, 2008). Several studies have addressed the effects of genetically modified potatoes on the activity and community structure of soil micro-organisms and found the effects to be dependent on the type of modification, the growth stage studied and soil characteristics (Heuer et al., 2002; Milling et al., 2004; Rasche et al., 2006; Gschwendtner et al., 2010; Weinert et al., 2010; Gschwendtner et al., 2011). However, so far little attention has been given to the fate of residues of GM crops that are left behind in the fields after harvest. Decomposition of residues of GM-crops could potentially influence ecosystem functions such as decomposition processes via impacts on fungal species diversity and composition (Deacon et al., 2006).

The physicochemical environment, litter quality and the composition of the decomposer community itself are the three main factors controlling litter decomposition (Hättenschwiler et al., 2005). It is well known that decomposition rates of litter vary among plants species (Hobbie, 1992; Berg and McClaugherty, 2008). Although the mechanisms by which plant diversity and species identity can affect ecosystem functioning are well documented, the link between plant genotype and litter decomposition remains elusive (Bernard et al., 2007). With regard to GM crops several studies have addressed the effects of Bt modification of a variety of crops on decomposition and decomposer community (Donegan et al., 1995; Wu et al., 2004; Castaldini et al., 2005; Flores et al., 2005; Lawhorn et al., 2009; Lu et al., 2010a; Lu et al., 2010b; Tan et al., 2010; Xue et al., 2011). A number of these studies have found the GM-trait to affect the fungal decomposer community possibly via unintended changes in the lignin content of the plant (Donegan et al., 1995; Wu et al., 2004; Castaldini et al., 2005; Lu et al., 2010b; Xue et al., 2011). Here we will, to our knowledge for the first time, evaluate the effects of starch-modified GM-potatoes on decomposer community. Earlier studies on GM-potatoes have found differences in rhizosphere microbial communities associated with GM-trait mostly at the senescent growth stage (Lottmann et al., 1999; Lottmann et al., 2000; Lukow et al., 2000) which increases the likelihood of prolonged effects of the genetic modification after the harvest.

Plant residues consists mainly of large biopolymers such as cellulose, hemicellulose, lignin and pectin. Microbes, in particular filamentous fungi, carry out most of the actual breakdown of these polymers by producing a suite of hydrolytic and oxidative extracellular enzymes (Møller et al., 1999; Aro et al., 2005; Romani et al., 2006). In forest soils, basidiomycetes are supposed to be the most important group of organisms responsible for litter degradation (Blackwood et al., 2007; Hofmockel et al., 2007; Osono, 2007). However, the fungal groups responsible for degradation of plant material in agricultural systems are not well known as the type and amount of litter entering these soils is different from that in forest ecosystems (chapter 3). DNA-based approaches used to describe fungal abundance, community composi-

tion and diversity mostly target ribosomal gene regions (Manter and Vivanco, 2007). However, there is an increasing trend to determine the amount and diversity of genes coding for enzymes involved in litter degradation e.g. laccases (Luis et al., 2004; Blackwood et al., 2007; Lauber et al., 2009), cellobiohydrolases (Edwards et al., 2008; Baldrian et al., 2012) and Mn-peroxidases (Bödeker et al., 2009). By targeting these genes information is obtained on functional aspects of the fungal community. In the current study we have analysed the abundance and diversity of genes encoding cellobiohydrolase (cbhI), a member of the GH7 glycoside hydrolase family (Edwards et al., 2008). Cellobiohydrolases are crucial for the degradation of cellulose by fungi as they are involved in the hydrolysis of microcrystalline cellulose (Lynd et al., 2002).

The objective of the present study was to compare the structure and functioning of soil fungal communities decomposing leaves and tubers of a GM-potato with the non-modified parental variety. The GM-variety used in this study is modified for its starch quality resulting in the reduction of amylose, in the tubers in order to reduce the processing costs. Therefore, an amylose-poor variety called 'Modena' was created by complete inhibition of the production of amylose via introduction of a RNAi construct of the granule-bound starch synthase, GBSS, gene inhibiting and amylose formation, which yields pure amylopectin. The modification was made without a marker gene as described by de Vetten et al. (2003). Although the modification is targeted towards starch composition in tubers, it may affect composition of other parts of the plant as well and, consequently, micro-organisms responsible for decomposition. In this study, we have taken the well-established approach of using litter bags (Bocock and Gilbert, 1957; Rubino et al., 2010) and measured the decomposition rate of both tubers and leaves of the GM-variety and its non-modified isoline variety, activities of some of the key enzymes involved as well as the abundance and diversity of cbhI genes. To complement the functional measurements the ITS copy numbers of *Basidiomycota* and *Ascomycota* were quantified and the community composition and diversity of these phyla were estimated.

6.2. Materials and methods

6.2.1. Experimental set-up and sampling

The GM-potato line 'Modena' and its parental cultivar 'Karnico' have been used earlier in field experiments (chapter 3). Potato plants of both the modified and isoline cultivar were grown under greenhouse conditions until the stage of senescence (EC90) (Hack et al., 2001) in soil (sieved < 4 mm) collected from an agricultural field in the Netherlands (sandy peat soil; pH = 5, organic matter content = 25 %, sand fraction = 94 %, water retention = 40 - 46 %). Details on growth conditions are given in chapter 5. Leaves were harvested, air dried, and approximately 4 grams or leaf material was put in mesh bags (mesh size 80 µm). The same procedure was done for potato tubers (weight 10-20 grams) after cutting them into halves to speed up the decomposition. The litterbags containing tissues from the GM potato or its isoline

were placed in the soil in which the same cultivar of potato had been grown. Nine replicates per treatment were made for each incubation period (see below) and were buried in 3 separate boxes (total of 6 boxes, each with 9 leaf samples and 9 tuber samples). Each box with a semi-transparent lid contained 24 kg of soil and was kept at 18-22 ^{o}C in the greenhouse and freed of weeds by hand hoeing. Water was provided whenever necessary. The soil moisture content was similar in all boxes in the beginning (30 % (w/w) moisture). At first sampling (one month), the moisture had dropped to 20 %. After one month, moisture level was kept stable at 20 % (w/w). pH and other measured parameters of the soil did not differ between the timepoints or the cultivars.

The litterbags were recovered after 1 month, 3 months and 6 months of the experiment; nine litterbags of each treatment were sampled each time. The last sampling time only 3 replicates of tubers per treatment were included in the analyses as the tubers in other litterbags had sprouted and their biomass thus increased. The residuesphere soil, i.e. the soil adjacent to decaying plant material, was collected by brushing the litterbags (Sengelov et al., 2000). The litterbags were dried, weighted and the residues of potatoes and leaves were collected. Parts of both the litter and residuesphere samples were instantly frozen and stored at -80 ^{o}C until nucleic acid based analyses were performed. The rest was stored at -20 ^{o}C and was used for enzyme activity measurements.

6.2.2. Plant chemical composition

Plant biomass was freeze-dried and finely milled using an analytical mill A10 (IKA, Germany) before analysis. The analysis was performed as described previously (Voříšková et al., 2011). First, water extractable compounds were extracted step by step in cold water (water : sample, 10 : 1, v/w, 30 min at 20°C) and hot water (water : sample, 10 : 1, v/w, 16 h at 80°C). The soluble compounds after extraction were used for the quantification of alkali-soluble polysaccharides (all polysaccharides except cellulose, i.e., mainly the hemicelluloses and starch), insoluble residue (mainly cellulose) and the acid-insoluble residues (mainly lignin). The content of acid-insoluble residues (the equivalent of "Klason lignin") was measured as dry mass of solids after hydrolysis with 72% (w/w) H2SO4 (Kirk and Obst, 1988). Alkali-soluble polysaccharides were quantified after a 24-h incubation of the sample with 0.5 KOH at 35°C (Kidby and Davidson, 1973; Sun and Tomkinson, 2002) and the amount of insoluble residues was calculated by subtracting the content of acid-insoluble residues and alkali-soluble polysaccharides from the initial mass of the substrate.

6.2.3. Fungal biomass and enzyme activities in the residuesphere

For all residuesphere samples ergosterol was quantified using an alkaline extraction method to give an estimate of fungal biomass (de Ridder-Duine et al., 2006). Activities of enzymes involved in decomposition of lignocellulose, i.e. laccases, cellulases and Mn-peroxidases, were quantified according to van der Wal et al. (2006). The amount of un-decomposed plant material inside litter bags was not sufficient to allow analysis of ergosterol and enzyme activities.

6.2.4. Molecular analyses

DNA was extracted from approx. 0.5 g of residuesphere soil and 0.1 g of litter using the method described by Griffiths et al. (2000). Terminal restriction fragment length polymorphism (T-RFLP) of the ITS regions of fungi was used as a fingerprinting method to assess the diversity and community development of the *Ascomycota* and the *Basidiomycota*. The diversity of the cbhI gene was determined using the same DNA extract. T-RFLP was performed for *Ascomycota* and *Basidiomycota* using primers and conditions presented in table 6.1 and restriction was done like in chapter 3.

Table 6.1. PCR primers, conditions and restriction enzymes to study *Ascomycota*, *Basidiomycota* and the cbhI gene. Same primers and conditions were used also for qPCR.

Target	Primers	PCR conditions	Restriction enzymes used for T-RFLP	Reference
Ascomycota	ITS1F: CTT GGT CAT TTA GAG GAA GTA A ITS4a: CGC CGT TAC TGG GGC AAT CCC TG	95°C 5 min, 35 cycles of (95 °C 15s, 62°C 30s, 72°C 90s), 72°C for 10 min	"HaeIII, HinfI"	Gardes & Bruns, 1993 Larena et al., 1999
Basidiomycota	ITS1F: CTT GGT CAT TTA GAG GAA GTA A ITS4b:CAG GAG ACT TGT ACA CGG TCC AG	95°C 5 min, 35 cycles of (95 °C 15s, 55°C 30s, 72°C 90s), 72°C for 10 min	"HaeIII, HinfI"	Gardes & Bruns, 1993
Cellobiohyrolases	fungcbhIF: ACC AAC/T TGC TAC/T ACI A/GGC/T AA fungcbhIR: GCC/T TCC CAI ATA/G TCC ATC	95°C 5 min, 35 cycles of (95 °C 15s, 48°C 30s, 72°C 90s), 72°C for 10 min	"HinfI, HincI"	Edwards et al. 2008

In order to test the primers and construct a clone library from selected samples, PCR products were purified using the Qiagen PCR purification kit. Purified fragments were cloned into Escherichia coli JM109 using the pGem-T Easy System II cloning kit (Promega, UK) with vector : insert ratio of 3:1. Successful transformants, 35-40 per sample, were amplified using vector-based M13 primers and further sequenced. The partial sequences of the cbhI gene were checked with MolQuest2 for putative exons comparing them with genome information of other fungi (Fgenesh). Further, the amino acid sequences were aligned using ClustalW and threshold of 95 % similarity was used to assign samples to groups of proteins. The proteins were compared to known sequences using BlastP in GenBank. The ITS sequences were checked for quality, aligned using ClustalW and clustered at a 97 % similarity level to obtain OTUs. BlastN was performed for the ITS sequences using GenBank database. All OTU types were further assigned into orders and classes. The GenBank accession numbers of the cbhI and ITS sequences are presented in tables S6.1 and S6.2. The cbhI sequences obtained were virtually digested in http://bis.zju.edu.cn/virs/index.html (Chen et al., 2009) with 100 different enzymes. Six enzymes (EcoRII, EagI, HindII, HaeIII, HinfI and AccI) cutting once at least 50 % (55- 82.5 %) of the sequences were selected to be tested in vitro. All clones obtained were analyzed with these restriction enzymes and enzymes HindII and HinfI (New England BioLabs) were selected for further analyses of soil samples. All restriction incubations were performed according to the manufactures instructions.

The quality of T-RFLP data was first visually inspected in GeneMapper Software v4.1 (Applied Biosystems) and then transferred to T-Rex (Culman et al., 2008). True peaks were identified as those of which the height exceeded the standard deviation (assuming zero mean) computed over all peaks and multiplied by

two (Abdo et al., 2006). All clones selected were analysed with the same restriction enzymes and used further to compare the peak patterns of the cloned samples to the environmental samples using TRAMP-R in the R statistical program (Fitzjohn and Dickie, 2007).

Quantitative PCR was performed for *Ascomycota*, *Basidiomycota* and the cbhI gene using ABsolute QPCR SYBR green mix (AbGene, Epsom, UK) on a Rotor-Gene 3000 (Gorbett Research, Sydney, Australia) with primers and conditions presented in Table 6.1. All samples and all standards were analyzed in at least two different runs to confirm the reproducibility of the quantification.

6.2.5. Statistics

Data of plant material weight loss and composition, copy numbers of ascomycetes, basidiomycetes and the cbhI gene were analyzed using univariate regression within the general linear mode (GLM) procedure in statistical program PAST (Hammer et al., 2001). The assumption of normality was tested with Shapiro-Wilk statistics and homogeneity of variances was assessed with Levene's test. Differences between time points of decomposition and between cultivars were tested for significance with Tukey's HSD test, or, when variances were unequal, with Tamhane's T2 test. All statistics were done with original non-transformed values.

Non-Metric Multidimensional Scaling (NMDS) with Jaccard as distance measure was used to assess the similarity of the fungal communities in the different fractions and between the cultivars. Similarly, principal component analysis (PCA) was used as a distance measure for the data of identified OTUs and classes. The effect of the treatments was tested using one- or two-way ANOSIM with Jaccard as a distance measure. Only presence-absence data were used.

The diversities of OTUs, assigned to classes and orders were compared with Shannon H' diversity index and Fisher's alpha. The diversity t-test was used to compare Shannon H' diversities at different levels in the statistical program PAST.

6.3. Results

6.3.1. Decomposed material and chemical composition of the plants

Initial decomposition rate (1 month) of both leaves and potatoes of the GM-variety was lower than that of the parental variety. Differences in decomposition rates between the two varieties were no longer apparent after 3 and 6 months of incubation (Figure 6.1). There were no significant differences between the cultivars in bulk polymer composition of the plant material that was put in the litter bags (Table 6.2). The lignin content of leaves (32 %) was higher than that of tubers which had more non-cellulosic polysaccharides (72 %). The composition of the tubers changed during the decomposition and after 6 months there was relatively more lignin and less non-cellulosic polysaccharides than in the beginning (Table 6.2).

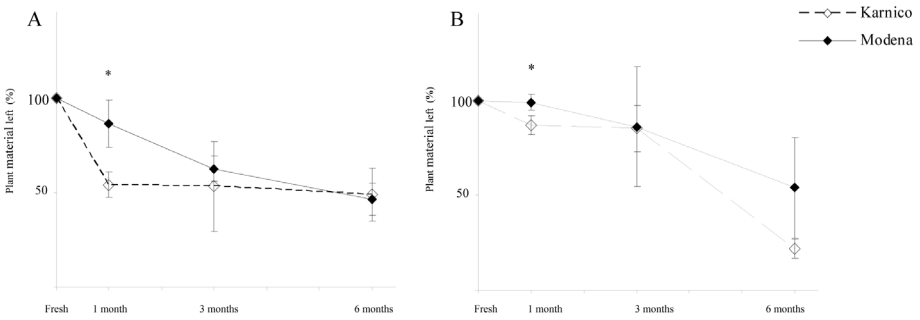


Figure 6.1. Weight loss of (A) potato leaves and (B) tubers during 6 months of decomposition in soil. The solid line and markers represent the data for the GM-variety 'Modena' and dotted lines and open symbols for the parental variety 'Karnico' . The error bars represent standard error. For all the treatments n=9 except n=4 for the potato plants (B) at 6 months.
*marks differences between cultivars at level p<0.05.

Table 6.2. Chemical composition of tubers and leaves from 'Karnico' and 'Modena' (GM-variety) used in this study. The potato material was followed further during its decomposition. For leaf material, this was not possible because of the limited amount of litter material left. The first value represents mean (± standard deviation) of each component and percentage of total composition. Differences between cultivars were tested for significance using ANOVA.

			Lignin (mg / g dw)	Cellulose (mg / g dw)	Non-cellulosic polysaccharides (mg / g dw)
Leaves	Initial material	Modena	248.66 (±32)	267.66 (±35)	258.14 (±12)
		%	32	35	33
		Karnico	250.64 (±61)	277.66 (±57)	269.54 (±63)
		%	32	35	33
		Sig.	*P=0.96*	*P=0.81*	*P=0.77*
Potatoes	Initial material	Modena	13.66 (±6.9)	215.67 (±10)	633.54 (±6.5)
		%	2	26	72
		Karnico	14.21(±10)	222.33 (±14)	628.30 (±14)
		%	2	26	72
		Sig.	*P=0.93*	*P=0.53*	*P=0.58*
	1 month	Modena	8.92 (±3.6)	242.00 (±11)	618.17 (±17)
		%	1	28	71
		Karnico	14.31 (±7.5)	238.33 (±16)	623.77 (±16)
		%	2	27	71
		Sig.	*P=0.33*	*P=0.76*	*P=0.69*
	3 months	Modena	17.47 (±6.5)	248.00 (±6.9)	566.57 (±30)
		%	2	30	68
		Karnico	16.16 (±12)	262.00 (±27)	572.78 (±22)
		%	2	31	67
		Sig.	*P=0.94*	*P=0.43*	*P=0.82*
	6 months	Modena	78.58 (±59)	262.33 (±34)	512.26 (±17)
		%	9	31	60
		Karnico	132.57 (±9.4)	223.00 (±2.8)	467.16 (±22)
		%	16	27	57
		Sig.	*P=0.37*	*P=0.22*	*P=0.08*

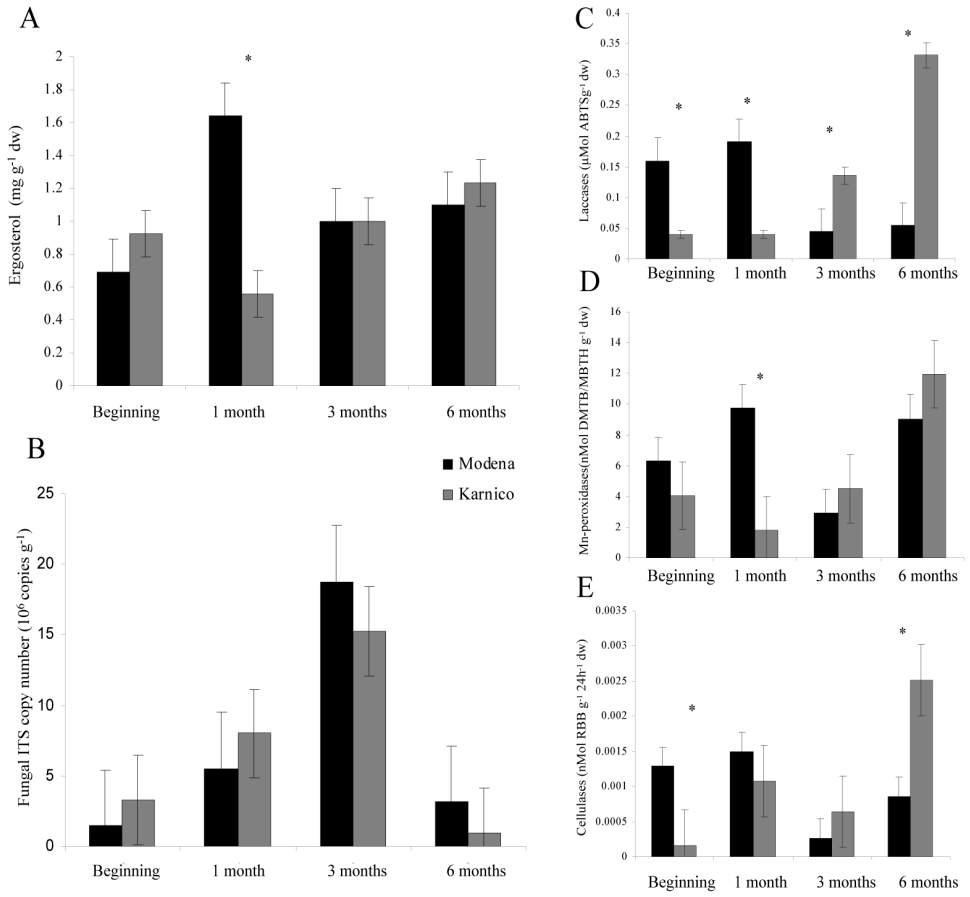


Figure 6.2. Fungal biomarker ergosterol (a), fungal copy numbers (b) and enzymatic activities (c-e) in the residuesphere, the soil surrounding the litter bags. The dark bars represent the GM-variety 'Modena' and the grey bars the parental variety 'Karnico'. The error bars represent standard error.
* marks differences between cultivars at level $p<0.05$

6.3.2. Fungal biomass and enzymatic activity

Ergosterol was used as an indicator of fungal biomass in the residuesphere. Measurements revealed that the GM-variety had more fungi in its residuesphere after one month of incubation (Fig 6.2a). The enzymatic measurements revealed that all enzymatic activities were higher in the beginning and after 1 month of decomposition in the soils where the GM-variety had been grown (Fig. 6.2c-e) while the activities after 3 and 6 months were higher in the soils where litter from the parental variety was decomposing. The amount of ergosterol correlated with both laccase and Mn-peroxidase activities (n=18, R= 0.56 and R=0.65, $p<0.005$). The amount or litter

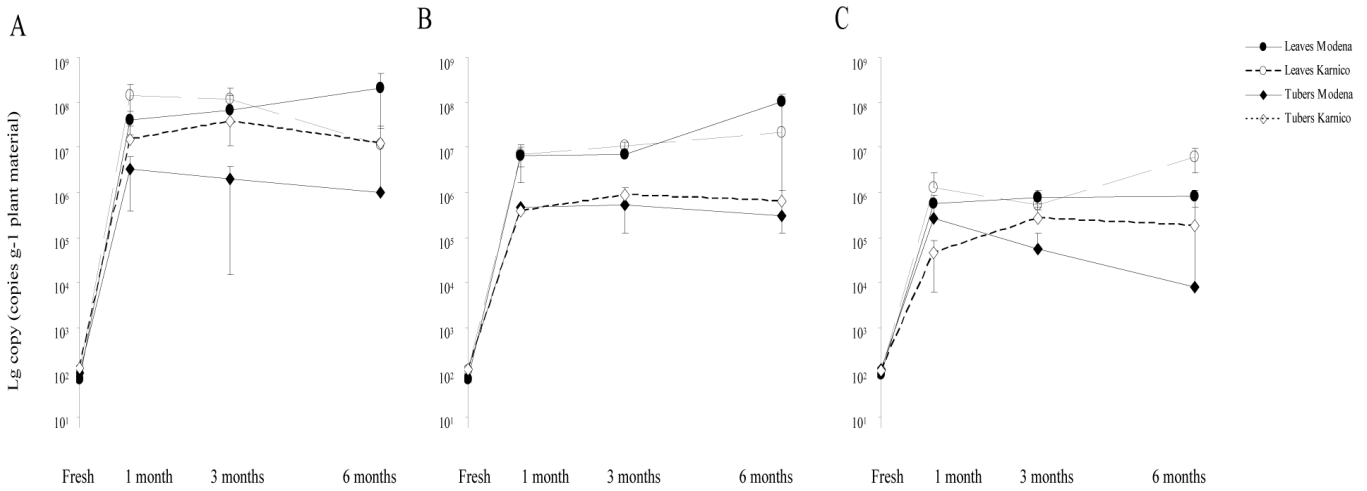


Figure 6.3. ITS copy numbers of ascomycetes (a) and basidiomycetes (b) and cellobiohydrolase gene fragment copy numbers (c) in decomposing potato leaves (○) and potato tubers (◊).The solid line and markers represent the GM-variety 'Modena' and dotted lines and open symbols parental variety 'Karnico'. The error bars represent standard error.

decomposed inside the litter bags did not correlate with the amount of ergosterol or extracellular enzymes outside the litter bag (n=18, R=0.22 – 0.04, p>0.17 for all). However, the amount of lignin in the litter correlated with Mn-peroxidases (n=18, R=0.77, p=0.044) and laccases (n=18, R=0.80, p=0.018) in the residuesphere, whereas cellulases (n=18, R=0.78, p=0.02) in the residuesphere correlated with the amount of cellulose in the litter.

6.3.3. Copy numbers and diversity of fungal communities

Correlation analysis revealed that the weight loss of leaves inside litter bags was weakly correlated with basidiomycete ITS copy numbers (n = 54, R= 0.49, P=0.06) and the weight loss of potatoes with cellobiohydrolase copy numbers (n = 44, R=-0.62, P<0.05). The copy numbers of ascomycetes in the residuesphere of leaves was positively correlated with the weight loss of the leaves inside the litter bags (n=54, R=0.70, P<0.05). Total fungal ITS copy numbers in the residuesphere were not influenced by the plant genotype (Fig. 6.2b). Inside the litterbags, ITS copy numbers of ascomycetes did not correlate with weight loss of the leaves. There was a significantly higher number of ascomycete copies inside litterbags with tubers from 'Karnico' after 1 and 3 months of decomposition as compared to tubers of 'Modena' (Fig. 6.3a). Basidiomycete ITS copy numbers in the litter bags were not affected by the origin of the litter (Fig. 6.3b). Cellobiohydrolase I -gene copy numbers were higher in the leaf litter of 'Karnico' after 1 months and 6 months of decomposition (Fig. 6.3c). The cbhI copy numbers in tubers were lower in 'Karnico' tubers after 1 month but significantly higher after 3 and 6 months.

After one month of decomposition there were 20 times and 6 times more ITS copy numbers of ascomycetes than basidiomycetes in the leaf litter of 'Karnico' and 'Modena', respectively. After three months there were around 10 times more ascomycetes in the litter of both cultivars and at the end of experiment there were similar amounts of basidiomycetal and ascomycetal ITS copy numbers. Richness (number of TRFs) of ascomycetes in the litter was correlated with weight loss in litter (n = 98, R=0.57, P=0.009). Diversity or richness of none of the individual orders in the sample explained the variation significantly. Numbers of basidiomycete TRFs (richness) did not correlate with number of ascomycete TRFs, copy numbers of any fungal group or weight loss of the litter.

In total 96 fungal OTUs were identified of which 72 were ascomycetes and 14 basidiomycetes (Supplementary table 6.2). Ascomycetes were the most diverse group of fungi inside the litter bags. The acomycete diversity inside the litter bags with leaves and potatoes was higher for 'Karnico' after 1 month (leaves t = 4.67 and potatoes t = 6.89, for both p<0.005) but lower after 3 months. After 6 months no differences in ascomycete diversity were found. At the level of orders, the tubers from 'Karnico' had significantly lower diversity of ascomycetes inside bags after one month (t = 3.42, p<0.005). There was a similar trend for the samples from leaves. However this was not significant (t=1.93, p=0.061). The differences in the ascomycete diversity between 'Modena' and 'Karnico' at the level of orders after 1 month

could be explained by absence of *Capnodiales*, *Chaetothyriales*, *Thelebolales* and *Xylariales* in the litter bags with material from 'Modena' and stronger dominance of Hypocreales and Sordariales in the litter from 'Modena'. At later stages of decomposition such differences were not observed. Basidiomycete diversity was less affected by the cultivar and no significant differences between the cultivars were found for any taxonomic level at any time point (table 6.3).

When both phyla were combined, the total diversity at the level of OTUs was higher in the litter bags of 'Karnico' than those of 'Modena' for both leaves ($t = 4.45$. $p<0.001$) and tubers ($t = 6.61$, $p<0.001$) (table 6.3). At later stages, the fungal diversity was not different between the cultivars although the litter bags with tubers from 'Modena' had slightly higher diversity after 6 months ($t = 1.93$, $p = 0.059$). At the level of orders similar differences were observed: after one month litter of Modena from both tubers and leaves had a less diverse fungal community ($t=1.8$, $p=0.07$ for leaves and $t=2.29$, $p<0.005$ for potatoes) than that of Karnico. At higher taxonomic level (classes), no differences in the diversity of fungi between 'Karnico' and 'Modena' were detected (table 6.3).

A total of 16 separate fragments all clustering to the GH7 cellobiohydrolase group were identified and had varying lengths and number of introns (table S6.1). 14 of these fragments clustered with ascomycetes while three showed similarity to basidiomycetes. The diversity of the cbhI gene (representing the functional diversity of the fungal decomposer community) was analyzed in a similar way as the phylogenetic community structure (table 6.3). There was a significant difference in the diversity of cbhI sequences in leaf litter ($t= 3.42$, $p<0.05$) between 'Karnico' and 'Modena' after 1 month of incubation which is consistent with results for taxonomic diversity. Furthermore, there was generally a more diverse functional decomposer community in litter bags with leaf litter than with potato litter (table 6.3). All the fragments identified from 1 month leaf litter samples of 'Modena' clustered with the Helotiales and ,consequently, analyses for all taxonomic levels revealed a significantly lower diversity of cbhI sequences than in the corresponding litter of 'Karnico'.

6.3.4. Fungal community composition

The NMDS of ascomycetes based directly on T-RFLP data revealed no differences between cultivars or incubation periods (ANOSIM $p>0.005$) but there was a significant difference between the litter type (table S6.2). However, as there were 31 OTUs that were present in the leaf litter of 'Karnico' but not in the litter of 'Modena' at the first sampling moment and the reverse (presence in Modena but not in Karnico) was only found for two OTUs, we continued the investigation with community fingerprints of the identified OTUs. The difference in the 31 OTUs was reflected in the PCA of the identified OTUs (Fig 6.4a.) and ANOSIM comparison revealed that samples of one month decomposing leaf litter were indeed significantly different (ANOSIM: $R=0.36$, $p<0.05$) between 'Modena' and 'Karnico'

The NMDS of basidiomycetes showed no significant differences in species composition between the GM- and its parental variety at any sampling point (ANOSIM: $p>0.05$) whereas the PCA of the identified basidiomycetes revealed that the

community was different after one month of decomposition between 'Modena' and 'Karnico (Fig 6.4b.) However, the cultivar identity did not have any overall effect on the community (table S6.3).

Also, for the cbhI gene, the only steering factor for the community structure was the type of plant material (leaves versus potatoes) and the origin of the samples (residuesphere versus litter) (ANOSIM: R=0.45 and R=0.48, $p<0.01$, data not shown). The samples taken from litter bags containing leaves were highly similar at different time points and between cultivars (Fig. 6.4c). The samples from tuber-containing litterbags were more dissimilar while the cultivars were still not significantly different (ANOSIM $p>0.05$) from each other at any time point (Fig. 6.4c). Combined over all time points the cultivars were highly similar (table S6.3).

6.4. Discussion

6.4.1. Effect of GM-trait on decomposition rates, enzymatic activities and fungal community

Both leaves and tubers of potato plants with a genetically modification in starch composition were initially decomposing slower than the corresponding plant parts of the parental potato variety. Previously, most studies on decomposition of GM-plant material did not reveal significant differences between GM and parental plants (Donegan et al., 1997; Daudu et al., 2009; Powell et al., 2009; Tan et al., 2010) with an exception of the study by Flores et al. (2005) who reported that decomposition of Bt-plant materials in soils was less than that of their parental isolines. The latter is most likely due to a difference in lignin content of the GM-plant affecting the decomposition rate. The tubers from the GM-line used in this study consisted of approximately 99-100 % amylopectin, while the percentage for the parental line is around 75 % (Gschwendtner et al., 2010). However, this did not coincide with apparent differences between the GM-variety and its parental isoline in the bulk polymer composition (content of cellulose, lignin and non-cellulosic polysaccharides) of both leaves and tubers. Yet the modification might have more subtle effects on the chemical composition of litter, e.g. the accessibility of cellulose, which can affect the decomposition rate (Berg and McClaugherty, 2008).

Although no differences in chemical composition of the present plant material were found, a significant difference in the composition of the fungal community colonizing the residues was observed between the GM variety and its parental isoline. Similarly, Xue et al. (2011) found an effect of Bt maize (modification Cry3Bb) on soil fungal communities using T-RFLP in one of the soils they tested. However, unlike our study they did not observe effects on decomposition rates. Furthermore, BT maize plant residues plowed under and mixed with soil for up to 4 months, affected soil respiration and mycorrhizal establishment and soil bacterial communities (Castaldini et al., 2005) whereas in another study significant effects of Cry3Bb and Cry1Ab crops on microbial activity and community composition were not observed(Icoz and Stotzky, 2008). A study on trees with a genetic modification of lignin biosynthesis

Table 6.3. Diversity and evenness of *Ascomycota*, *Basidiomycota* and cellobiohydrolases in the litter and in the residuesphere. The 'Leaf in' marks leaf litter, 'Potato in' potato litter and 'Out' combined samples of sampling periods of the residuesphere. Higher fungi marks a combination of detected basidiomycetes and ascomycetes. Different letters in rows indicate significant differences in diversity ($p<0.05$;diversity t-test).

		1 month				3 months	
		Karnico		Modena		Karnico	
		Leaf In	Potato in	Leaf In	Potato in	Leaf In	Potato in
Ascomycota	# of OTUs	48	52	20	10	42	28
	Shannon_H	3.871ab	3.951ab	2.996d	2.303e	3.738bc	3.332cd
	# of orders	15	15	9	5	14	7
	Shannon_H	2.275abc	2.285ab	1.817cd	1.471de	2.342ab	1.509de
	Evenness_e^H/S	0.649	0.655	0.684	0.871	0.743	0.646
	Fisher_alpha	7.49	7.063	6.296	3.98	7.217	2.996
	# of classes	6	5	5	3	6	2
	Shannon_H	1.298a	1.025ab	1.010ab	0.9503ab	1.318a	0.2573c
	Evenness_e^H/S	0.610	0.557	0.549	0.862	0.623	0.647
	Fisher_alpha	1.81	1.363	2.14	1.453	1.896	0.493
Basidiomycota	# of OTUs	6	4	5	4	9	6
	Shannon_H	1.792ab	1.386a	1.609a	1.386a	2.197ab	1.792ab
	# of orders	2	2	2	2	4	4
	Shannon_H	0.450a	0.562ab	0.500a	0.562ab	1.273abc	1.242abc
	Evenness_e^H/S	0.785	0.877	0.825	0.877	0.893	0.866
	Fisher_alpha	1.051	1.592	1.235	1.592	2.759	5.245
	# of classes	2	2	2	2	2	2
	Shannon_H	0.451a	0.562a	0.500a	0.562a	0.687a	0.693a
	Evenness_e^H/S	0.785	0.877	0.825	0.877	0.994	1.000
	Fisher_alpha	1.051	1.592	1.235	1.592	0.797	1.051
Higher Fungi	# of OTUs	54	56	25	14	51	34
	Shannon_H	3.989b	4.025b	3.219c	2.639d	3.932b	3.526bc
	# of orders	17	17	11	7	18	11
	Shannon_H	2.421a	2.420ab	2.054bc	1.810c	2.618a	1.928c
	Evenness_e^H/S	0.662	0.661	0.709	0.873	0.761	0.625
	Fisher_alpha	8.537	8.306	7.504	5.571	9.753	5.642
	# of classes	8	7	7	5	8	4
	Shannon_H	1.552ab	1.249bc	1.408ab	1.438ab	1.670a	0.800c
	Evenness_e^H/S	0.590	0.498	0.584	0.842	0.664	0.557
	Fisher_alpha	2.596	2.112	3.228	2.782	2.64	1.178
Cellobiohydrolases	# of OTUs	10	5	2	3	7	5
	Shannon_H	2.303a	1.609abc	0.6931c	1.099bc	1.946ab	1.609abc
	# of groups	5	3	1	2	6	4
	Shannon_H	1.359a	0.9503a	-	0.6365a	1.748a	1.332a
	Evenness_e^H/S	0.779	0.862	1	0.945	0.957	0.947
	Fisher_alpha	3.980	3.167	0.796	2.622	19.950	9.284

Table 6.3. continues

		6 months					
Modena		Karnico		Modena		Karnico	Modena
Leaf In	Potato in	Leaf In	Potato in	Leaf In	Potato in	Residuesphere	
47	6	61	22	58	34	5	7
3.85ab	1.792e	4.111a	3.091d	4.060a	3.526c	1.840e	1.920e
15	4	17	8	17	13	5	6
2.335ab	1.277e	2.443a	1.780d	2.381ab	2.015bcd	1.45de	1.556de
0.688	0.897	0.677	0.741	0.636	0.577	0.852	0.683
7.375	3.878	7.645	4.523	7.905	7.691	14.12	8.007
4	2	6	4	6	5	3	4
1.022ab	0.410c	1.177a	0.776bc	1.235a	1.048ab	0.796abc	0.952ab
0.695	0.754	0.541	0.543	0.573	0.571	0.739	0.648
1.03	0.9354	1.66	1.431	1.631	1.617	1.989	1.128
12	7	9	5	9	4	10	10
2.485b	1.946ab	2.197ab	1.609a	2.197ab	1.386a	2.303ab	2.303ab
6	5	5	2	5	2	5	5
1.605bc	1.494bc	1.505bc	0.500a	1.505bc	0.562ab	1.414bc	1.414bc
0.830	0.891	0.901	0.825	0.901	0.877	0.823	0.823
4.322	5.705	3.980	1.235	3.980	1.592	3.538	3.538
2	2	2	2	2	2	2	2
0.617a	0.662a	0.611a	0.500a	0.611a	0.562a	0.689a	0.689a
0.927	0.969	0.921	0.825	0.921	0.877	0.996	0.996
0.660	0.856	0.752	1.235	0.752	1.592	0.715	0.715
59	13	70	27	67	38	15	17
4.078ab	2.565d	4.248a	3.296c	4.205a	3.638c	2.821d	2.95d
21	9	22	10	22	15	10	11
2.692a	2.069c	2.705a	2.022c	2.656a	2.199bc	2.085c	2.03c
0.703	0.880	0.680	0.756	0.648	0.601	0.805	0.697
11.33	10.88	10.8	5.746	11.16	9.147	11.41	11.62
6	4	8	6	8	7	5	6
1.442ab	1.240bc	1.537ab	1.204bc	1.494ab	1.334ab	1.413ab	1.510ab
0.705	0.864	0.581	0.556	0.557	0.542	0.822	0.683
1.65	1.871	2.303	2.392	2.341	2.52	2.387	1.81
10	11	5	1	8	1	1	5
2.303a	2.398a	1.609abc	-	2.079ab	-	-	1.609abc
6	5	4	1	5	1	1	5
1.498a	1.414a	1.332a	-	1.494a	-	-	1.609a
0.745	0.823	0.947	1	0.891	1	1	1
6.333	3.538	9.284	0	5.705	0	0	0

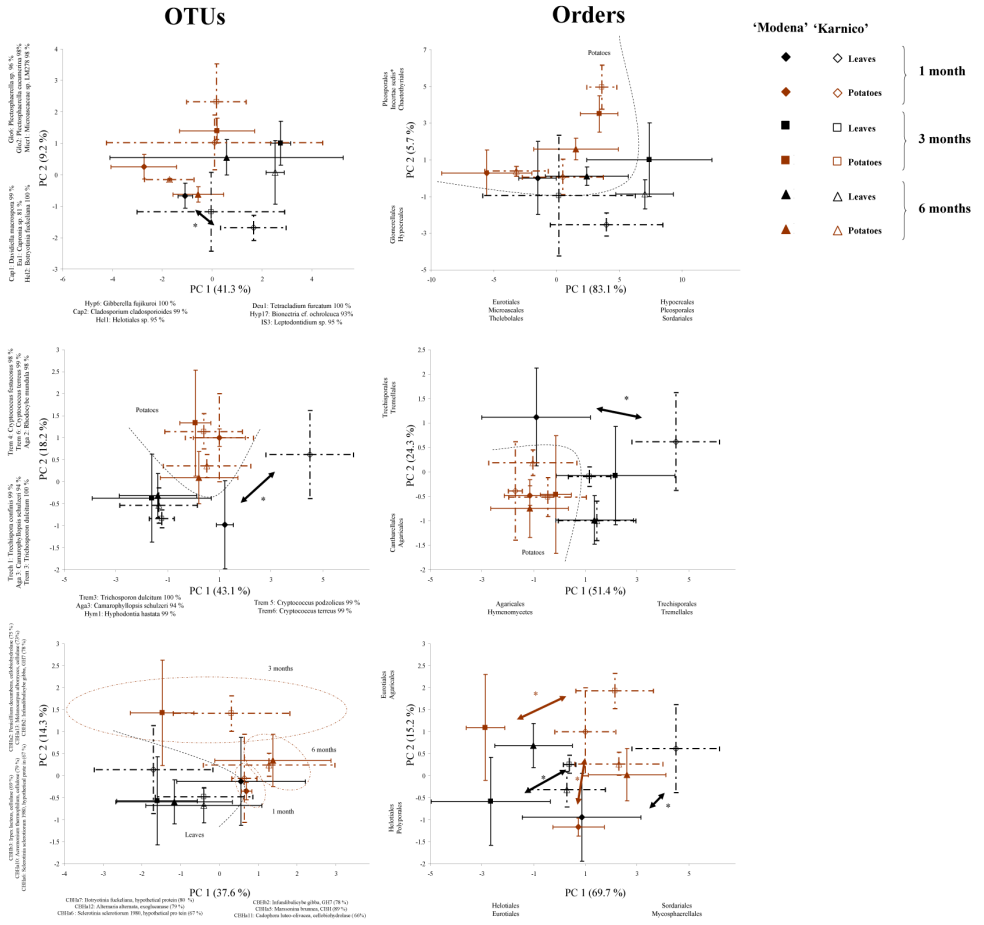


Figure 6.4. Community fingerprints as PCA analyses at the level of OTUs and orders of the ascomycetes (a), basidiomycetes (b) and identified partial cbhI sequences (c). Brown symbols represent decomposing potato tubers and black symbols decomposing potato leaves. The closed symbols represent 'Modena' and the open symbols 'Karnico'. For NMDS analyses the two dimensional stress value is given in the figure and for PCA analyses the PC-scores and the OTUs and orders most explaining the axis. For additional statistical analysis, see table S6.3.

found that modified trees decomposed faster than unmodified isolines and that fungi (measured by PLFAs) were most responsive to the different genotypes (Henault et al., 2006). This is obviously contrary to our findings and highlights the importance of evaluating the effects of genetic modification on a case-by-case basis.

Recently, Weinert et al. (2010), studied bacterial communities at potato tuber

surfaces at the stage of senescence (EC90) and observed that the bacterial community structure as well as the frequency of antagonistic activities were influenced by potato genotype. Thus, shifts in community composition coincided with shifts in functioning of those communities. Our results may also point at co-occurrence of shifts in functioning (decomposition rates) and community composition. We could detect differences in the diversity and composition of ascomycetes as well as the cbhI gene, which corresponded to the slower initial decay of GM material.

The enzyme activities in the residuesphere reflected the corresponding chemical composition of the plant material inside the litterbag. This may be a reflection of the enzyme production inside the bags as it is not clear if the enzymes in the residuesphere are produced there or are leaching out of the bags. However, the community structure and diversity of fungi in the residuesphere seemed to be little affected by the plant genotype and the type of decomposing material (Fig. 6.2) and represented a fraction of the fungal diversity inside the litter bags. The fungi that do grow out of the litterbags have a relatively low sensitivity to soil fungistasis (Garbeva et al., 2011).

6.4.2. Fungal community dynamics during litter decomposition

During litter decomposition, total fungal diversity inclined considerably. However, *Ascomycota* and *Basidiomycota* responded very differently. Differences in diversity at the first sampling between GM- and its parental variety could be specifically explained by differences in ascomycete community diversity. Decomposition of litter is usually initiated by generalist primary colonizers consisting of fungi and bacteria that can utilize simple sugars and easy accessible cellulose (Kubartová et al., 2007). Primary colonizers may be the most important group of decomposer organisms in our relatively short-term study. We could, however, observe a clear shift in the fungal communities as the copy numbers of ascomycetes were highest in the beginning of the experiment while the basidiomycete copy numbers increased during the experiment. This can be explained by the succession of microbial communities during decomposition and the changes in the chemical litter composition as a result of microbial activity (Berg and McClaugherty, 2008); first cellulose accessible to all fungi is utilized leaving the more recalcitrant material which can be used only by certain fungi (mostly basidiomycetes). Indeed, earlier studies on ascomycetes responsible of degradation of litter have shown that these fungi prefer more simple carbohydrates (Kluczek-Turpeinen et al., 2007) which makes the ascomycetes good competitors for the simple sugars present in the early stages of decomposition while basidiomycetes are known for their abilities to degrade more recalcitrant matter (Leonowicz et al., 1999). However, the diversity of ascomycetes was highest in the leaf litter at later stages which indicates that the diversity of ascomycetal functions is large and that part of the ascomycete community is adapted to degradation of more complex substrates. For example, the xylariaceous ascomycetes are well-known lignin degraders while the litter microfungi such as Aspergillus and Penicillium are thought to have much lower ligninolytic activities (Osono, 2007) and are considered as primary colonizers.

Despite the dominance in numbers and diversity of ascomycetes, the copy

numbers of basidiomycetes rather than that of ascomycetes seemed to link to the amount of leaf litter lost inside the litterbags. The connection between basidiomycetes and leaf litter loss may be explained by the higher amount of lignin in the leaf litter than in the litter of tubers as this connection did not hold for the lignin-poor tuber litter. The basidiomycete community based on ITS sequence was dominated by yeast species such as *Cryptococcus podzolicus* and *Cryptococcus festucosus*. The importance of yeasts in degradation processes in soils is largely unknown although some studies have shown that they are common in decomposer communities. They are identified as r-strategist decomposers (van der Wal et al., 2006; Sampaio et al., 2007), while at the same time there is evidence for their importance in rhizosphere (Botha, 2011; Mestre et al., 2011). It is known that some yeast species belonging to the genus Trichosporon can utilize phenolic compounds which can be related to lignin degradation (Middelhoven, 2006). As the phyllosphere is known to be a common niche for yeasts they are probably already present at the start of the litter incubation (Sampaio et al., 2007). There were, however, yeasts, such as *Cryptococcus festucosus* and *Trichosporon dulcitum* that were present only in the litter at later stages confirming the complexity of categorizing decomposers (Crawford et al., 1990).

Analyses of the cbhI gene further confirmed the finding of the importance of ascomycetes in cellulose decomposition in this study. Of the 16 identified fragments 13 were related to ascomycetes and only 3 to basidiomycetes despite their high representation in the available databases (Edwards et al., 2008). We believe that this is not a technical artifact but rather reflecting the real dominance of ascomycetes in cellulose decomposition in this relatively short-term decomposition experiment. Further, investigating the functionality of community together with community structure is an important step towards understanding the decomposition processes (Blackwood et al., 2007; Edwards et al., 2008). We did find similarities in the abundance and community of cbhI genes and the community of fungi measured by ITS markers.

Our results showed only a minor difference between the potato and leaf litter in copy numbers of fungi while diversity was stronger affected by the type of litter although at different levels for each phylum (Fig. 6.4). The same was observed in a study on maize where no strong differences in fungal communities colonizing different types of residues were found (Xue et al., 2011). In our study, the observed difference between identified OTUs indicates that there is no effect on the total community while the dominant OTUs vary more. The observed difference in the initial chemical composition between the tubers and leaves are likely to explain the differences in the decomposer community (Daudu et al., 2009).

From risk assessment perspective, finding differences in the initial decomposer community and function is alarming as there is a real possibility of plant parts left behind in the fields during harvest. These effects were, however, transient and after three months both the community and amount of material lost from the leaves between cultivars are similar. Caution is nevertheless required before extrapolating these findings to field situation and there is a need for further confirmation of results obtained here using a wider range of modifications and crop species.

								1 month				3 months				6 months					
					Introns			Karnico		Modena		Karnico		Modena		Karnico		Modena		Karnico	Modena
Name	Phyla	Order	GenBank Accession	Protein size (aa)	Number	Length(s) (bp)	Blast P match (% identity)	Leaf In	Potato in	Leaf In	Potato in	Leaf In	Potato in	Leaf In	Potato in	Leaf In	Potato in	Leaf In	Potato in	Out	Out
CBHa1	Ascomycete	Eurotiales	JQ585572	142	0	-	*Aspergillus terreus*, cellobiohydrolase, AAW68437 (85 %)	x				x	x	x	x	x		x			x
CBHa2	Ascomycete	Eurotiales	JQ585573	176	1	48	*Penicillium decumbens*, cellobiohydrolase, ACV95805 (75 %)	x					x		x						
CBHa3	Ascomycete	Eurotiales	JQ585574	165	0	-	*Aspergillus nidulans*, hypothetical protein AN5176.2, XP_662780 (69 %)											x			
CBHa4	Ascomycete	Helotiales	JQ585576	168	2	54, 58	*Botryotinia fuckeliana*, hypothetical protein, XP_001555330 (96 %)	x	x	x		x		x	x			x			
CBHa5	Ascomycete	Helotiales	JQ585568	87	2	95, 74	*Marssonina brunnea* f. sp. 'multigermtubi', CBH, ADB23428 (89 %)		x												
CBHa6	Ascomycete	Helotiales	JQ585577	182	2	41, 72	*Sclerotinia sclerotiorum* 1980, hypothetical protein, XP_001596118 (67 %)	x		x		x		x	x	x		x			
CBHa7	Ascomycete	Helotiales	JQ585578	157	1	67	*Botryotinia fuckeliana*, hypothetical protein, XP_001555330 (80 %)"	x						x	x	x		x			
CBHa8	Ascomycete	Helotiales	JQ585579	176	2	96, 76	*Botryotinia fuckeliana*, hypothetical protein, XP_001555330 (90 %)"	x	x		x			x	x						x
CBHa9	Ascomycete	Helotiales	JQ585580	169	2	98, 89	*Sclerotinia sclerotiorum* 1980, hypothetical protein, XP_001596118 (78 %)"	x			x			x	x						
CBHa10	Ascomycete	Hypocreales	JQ585569	130	0	-	*Acremonium thermophilum*, cellulose 1,4-beta-cellobiosidase, CAM98445 (79 %)					x		x							x
CBHa11	Ascomycete	Mycosphaerellales	JQ585570	168	1	66	*Cadophora luteo-olivacea*, cellobiohydrolase, ADY89599 (66%)					x	x								
CBHa12	Ascomycete	Pleosporales	JQ585581	178	1	56	*Alternaria alternata*, exoglucanase, AAF05699 (79 %)	x						x	x	x		x			
CBHa13	Ascomycete	Sordariales	JQ585582	168	1	56	*Melanocarpus albomyces*, cellulase, CAD56666 (73%)		x				x		x						
CBHb1	Basidiomycete	Agaricales	JQ585583	177	1	49	*Schizophyllum commune*, cellobiohydrolase, AAX55505 (80 %)	x				x	x	x	x			x			
CBHb2	Basidiomycete	Agaricales	JQ585584	158	1	68	*Infundibulicybe gibba*, GH7, ABY61330 (78 %)								x		x		x		x
CBHb3	Basidiomycete	Polyporales	JQ585585	169	0	-	*Irpex lacteus*, cellulase, BAA76364 (69 %)	x	x		x	x		x		x		x		x	

Table S6.1. The identified cbhI sequences in this study and their presence in the samples. All the fragments were checked in MolQuest for protein sequence and the size of the protein (in aminoacids) and number and length of introns is presented. The closest hit was obtained with BlastP and similarity is presented in brackets after the name. The 'Leaf in' marks leaf litter, 'Potato in' potato litter and 'Out' combined samples from the residuesphere.

Table S6.2. Presence of identified fungal species in samples in the litter and in the residuesphere. The 'Leaf in' marks leaf litter, 'Potato in' potato litter and 'residuesphere' combined samples of all sampling periods from the residuesphere.

					1 month			
					Karnico		Modena	
	Name	Order	GenBank Accession	Closest Hit (% idenity)	Leaf In	Potato in	Leaf In	Potato in
Ascomycota	Cap1	Capnodiales	HM037657	Davidiella macrospora (EU167591) (99)	x			
	Cap2	Capnodiales	HM037664	Cladosporium cladosporioides (AY251074) (99)				
	Cap3	Capnodiales	JQ585531	Cladosporium herbarum (AF177734) (80)				
	Cap4	Capnodiales	JQ585532	Zasmidium nocooxi (CQ852842) (83)				
	Cap5	Capnodiales	JQ585533	Devriesia sp. NG_p52 (HQ115717) (100)	x	x		
	Chae1	Chaetothyriales	HM037655	Uncultured Herpotrichiellaceae (FJ554453) (98)	x	x		
	Chae2	Chaetothyriales	JQ585534	Cladophialophora chaetospira strain (EU035406) (100)	x	x		
	Chae3	Chaetothyriales	JQ585535	Exophiala sp. Ppf18 (GQ302685) (97)	x	x		
	Deu1	Deuteromycota	HM037654	Tetracladium furcatum strain CCM F-11883 (FJ000375) (100)	x	x		
	Deu2	Deuteromycota	JQ618502	Leptodontidium sp. 3435 (FN393420) (99)	x			
	Deu3	Deuteromycota	JQ618503	Scytalidium lignicola (FJ914697) (100)		x		
	Deu4	Deuteromycota	JQ618504	Tetracladium sp. (95)	x	x	x	x
	Deu5	Deuteromycota	HM037644	Microsphaeropsis sp. MTFD09 (DQ132840) (99)		x	x	
	Eur1	Eurotiomycetes	JQ585537	Capronia sp. 94003b (EU129158) (81)	x			x
	Glo1	Glomerellales	HM037642	Verticillium dahliae(HQ839784) (90)		x		
	Glo2	Glomerellales	HM037643	Plectosphaerella cucumerina (AJ492873)(98)		x		
	Glo3	Glomerellales	HM037661	Verticillium dahliae(HQ839784) (97)		x		
	Glo4	Glomerellales	JQ618505	Gibellulopsis nigrescens(HQ115693)(98)	x	x	x	x
	Glo5	Glomerellales	JQ585540	Gibellulopsis nigrescens (HQ115693) (100)		x		
	Glo6	Glomerellales	HM037643	Plectosphaerella sp. (96)		x		
	Hel1	Helotiales	HM037648	Botryotinia fuckeliana isolate Bot. 1283 (EF207415) (100)	x	x	x	x
	Hel2	Helotiales	HM037646	Unknown Helotiales sp. (<95)	x	x	x	x
	Hel3	Helotiales	JQ585538	Meliniomyces variabilis (EF093178) (95)	x	x		

3 months				6 months					
Karnico		Modena		Karnico		Modena		Karnico	Modena
Leaf In	Potato in	Leaf In	Potato in	Leaf In	Potato in	Leaf In	Potato in	Residue	sphere
x				x			x		
		x		x		x	x		
x				x	x				x
				x					
x				x	x	x			x
x		x		x		x			x
x		x		x		x	x		
x		x		x		x		x	x
x		x		x		x			
x				x		x			
x		x		x		x	x		x
							x		
x				x		x	x		
	x	x		x		x			
	x	x		x		x			x
	x	x		x		x			x
x	x	x	x	x	x	x	x		x
	x	x		x		x			x
	x	x		x		x			x
x		x		x	x	x	x		x
x		x	x	x	x	x	x		x
x		x				x	x		

Table S6.2. Continues

					1 month			
					Karnico		Modena	
	Name	Order	GenBank Acceccion	Closest hit (% indentity)	Leaf in	Potato in	Leaf in	Potato in
Ascomycota	Hel4	Helotiales	HM037654	Mollisia cinerea(FR668005)(90)				
	Hel5	Helotiales	HM037647	Helotiales sp.(AJ879686)(89)		x		
	Hyp1	Hypocreales	HM037640	Fusarium sp. (96)	x			
	Hyp2	Hypocreales	HM037641	Fusarium sp. 14018 (EU750682) (99)	x	x		x
	Hyp3	Hypocreales	HM037650	Clonostachys miodochialis (AF210674) (99)	x	x		
	Hyp4	Hypocreales	HM037672	Bionectria cf. ochroleuca (EU552110) (98)	x	x		
	Hyp5	Hypocreales	HM037668	Fusarium sp. 5/97?45 (AJ279478) (97)	x			
	Hyp6	Hypocreales	JQ585539	Gibberella fujikuroi strain SH-f13 (HM165488) (100)				
	Hyp7	Hypocreales	HM037656	Nectria sp. ASIN2 (DQ779785) (100)	x	x	x	x
	Hyp8	Hypocreales	JQ618508	Gibberella zeae (DQ459827) (100)	x	x		x
	Hyp9	Hypocreales	HM037641	Fusarium equiseti (GQ50572) (100)	x	x		x
	Hyp10	Hypocreales	JQ618506	Fusarium merismoides var. merismoides (EU860057) (100)	x	x	x	x
	Hyp11	Hypocreales	HM037670	Gliomastix murorum(AB540557) (98)	x	x		
	Hyp12	Hypocreales	JQ618507	Fusarium sp. HMA-16 (GU480953) (100)	x	x	x	x
	Hyp13	Hypocreales	JQ618511	Gibberella sp. (FJ466712) (98)				
	Hyp14	Hypocreales	JQ618512	Hypocreales sp. r382(HQ649873) (99)	x	x		
	Hyp15	Hypocreales	JQ618513	Neonectria radicicola (GU934581) (98)				
	Hyp16	Hypocreales	HM037668	Fusarium oxysporum voucher (FJ466709)(98)		x		
	Hyp17	Hypocreales	JQ618509	Bionectria cf. ochroleuca (EU552110)(93)	x	x		
	Hyp18	Hypocreales	JQ618510	Fusarium sp. RGT-S4(HQ674657) (96)	x	x	x	x
	Hyp19	Hypocreales	HM037667	Bionectria ochroleuca strain G11(GU566253)(98)	x	x		
	IS1	Incertae sedis	HM037637	Pseudeurotium bakeri (DQ068995) (100)	x			
	IS2	Incertae sedis	HM037638	Pseudeurotium bakeri (GU934582)(100)	x	x		
	IS3	Incertae sedis	HM037663	Leptodontidium sp. (95)	x	x		x
	IS4	Incertae sedis	HM037666	Ascochyta pisi var. pisi (EU167557) (98)	x	x		

3 months				6 months					
Karnico		Modena		Karnico		Modena		Karnico	Modena
Leaf in	Potato in	Leaf in	Potato in	Leaf in	Potato in	Leaf in	Potato in	Residue	sphere
				x				x	
		x		x		x			
x		x		x		x			x
x	x	x		x	x	x	x		x
				x		x	x		x
x	x			x		x	x		x
		x					x		
x	x	x	x	x	x	x	x	x	x
x	x	x		x	x	x	x		x
x	x	x		x	x	x	x		x
x	x	x	x	x	x	x	x	x	x
				x		x			
x	x	x		x	x	x	x		x
	x	x		x		x			
						x			
	x	x		x		x			
x	x	x		x		x	x	x	x
x	x	x	x	x	x	x	x		x
x	x	x		x		x	x		x
x	x	x			x	x	x		x
x	x	x			x	x	x		x
x		x		x		x	x		x
x				x		x			

Table S6.2. Continues

					1 month			
					Karnico		Modena	
	Name	Order	GenBank Accession	Closest hit (% indentity)	Leaf in	Potato in	Leaf in	Potato in
Ascomycota	Lec1	Lecanorales	JQ618514	Parmelia sp. (HQ671309) (85)	x	x		
	Mag1	Magnaporthales	JQ585541	Phialophora sp. DF36 (EU314710) (99)	x	x		x
	Mag2	Magnaporthales	JQ618515	Mycoleptodiscus indicus isolate UM28(HQ148095)(100)	x	x		
	Micr1	Microascales	HM037651	Microascaceae sp. LM278 (EF060607) (98)		x		
	Mit1	Mitosporic Pezizomycotina	HM037639	Trichocladium asperum (AM292050) (99)	x	x		
	Pleo1	Pleosporales	HM037652	Uncultured Ampelomyces clone IIP2?29 (EU516670) (98)	x			
	Pleo2	Pleosporales	HM037669	Aff. Drechslera MT0008 (AB199583) (99)	x			
	Pleo3	Pleosporales	JQ585542	Dendryphion nanum (AY387657) (98)	x	x		
	Pleo4	Pleosporales	JQ618516	Coniothyrium sp. 229 (FJ228186) (93)	x	x		
	Pleo5	Pleosporales	JQ585543	Pyrenochaeta sp. ZLY-2010b (HM5955516) (90)	x	x		x
	Pleo6	Pleosporales	JQ585542	Dendryphion nanum (GU934517) (100%)	x			
	Sor1	Sordariales	JQ585544	Podospora miniglutinans (FJ946483) (94)	x	x		x
	Sor2	Sordariales	JQ618517	Podospora setosa (GU391421) (95)		x		x
	Sor3	Sordariales	JQ585545	Podospora glutinans (AY615208) (96)		x		
	Sor4	Sordariales	JQ585546	Podospora sp. (80)	x	x		x
	Sor5	Sordariales	JQ585571	Chaetomium sp. 15003 (EU750691) (98)	x	x	x	x
	Thel1	Thelebolales	JQ585547	Thelebolus sp. (FJ613125) (99)	x			
	Unk1	Unknown	HM037658	Ascomycete sp.(83)				
	Xyl1	Xylariales	JQ585548	Sarcostroma bisetulatum (EU552155) (80)	x	x		
Basidiomycota	Aga1	Agaricales	JQ618518	Clitocybe trulliformis (JF907809) (100)	x			
	Cant1	Cantharellales	HM037684	Ceratobasidium sp. aurim1217 (DQ093646) (98)				
	Cant2	Cantharellales	HM037678	Ceratobasidium sp. FO 38200 (DQ520098) (93)				
	Hym1	Hymenochaetales	JQ618519	Leifia flabelliradiata (DQ873635) (97)				x

3 months				6 months					
Karnico		Modena		Karnico		Modena		Karnico	Modena
Leaf in	Potato in	Leaf in	Potato in	Leaf in	Potato in	Leaf in	Potato in	Residue	sphere
x				x		x			
x	x	x		x	x	x	x	x	x
x	x	x		x	x	x	x		x
	x	x		x		x			x
		x		x			x		x
x				x		x			
x		x		x					x
				x		x			
		x		x		x		x	
x		x		x		x	x		
				x		x			
x	x	x		x	x	x	x		x
				x	x	x	x		
x		x		x	x				
x	x	x		x	x	x			x
x	x	x	x	x	x	x	x		
x		x		x		x			x
						x			
x		x		x		x	x		
		x							
x		x		x		x			
x	x	x	x	x	x	x	x	x	x
x		x			x		x	x	x

Table S6.2. Continues

					1 month			
					Karnico		Modena	
	Name	Order	GenBank Accession	Closest hit (% indentity)	Leaf in	Potato in	Leaf in	Potato in
Basidiomycota	Pol1	Polyporales	JQ618520	Fomitopsis cf. meliae (AB540581) (97)				
	Trech1	Trechisporales	JQ618521	Trechispora confinis (AF347081) (92)		x		
	Trem1	Tremellales	JQ585560	Cryptococcus podzolicus (FN428938) (97)	x	x	x	x
	Trem2	Tremellales	HM037680	Uncultured Cryptococcus (EU516999) (99)	x			
	Trem3	Tremellales	JQ585559	Trichosporon dulcitum strain HB940 (AJ507663) (100)				
	Trem4	Tremellales	HM037676	Cryptococcus festucosus (FR717832) (98)	x			x
	Trem5	Tremellales	JQ585556	Cryptococcus podzolicus (FN428940) (99)	x	x	x	x

3 months				6 months					
Karnico		Modena		Karnico		Modena		Karnico	Modena
Leaf in	Potato in	Leaf in	Potato in	Leaf in	Potato in	Leaf in	Potato in	Residuesphere	
	x	x		x		x		x	x
	x	x	x					x	x
x	x	x	x	x	x	x	x	x	x
x		x			x	x	x	x	x
x	x	x	x	x	x	x	x	x	x
x		x		x				x	x
	x		x		x			x	x

Table S3. ANOSIM comparisons of cultivar, type of litter, time and interaction effects of ascomycetes, basidiomycetes and CBHI. The R values indicate differences between "within group" and "between group" distances so that R=1 means the groups are completely different and no overlap exist while R=0 implies that the two groups are very similar. Significant P-values ($p<0.05$) are marked with bold.

		Cultivar (overall)		Type (potato vs. leaf)		Time		Type and time (interaction)		Cultivar and type (interaction)		Cultivar and time (interaction)		Time, type and cultivar (interaction)	
		R	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*
Ascomycota	TRFs	-0.02	0.51	**0.29**	**0.005**	-0.03	0.62	0.13	0.11	**0.22**	**0.02**	-0.06	0.71	0.17	0.10
	OTUs	0.02	0.29	0.07	0.13	-0.03	0.58	0.15	0.10	**0.14**	**0.05**	-0.02	0.57	0.2	0.12
	Orders	0.00	0.39	0.09	0.07	-0.08	0.89	-0.01	0.51	0.15	0.08	-0.12	0.72	-0.02	0.54
Basidiomycota	TRFs	0.08	0.36	-0.18	0.94	0.04	0.28	0.03	0.38	-0.12	0.87	0.25	0.09	0.16	0.11
	OTUs	-0.18	0.97	**0.33**	**0.004**	0.61	0.003	**0.93**	**<0.001**	-0.02	0.52	0.32	0.11	**0.22**	**0.043**
	Orders	-0.13	0.86	**0.42**	**0.002**	0.15	0.18	**0.78**	**<0.001**	0.14	0.19	-0.23	0.80	0.15	0.12
CBH	TRFs	-0.06	0.74	**0.44**	**<0.001**	0.06	0.23	**0.34**	**0.002**	**0.36**	**0.001**	0.00	0.47	**0.36**	**0.008**
	OTUs	-0.11	0.89	**0.42**	**<0.001**	0.2	0.06	**0.69**	**0.001**	**0.15**	**0.026**	-0.08	0.65	**0.24**	**0.013**
	Orders	-0.11	0.88	0.12	0.15	**0.22**	**0.05**	**0.56**	**0.001**	-0.12	0.79	0.14	0.11	**0.28**	**0.042**

7

General discussion

Chapter 7

The aim of this thesis was to investigate the effects of genetic modification of the starch quality in potato on the structure and function of the soil fungal community. This was assessed in a three-year field experiment monitoring the normal variation in impact of growing potatoes on soil fungi, and in two greenhouse experiments focusing on both the effects of rhizodeposition and plant tissue composition (litter). In order to study the fungal community composition and functionality, an integrated approach of molecular fingerprinting methods and measurements of fungal enzymes involved in degradation of organic matter in the soil was employed. Firstly, I will discuss the methodology of studying fungi in soil and possible indicators which could be used in further studies to assess the effects of GM-crops. Secondly, I will discuss the variability of agricultural practice related factors measured during the field experiments which have the potential to mask the effects of the genetic modification. Thirdly, I will concentrate on the greenhouse experiment which elucidated the role of root-exudates in shaping the fungal communities in the soils, and how this information can be extrapolated to field experiments. In the section dealing with decomposer fungi that were possibly affected by GM-crops, I will discuss the implications of plant parts left in the field after harvest, and their effects on fungal decomposer communities. Finally, I will discuss ideas and directions for future research on GM-crops.

7.1. Methods – The quest for indicators to assess the effects of genetically modified crops on soil communities and functionality

Previously, many studies have focused on bacteria and much less on fungi when evaluating the effects of GM-crops on soil microbial community structure and function (Bruinsma et al., 2003; Kowalchuk et al., 2003; Liu et al., 2005) despite the increasingly recognized importance of fungi in the rhizosphere (Carlile et al., 2001; Buée et al., 2009a). Moreover, the studies performed to evaluate the effects of various GM-traits on fungi have in large part investigated the diversity of fungal communities using cultivation based methodologies (Donegan et al., 1996; Donegan et al., 1999; Girlanda et al., 2008; Li et al., 2011) and have often failed to investigate the relationship between function and structure of these communities. A recent study on the potato rhizosphere indicated that the fungi are the most abundant group of organisms in this environment with approximately 1012 intergenic transcribed spacer (ITS) copies in a gram of soil (Gschwendtner et al., 2010). However, yet little is known about the function of the fungal community.

The estimates of fungal biomass based on ergosterol concentration revealed that the fungal biomass in the bulk soil of the two soils investigated were in the range of those found for other intensively managed agricultural soils. The highest concentrations were found in the organic-rich soil, which is in line with the role of fungi in decomposition of recalcitrant organic matter (de Boer et al., 2006). Further, I showed in chapter 4 the existence of a positive correlation between the diversity of higher fungi measured by terminal restriction fragment length polymorphism (TRFLP) and ergosterol concentration. This relationship between biomass and diversity has been earlier detected in many natural ecosystems (Nielsen et al., 2011) and even though the system studied here is a disturbed one, this relationship seems to hold. To add to evidence, the decomposition related enzymes (laccases, Mn-peroxidases and cellulases) were strongly correlated with the soil fungal biomass indicating that in these intensively managed agricultural soils, ergosterol levels in the potato rhizosphere could be used as an indicator of both fungal abundance and decomposing activities. A more detailed view on the dynamics of the fungi was obtained by employing an approach of analyzing the three most important soil fungal phyla (*Ascomycota*, *Basidiomycota* and *Glomeromycota*) parallel. This approach of analyzing multiple phyla or groups of organisms simultaneously has been widely used in studies of bacteria (Fierer et al., 2005; Yergeau et al., 2007) but only rarely in studies of fungi. Development and application of high-throughput sequencing approaches for analysis of fungal communities will undoubtedly confirm and extend these findings and aid to elucidate the role of different fungi and fungal phyla in the rhizosphere processes (Buée et al., 2009b; Öpik et al., 2009; Jumpponen et al., 2010).

The effects of GM-crops on *Glomeromycota* communities have been rarely investigated with molecular methods. Earlier studies revealed a functional response of AMF by microscopic measurements of colonization percentages (Kaldorf et al., 2002; Castaldini et al., 2005; de Vaufleury et al., 2007; Powell et al., 2007; Girlanda et al., 2008;

Knox et al., 2008; Cheeke et al., 2011). These measurements are, however, very labor intensive and can be performed only by well trained individuals (McGonigle et al., 1990). There is, thus, a need for indicators of AMF community responses and methods that can be applied more widely. By using molecular methods I observed that the community of AMF in the rhizosphere of potato is more diverse than thought earlier (Cesaro et al., 2008; Oehl et al., 2010; Verbruggen et al., 2010).
This relatively high diversity of AMF is discussed in more detail later.

The efforts to identify individual OTUs in the rhizosphere did not reveal any potential indicator species or groups that could distinguish potato cultivars, including the GM- cultivar. Nevertheless, some species were more dominant in the rhizosphere of certain cultivars at specific time points but no overall trend in the dominance was found. However, most functions are not conserved in phylogeny and, therefore, the functionality of the OTUs is often not known. , Hence, conclusions on the role of single species can unfortunately not be drawn (James et al., 2006). The community composition and diversity at the level of orders and phyla has, however, potential to be used as an indicator on functioning of soil fungi together with ergosterol.

Based on the evidence presented in chapters 3 and 4, the recommendation for methods to analyze possible ecologically relevant effects of GM-crops would be to investigate changes in fungal biomass by measuring ergosterol content in the rhizosphere and further investigate changes in fungal community at the phylum level. Besides the common use of fungal : bacterial ratio as an indicator of disturbance levels in soils (Bååth and Anderson, 2003; Marschner et al., 2003; Lauber et al., 2008), an approach of evaluating ratios of different fungal phyla and orders in the soils is recommended. I showed that the ascomycete : basidiomycete ratio altered between plant growth stages, between years and between fields. Moreover, changes in this ratio were detected also in chapters 5 and 6 which highlight the potential of this approach (Figure 7.1). The development of next-generation high-throughput sequencing methods and using them more widely in different soils will hopefully further confirm the usefulness of this indicator (Buée et al., 2009b; Jumpponen and Jones, 2009; Jumpponen et al., 2010). Besides analyses of the higher fungi, the AMF community should be analyzed separately but still parallel as it seems to respond to different environmental cues than the communities of ascomycetes and basidiomycetes (table 7.1).

The approach of combining the information of community diversity and function is essential when evaluating the effects GM-crops in natural soils. Due to functional redundancy it is possible that a very different microbial community can have the same functioning (Nielsen et al., 2011). Thus, it is important to combine the information about structure and function of the communities. As higher fungi are well known agents in decomposition of organic matter in general and of cellulosic substrates in particular (Carlile et al., 2001; Lynd et al., 2002) and almost all fungal phyla contain cellolytic species, some key enzymes in degradation were selected as targets of the fungal activity. Here, I measured extracellular enzymes and combined that information with data on community structure. Recent methodological

developments allow for the assessment of the abundance of the genes involved in production of these enzymes and to relate this to species (Luis et al., 2005; Kellner et al., 2007; Edwards et al., 2008; Bödeker et al., 2009), which may prove to be a more sensitive approach.

7.2. Large variation in the field experiments – significant differences in the greenhouse

The results presented in chapters 3 and 4 showed that the fungal communities in the potato rhizosphere were not affected by the amylopectin accumulating potatoes in the field. By contrast, a small but significant effect of cultivar was detected in both the greenhouse studies (chapters 5 and 6). This apparent contrast in experimental results can be explained by selective effects of other factors than the GM-trait in the field versus the greenhouse (Griffiths et al., 2000). Direct comparison of the experiments with each other using the above discussed ascomycete-basidiomycete ratio revealed that the fungal community composition was affected by cultivation in the greenhouse although senescent growth stages in the greenhouse clustered more closely with that in the field and than with other field growth stages and bulk soil (Fig. 7.1.).

The abiotic conditions in greenhouse studies were controlled and resulted in a lower fungal biomass and corresponding low enzymatic activities as compared to the field experiments. In this controlled situation larger differences between the GM- and its parental variety were detected, whereas in the field other factors affected the biomass and communities much more than the genetic modification did. This masking effect of environmental factors has been observed earlier by Griffiths et al. (2000) who compared the effect of GM potatoes that produce lectins on non-target soil organisms in the greenhouse and field and found differences between GM- and its parental variety in the greenhouse experiment but not in the field experiment. Gschwendtner et al. (2010) confirmed their findings and suggested that these differences could be due to management practices such as application of fungicides in the fields besides the obvious explanation of the climatic conditions.

This information supports the hypothesis that soil fungal communities are more responsive to other factors in soils than the GM-trait. Indeed, in other field and greenhouse studies where effects of GM-plants on soil fungal communities have been studied, plant identity (GM versus non-GM) had no effect at all (Milling et al., 2004; Turrini et al., 2004; Götz et al., 2006; Girlanda et al., 2008; Hart et al., 2009; Wang et al., 2009) or had a minor influence as compared to other factors affecting the soil fungal community (Donegan et al., 1996; Dunfield and Germida, 2003; Icoz et al., 2008; O'Callaghan et al., 2008; Oliveira et al., 2008; Weinert et al., 2009).

Agricultural management and soil properties like organic matter content, pH, nutrients and water holding capacity have been identified as major abiotic factors affecting soil fungal communities (Kent and Triplett, 2002; Larkin, 2003; Lauber et al., 2008; Singh et al., 2009). Similarly, several biotic factors, including plant spe-

cies identity and plant community diversity have also been shown to influence soil-borne fungal communities (Garbeva et al., 2004; Kasel et al., 2008; Berg and Smalla, 2009; Buée et al., 2009a). This complexity of factors affecting the soil communities precludes a detailed understanding of the impacts of single factors. In previous studies, the effect of the soil type was identified as a key factor influencing the microbial communities in the rhizosphere (Sessitsch et al., 2001; Costa et al., 2006; Oehl et al., 2010; Santos-González et al., 2011) whereas other studies have shown the plant species (Berg et al., 2002; Kowalchuk et al., 2002; Garbeva et al., 2006; Bharadwaj et al., 2007; Singh et al., 2007; Garbeva et al., 2008; Berg and Smalla, 2009; Singh et al., 2009) and growth stage to be the most important factors determining the community (Butler et al., 2003; Sessitsch et al., 2004; van Overbeek and van Elsas, 2008; Wang et al., 2009; Inceoglu et al., 2010). The main conclusions on the relative effects of soil and plant factors on the fungal community structure and activity derived from this

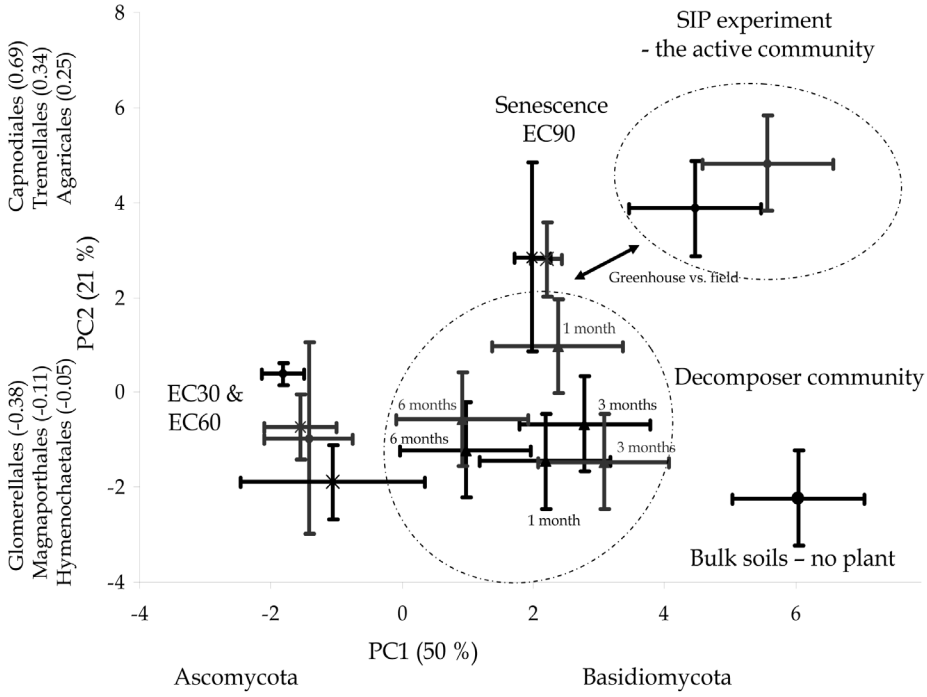


Figure 7.1. The community structure of ascomycetes and basidiomycetes at the level of orders in all the experiments performed. The GM-variety 'Modena' is marked with black symbols and error bars and parental isoline 'Karnico' with gray symbols and error bars. The growth stage or the respective experiment are marked next to the symbols. For PC1 the fungal phyla were the most important explaining factors while for PC2 individual orders of fungi explained most of the variation

7.2.1. Soil type

Earlier studies have found soil type as one of the most explanatory factor affecting the soil microbial communities (Sessitsch et al., 2001; Buyer et al., 2002; Garbeva et al., 2004; Costa et al., 2006; Berg and Smalla, 2009; Wang et al., 2009; Weinert et al., 2009; Santos-González et al., 2011; Kuramae et al., 2012). Results from my study showed, indeed, that the field site had an effect on fungal communities in the bulk soils. Yet, the data on rhizosphere samples indicated that the effect of plant growth stage and the presence of the plant (rhizosphere effect) were much larger than the effect of field site and thus soil type. Of the functions, only cellulases were affected by field site as field BUI had a higher activity of cellulases than field VMD through out the years and growth stages. None of the other functions nor soil fungal biomass were affected and it is unclear why only cellulase production responded to the field site.

A study done in the same framework and in the same fields on bacterial communities found the soil type to be the key determinant of the bacterial communities in both the bulk soil and rhizosphere (Inceoglu et al., 2010). They hypothesized that this was due to the higher organic matter content in field VMD. Indeed, we detected differences in community structure of all the fungal phyla, but no effect on function or diversity in other groups than AMF. The observed difference in community structure but the lack of differences in diversity or community function of the higher fungi can be explained by functional redundancy: different communities might still perform similar functions in both soils (Nielsen et al., 2011).

Previous studies have found AMF to be strongly affected by the soil type (Oehl et al., 2010). The factors in soils potentially affecting the AMF communities and thus explaining differences between fields are pH (Clark, 1997), soil P availability (Oehl et al., 2004; Cheeke et al., 2011) and organic matter content (Sieverding, 1989; Verbruggen et al., 2010). The pH was similar (around 5) in both soils studied in this thesis, P content of the soil was slightly higher in field BUI and the organic matter was much higher in field VMD. The observed lower diversity of AMF in field BUI can thus, be explained by either higher P content or lower organic matter content. The latter option is discussed in more detail in the section about yearly variation.

AMF species in soils can be divided into 'generalist' types which are present in all the soils and 'specialist' which only occur in certain types of soils irrespective of the plant species (Öpik et al., 2006; Oehl et al., 2010). The differences between the soils studied here could indeed, be explained by the presence of certain OTUs of AMF in one field and absence of others. For example certain Paraglomus sp. were abundant in VMD but completely absent in BUI, whereas Glomus versiforme was absent in VMD but abundant in BUI.

7.2.2. Growth stage

Plants are thought to selectively attract certain soil microbes to their rhizosphere by secreting compounds in their root-exudates (Garbeva et al., 2004; Costa et al., 2006).

Table 7.1. The responses of measured functional and structural parameters of soil fungal communities in the potato rhizosphere to the field site, year, growth stage of the plants, genotype of the plant and the GM-trait. The significant effects are written with bold font

History of the site

Soil type

Management practices

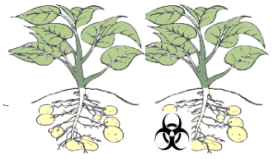
Field location

Season and climate

Year

Fungal biomass	No significant overall effect of field site on soil fungal biomass	**Significant decrease in fungal biomass from 2008-2010**
Laccases	No significant overall effect of field site on soil laccase activity	**Significant decrease in laccases produced from 2008-2010**
Mn-peroxidases	No significant overall effect of field site on soil Mn-peroxidase activity	No significant overall effect of year on Mn-peroxidases
Cellulases	**Significantly higher cellulase activity in field BUI**	**Significant decrease in cellulases produced from 2008-2010**
Ascomycota diversity / community	Ascomycete diversity not affected by the field site / community structure weakly affected	**Significant increase in ascomycete diversity from 2008-2010**
Basidiomycete diversity / community	Basidiomycete diversity not affected by the field site / community structure weakly affected	**Significant decrease in basidiomycete diversity from 2008-2010**
Glomeromycete diversity / community	**Glomeromycete diversity and community structure affected by the field site**	**Significantly more diverse AMF community in 2010**

Table 7.1. continues

 Growth stage	 Genotype/ Cultivar	 GM- trait
Significant effect of growth stage on rhizosphere fungal biomass. Highest biomass in senescence.	No significant overall effect of cultivar on rhizosphere fungal biomass a	No significant overall effect of GM-trait on rhizosphere fungal biomass
Significant effect of growth stage on rhizosphere laccases produced. Highest activity in senescence.	No significant overall effect of cultivar on the laccases produced in the rhizosphere	No significant overall effect of GM-trait on rhizosphere laccase activity
Significant effect of growth stage on rhizosphere Mn-peroxidases produced. Highest activity in senescence.	**Less Mn-peroxidases in the rhizosphere of Aveka and Premiere compared to Modena**	No significant overall effect of GM-trait on rhizosphere Mn-peroxidase activity
Significant effect of growth stage on rhizosphere cellulases produced. Highest activity in senescence.	No significant overall effect of cultivar on the cellulases produced in the rhizosphere	No significant overall effect of GM-trait on rhizosphere cellulase activity
Significant effect of growth stage on ascomycete diversity and community structure. Most diverse community during flowering.	No significant effect of cultivar on ascomycete community structure or diversity	No significant overall effect of GM-trait on community structure or diversity of ascomycetes in the rhizosphere
Significant effect of growth stage on basidiomycete diversity and community structure. Most diverse community in senescence.	No significant effect of cultivar on basidiomycete community structure or diversity	No significant overall effect of GM-trait on community structure or diversity of basidiomycetes in the rhizosphere
Minor effect of growth stage on glomeromycete diversity and community structure.	No significant effect of cultivar on glomeromycete community structure or diversity	No significant overall effect of GM-trait on community structure or diversity of glomeromycetes in the rhizosphere

Furthermore, it is known that both the quantity and quality changes during plant growth which can further shape the rhizosphere microbial communities (Duineveld et al., 2001; Marschner et al., 2002; Jones et al., 2004). Moreover, there are changes in root morphology during growth of the plant which might also promote changes in rhizosphere fungal communities (Berg and Smalla, 2009). Earlier studies have pinpointed that the effect of growth stage could derive from either changes in abiotic and environmental conditions such as soil moisture and temperature during the growing season or from changes in biotic factors (Wang et al., 2009). Since the strong effect of growth stages on all measured parameters was seen during three years and in two locations with changing environmental conditions, I conclude that the observed effect of plant growth stage is mostly due to changes in plant physiology during its growth.

In chapter 4 I demonstrated that the growth stage of the potato is the most important factor affecting the soil fungal community in the potato rhizosphere. Earlier studies indicated that bacterial and fungal communities in the rhizosphere would either decrease (Milling et al., 2004; Acosta-Martínez et al., 2008; Wang et al., 2009; Gschwendtner et al., 2010) or increase (Lottmann et al., 2000; Gomes et al., 2001; Smalla et al., 2001; Gomes et al., 2003; Sessitsch et al., 2004) during plant maturation. Results presented in this thesis clearly indicate that the plants at the senescence stage (EC90) harbor the most diverse, active and abundant fungal communities in their rhizosphere (table 7.1). Moreover, the strong increase of fungal biomass and ligno-cellulolytic enzyme activities during the later growth stages of potato suggest that fungi play a major role in the decomposition of rhizodeposits derived from more mature roots and/or consume slough off root cells from older roots (Rangel-Castro et al., 2005; Dennis et al., 2010). It has been shown that the amount of carbon allocated to the potato roots increases with increased age of the plant and initiation of carbon storage structures (i.e. tubers in potato) (Timlin et al., 2006). These age-dependent exudation patterns might explain the differences in outcome of earlier studies conducted on GM-plants compared to studies presented in this thesis as they have been done for different growth stages (Rossi et al., 2007; Wu et al., 2009; Gschwendtner et al., 2011). This points at the importance of considering the plant phenological state when designing (greenhouse) experiments (van Overbeek and van Elsas, 2008; Weinert et al., 2010).

Moreover, the senescence (EC90) was the only growth stage in which significant differences were found between the GM crop 'Modena' and its parental variety 'Karnico'. However, this was likely due to soil-cultivar interaction effects as it could only be detected at one growth stage and in one of the soils (VMD). Not all groups of fungi responded similarly to the presence and growth stage of the plant (chapter 3). In the absence of plants, the phylum *Basidiomycota* showed greatest diversity, probably due to the basidiomycete ability to degrade the more complex C derived from remainders of the previous crops. Members of the phylum *Ascomycota* were the most diverse during flowering, and, at the stage of senescence, the *Basidiomycota* again displayed greater diversity, suggesting that both phyla play important, yet temporally distinct roles in the rhizosphere. This also explains the observation of

highest activity and biomass in senescence stage when the amount of more recalcitrant decomposable material is at its maximum while root exudation is still continuing thereby broadening the spectrum of substrate availability (Rangel-Castro et al., 2005; Broeckling et al., 2008; Dennis et al., 2010). This succession of ascomycetes and basidiomycetes iterated every year and is a very interesting phenomenon which deserves further investigation.

Unlike the higher fungi, the AMF community did not respond to the plant growth stage. This was in line with earlier observations that AMF colonize the roots of the young plants and the colonization continues at a stable level during the growth of the plants as long as plant provides carbon to the fungal partner (Smith and Read, 1997).

7.2.3. Climatic conditions and agricultural practices over the years

Temporal changes in fungal abundance and community composition can be partly attributed to climatic factors and especially moisture content in the soils (Dunfield and Germida, 2003; Icoz et al., 2008; Oliveira et al., 2008). The total fungal biomass and enzymatic activities decreased each year, during the 3-yeard field study, while the diversity of ascomycetes increased and the diversity of basidiomycetes decreased. This was observed in both fields and might be due to changes in land management such as the addition of an organic fertilizer (pig manure) in 2010. Not much is known on the responses of fungal phyla to yearly variation in both weather and agricultural practices. There were no single factors in the weather conditions which could explain the observed differences; the summer of 2008 was wet and the average temperature was 17.3 ^{o}C, the summer of 2009 was fairly dry and sunny with average temperature of 17.4 ^{o}C and the summer of 2010 was warm and sunny but also wet with average temperature of 17.7 ^{o}C (data from: KNMI). Thus, there were no clear trends in the weather which could explain this dynamics of fungal populations.

It has been demonstrated that the fungal biomass increases after abandonment of agricultural fields (Van der Wal et al., 2006b) and it can thus be proposed as another alternative explanation that in the fields under intensive agriculture the fungal biomass would decrease naturally each year. The third option to explain this decrease in fungi is the change in fertilizers between 2009 and 2010. However, as the decrease was steady between years and not only in 2010, this might not be the case for higher fungi. Little is known about responses of fungi in general and basidiomycetes and ascomycetes to organic fertilizers although it is thought that organic agricultural practices can increase fungal diversity (Bengtsson et al., 2005). However, as parameters such as organic matter content or ammonia production after addition of manure were not measured yearly in this study, it is not possible to speculate the causal of this decrease in fungal biomass.

Furthermore, it seemed that the community structure would shift from a basidiomycete rich community towards a community relatively dominant in ascomycetes. However, as the functions of these phyla are not known, the relevance of this shift remains to be determined (James et al., 2006).An increase in AMF diversity

and a shift in community structure was observed in 2010 after change from mineral to an organic fertilizer which in correspondence with results on the positive effects of organic fertilizers and organic farming on AMF diversity (Oehl et al., 2010; Verbruggen et al., 2010).

7.2.4. Cultivar

An important factor for the evaluation of GM crops is the possible selective effects by cultivars on fungal abundance and functioning. Some transient effects have been observed in studies that have compared multiple GM-varieties (Cowgill et al., 2002; Turrini et al., 2004; Castaldini et al., 2005; Xue et al., 2005; Powell et al., 2007; Icoz et al., 2008; Knox et al., 2008; Oliveira et al., 2008; Kremer and Means, 2009; Wu et al., 2009) or multiple 'normal' varieties against the GM-variety as done in this thesis (Milling et al., 2004; Turrini et al., 2004; Weinert et al., 2009). Most of the studies performed with multiple non-modified varieties found some degree of cultivar dependence of soil fungal community composition (Turrini et al., 2004; Weinert et al., 2009). There was some indication of cultivar dependence, for instance the cultivar 'Premiere' had a lower amount of fungi measured by ergosterol in its rhizosphere than two other cultivars 'Aveka' and 'Désirée'. However, despite the differences in enzymatic activities, diversity of fungal community was not affected by the cultivar-type. Thus, differences observed between cultivars on soil fungi can be deemed transient.

7.2.5. Baseline of decomposers

Besides a baseline for the plants grown in the field, a baseline for litter decomposition is required as well, as there are many factors such as burying depth, soil parameters, temperature and moisture and the exposed time which may affect the outcome of the decomposition analyses (Holland and Coleman, 1987; Burgess et al., 2002; Zwahlen et al., 2003; Xue et al., 2011). In a study by Xue et al. (2011) the authors found that there was little or no effect of the cultivar and GM-trait on functioning and structure of decomposer communities when compared to the other factors such as soil type and history, year (climate) and type of litter. I did not test this here in the decomposer experiment directly, but there was no significant effect of cultivar in the field situation on the fungal communities sampled after the growth seasons and in the rhizosphere of the next crop. This indicates the lack of an ecologically significant effect of amylopectin accumulating potatoes on soil fungal communities decomposing soil organic matter.

7.3. Tracking the carbon flow in the rhizosphere reveals some differences between the GM- and its parental cultivar but also succession of fungi

On the basis of the field studies, I hypothesized that the strong seasonal effect on the fungal biomass, diversity and enzyme activity might have been due to quantitative or qualitative changes in rhizodeposits. Earlier studies have indeed observed changes in the community structure of microbes due to changes in the proportion of phytosynthates released in the rhizosphere and the composition of rhizodeposits is known to vary during plant's life cycle according to changes in plant health status and physiology (Sessitsch et al., 2004; Mougel et al., 2006; Artz et al., 2007; Singh et al., 2007; Hart et al., 2009). In chapter 5 I tested this hypothesis by 13C -tracing of the carbon photosynthesized by the modified cultivar and its parental cultivar to assess intraspecific differences in carbon partitioning into plant parts and further to microorganisms. Based on results obtained in chapters 3 & 4, the senescent stage and the soil from field VMD were selected to be studied in more detail in the greenhouse. Figure 7.2 shows the PLFA-SIP and RNA-SIP based results on carbon partitioning from the plants to soil microbial communities. The most striking result is that the fungal communities in these intensively managed agricultural ecosystems are a more important sink for carbon than thought initially. There is evidence from earlier stable isotope experiments that fungi are a very important group of organotrophic

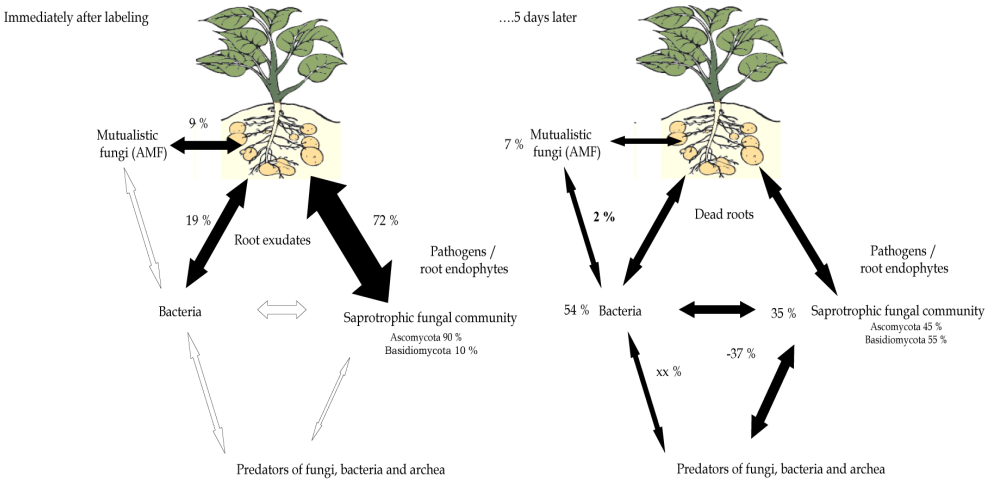


Figure 7.2. Carbon flow from the plants to soil microbial communities. The amount of carbon allocated to each partner could be calculated exactly immediately after labeling but 5 days later the numbers are estimates on transfer.

organisms in the rhizosphere receiving considerable amount of plant derived carbon (Butler et al., 2003; Lu et al., 2004; Wu et al., 2009), and that fungi can respond rapidly to addition of easily degradable substrates to soil (Broeckling et al., 2008; De Graaff et al., 2010). However, this was not so much expected for intensive agricultural systems where fungi are thought to be of minor importance because of soil disturbance, high fertilization and use of fungicides.

As seen from figure 7.2., majority of the carbon allocated to microbes was immediately after labeling found in the PLFA marker 18:2ω6.9 which is thought to be indicator of fungal biomass (Frostegård et al., 2011). This has been noted also in earlier studies which have shown that fungi are quickly incorporating carbon from the plants into their phospholipids (Lu et al., 2007; Wu et al., 2009; Drigo et al., 2010; Gschwendtner et al., 2011). Another interesting phospholipid representing almost 10 % of the microbial labeled carbon is PLFA 16:1ω5 mainly representing AMF (Olsson and Johnson, 2005; Denef et al., 2007). This is notable, as it has been thought that despite the importance of mycorrhiza in nutrient uptake, their importance would be minor in a high nutrient environment like intensively managed agricultural soils as discussed earlier (Cesaro et al., 2008; Cheeke et al., 2011). Yet, results obtained from earlier developmental stages of potato showed similar results with 6.3 % of the 13C allocated to the AMF specific PLFA marker (Gschwendtner et al., 2011). Furthermore, the majority of bacteria received the labeled carbon later than fungi, possibly through fungal related exudation processes (Vandenkoornhuyse et al., 2007; Drigo et al., 2010) or due to their inability to have access inside the root. Also the PLFA marker indicative of protozoa (20:4ω6), not known to be able to use plant derived carbon readily, revealed delayed response to the 13C addition possibly because they were feeding on labeled bacteria or fungi.

When root derived products enter the soil, they are rapidly metabolized and the microbial community is likely to shift in favor of those species that are able to compete for these resources (Dennis et al., 2010). Indeed, I could show that the fungal species in the rhizosphere differed in their strategy. Certain species and groups such as ascomycetes, glomeromycetes and some basidiomycetal yeasts received carbon immediately released by the plant into its rhizosphere while later fungal community changed in favor of (basidiomycetal and ascomycetal) species better adapted to different carbon source or secondary carbon from dead plant parts or from other organisms (Lu et al., 2004; Rangel-Castro et al., 2005; Lu et al., 2007; Vandenkoornhuyse et al., 2007; Dennis et al., 2010).

Significant differences between GM-cultivar 'Modena' and its parental isoline 'Karnico' were observed in the carbon flow which resulted in differences in both diversity and copy numbers of different fungi. A recent study done for the same genetic modification (although in different soil) using PLFA markers revealed no significant effect of GM-trait in associated fungal communities or the plant exudation patterns (Gschwendtner et al., 2011). This difference with my findings could be explained by differences in the growth stage sampled which is discussed earlier (chapter 5).

The approach of using RNA-SIP to study active members of fungal com-

munities is a promising tool to investigate side-effects of GM-plants. Earlier Rasche et al. (2009) investigated differences in shoot endophytic bacteria between two cultivars of potato using DNA-SIP and found cultivar related shifts in bacterial communities after 4 days of labeling very similar to the differences that were observed in chapter 5. Contrastingly, by using PLFA-SIP as an indicator of microbial communities under Bt-rice and its parental isoline, Wu et al. (2009) did not find differences in 13C distribution in roots or rhizosphere. This is an anticipated result since no modification in carbon allocation with the plant was made by inserting the cry1Ab gene. This further highlights the selection of relevant methods to study each modification separately.

7.4. Decomposition as an indicator of community function

The largest differences between parental and GM-variety were observed at the stage of senescence. So, there is a possibility that this effect could persist over the year due to differences in litter and plant residue quality. Thus, leaf- and tuber litter decomposition was chosen as a key indicator of ecosystem function in this thesis. Although no differences in chemical composition of 'Karnico' and 'Modena' were found, there was a significant effect of the cultivar on initial decomposition rate of both leaves and tubers. It seems that for yet unknown reason the material from GM-variety decomposed slower than its parental variety in the same soil. This can be attributed to either subtle differences in composition of the plant material or differences in soil communities present. Another study done with leaves of nematode resistant potato plants found no differences in decomposition rates of the leaves after 3 weeks when in their soils 64 % of the leaf litter mass was lost (Cowgill et al., 2002). Notably, the differences in many of the experiments could be explained by the artifact that the genetic modification gad lead to a different composition of the plant (i.e. higher lignin content in Bt-modified maize) (Flores et al., 2005; Fang et al., 2007; Daudu et al., 2009). I did not find any difference in lignin, cellulose or hemicellulose content of the leaves or tubers and thus the chemical bulk composition of the plant did not explain the observed differences in decomposition and decomposer community. However, as the tubers from the GM-line used in this study consists of approximately 99-100 % amylopectin , while the percentage for the parental line is around 75 % , differences in the decomposition of tuber material were expected. Furthermore, there is a possibility of unintended effects of this modification on the leaves, thereby also affecting the leave decomposition.

The results presented in chapter 6 point at co-occurrence of shifts in functioning (decomposition rates) and community composition. I could detect differences in the diversity and composition of ascomycetes as well as functional gene (cbhI) between cultivars, which corresponded to the delayed loss of material in the soil from the GM-variety. Nonetheless, as the decomposer fungi are vastly diverse both taxonomically and functionally, it was not possible to pinpoint any specific species nor type or class of functional gene to be responsible for the differences observed.

Similarly to the field seasons, the fungal communities changed during the decomposition. Decomposition of litter is usually initiated by generalist primary colonizers involving a diverse community of fungi and bacteria that can utilize simple sugars and other low molecular weight compounds (Kubartová et al., 2007). As the litter quality of potato was high certain fungi (such as ascomycetes and basidiomycetal yeasts) together with bacteria might be the most important group of decomposer organisms in this system. Indeed, there was a shift in the fungal community in decomposing litter which coincided with changes in litter chemical composition.

I showed in chapter 6 that yeasts played an important role in decomposition processes in the agricultural soils. The importance of yeasts in degradation processes in soils has been largely unknown although some studies have shown them to be common in decomposer communities and identified them as r-strategist decomposers (van der Wal et al., 2006a; Sampaio et al., 2007), while at the same time there is mounting evidence of their importance in rhizosphere as also shown in chapter 5 (Botha, 2011; Mestre et al., 2011). The decomposer community clearly separated from the growing plants and its community was between the community of bulk soils in the field and senescent growth stage (Fig. 7.1.). The difference in decomposer communities between the GM- and its parental community was also clearly seen in this figure and were separated along the PC2 while the experiments and growth stages were separated along PC1 thus affecting their ascomycete-basidiomycete ratio as discussed earlier.

7.5. Final conclusions and future perspectives

I showed that in most studies on the effects of various GM-traits and crop species on soil fungal communities significant effects were not observed (Fig. 2.1). However, not all possible combinations of crops and modifications have been investigated and it is clear that generalizations are difficult to make and a case-by-case approach is still the only option to assess GM crop effects. Thus, in future when new traits are introduced, an investigation on effects of this specific modification on relevant soil biota has to be conducted.

The 'baseline' approach presented in this thesis urged that many factors are to be considered as there are many that affect soil biota more than the modification. Thus, the broader sampling scheme proposed in this thesis should be used to assess the impacts of GM-crops on soil biota. However, fungi seem not to be the most responsive group of organisms to perturbations and thus investigations on other (relevant) organisms should be carried out.

Furthermore several differences for instance in the decomposer community and decomposition rate between the GM crop and other cultivars may be alarming but these findings appeared to be transient. Thus, no actual ecosystem level risk is present in the studied starch modified potato production. However, although no long term risk was detected in this thesis, it is wise to include decomposition and

long term after season evaluations into the sampling scheme and risk evaluation. Thus, the results available on the impact of GM plants on natural and agricultural ecosystems show that specific effects of single transformation events should be tested on a case-by-case basis in a natural setting where the baseline factors are all taken into the consideration, taking into account biochemical, physiological, and molecular parameters. In order to properly assess the baseline noise in the system there is a need for statistical methods which can evaluate the effects of GM-trait. The new techniques such as SIP-experiments and high throughput sequencing and metatranscriptomics should be used in parallel with well designed field experiments which consider as many 'baseline' factors as possible.

Chapter 7

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Summary

During the past decades, transgenic techniques have become an accepted way of crop improvement. Despite the increased surface of land allocated to cultivation of genetically modified (GM) crops, the effects of the modified crops on soil fungal communities and functionality are largely unknown. The aim of this thesis was to evaluate the effects of genetically starch-modified (GM)-potatoes on soil fungal communities via changes in root-exudates and plant tissue (litter) composition, and to compare the observed differences between the GM- and its parental variety in the context of the 'normal' variation (such as year, climate, field site, plant growth stage and differences among conventionally produced cultivars). For this, we initially developed methods that were subsequently employed in a three-year field experiment monitoring the normal variation in impact of growing potatoes on soil fungi, and in two greenhouse experiments focusing on both the effects of rhizodeposition and plant tissue composition (litter).

In order to evaluate the effects of GM-crops on soil fungi combined methods for molecular fingerprinting and measurements of fungal enzymes involved in degradation of organic matter in the soil were developed and used throughout this thesis. As a proof of principle for the methods developed, the dynamics of fungi in fields cropped with the GM, parental- and four other cultivars were evaluated in a one-year field experiment. These experiments revealed that the effects of the GM-cultivar 'Modena' on rhizosphere fungal communities and functionality were similar to that of its parental isoline 'Karnico' in most of the measured parameters.

In chapter 4 the normal variability of agricultural practice related factors measured during the field experiments were evaluated. The plant growth stage was found to have the largest effect on rhizosphere fungal communities. The highest biomass of fungi was found in the senescence stage of growth, although different fungal groups had different patterns in their abundance. The second most influential factor for the soil fungi was the year when fields were sampled. This factor comprises both climatic factors as well as agricultural practices. Notably, in our study we detected more ascomycetes and less basidiomycetes and fungi in general in 2010 compared to 2008 in both fields which might be an indication of changed community structure due to an altered regime of fertilizer treatment.

Surprisingly, despite the strong differences in soil organic matter content, the field location did not substantially affect the community function or diversity of the higher fungi and results from the two fields could be even combined for baseline purposes. Despite some differences in fungal-related parameters between individual cultivars, there were no directional effects and most of the differences observed were not consistent between fields and years. Furthermore, the GM-variety 'Modena' was not significantly different from its parental variety 'Karnico' in any measured parameter and it seemed that these cultivars had a very similar effect on both the structure and function of soil fungal communities. The only more lasting effect was the difference in the amount of fungi in the rhizosphere of these cultivars in one field in the stage of senescence in 2008, 2009 and 2010. This phenomenon was further investigated in detail in chapter 5.

The aim of chapter 5 was to gain insight in the carbon flow from the roots of a GM-

potato cultivar and its parental isoline to the soil fungal community using stable isotope probing (SIP). This experiment was carried out with soil collected from one of the fields presented in chapters 3 & 4 and growth stage of senescence was investigated to confirm the observed differences in the field experiment. The microbes receiving ^{13}C from the plant were assessed temporarily through RNA/PLFA-SIP at three time points and the communities of Ascomycota, Basidiomycota and Glomeromycota were analysed separately. Ascomycetes and glomeromycetes received carbon from the plant already 1 and 5 days after labeling, respectively, while basidiomycetes were slower in accumulating the labeled carbon. The rate of carbon allocation by the GM-variety differed from its parental variety, thereby affecting the soil fungal communities. Both saprotrophic and mycorrhizal fungi are rapidly metabolizing organic substrates flowing from the root into the rhizosphere, there are large differences in utilization of root-derived compounds at a lower phylogenetic level within the investigated fungal phyla, and active communities in the rhizosphere differ between GM-plant and its parental cultivar through effects of differential carbon flow from the plant.

The effects of potential unintended changes in plant composition, which could affect the soil fungal decomposer web, were evaluated in chapter 6. The potato plants grown in chapter 5 in a greenhouse were used, and tissues (tubers and leaves) were placed in litterbags into soil collected from the agricultural fields. The weight loss of both leaves and tubers in litterbags were analysed after 1, 3 and 6 months of incubation in soils and combined with measurements of fungal extracellular enzyme activities (laccases, Mn-peroxidases and cellulases) as well as molecular analyses of the fungal community functional and phylogenetic diversity. Chapter 6 revealed that initial decomposition of both tubers and leaves of the parental isoline was significantly faster than that of the GM-variety. This coincided with differences in fungal community composition. After this initial difference, no significant differences in any of the parameters measured could be detected after 3 and 6 months of decomposition, illustrating the transient nature of the initial difference between the cultivars. Hence, it can be concluded that the starch modified tubers do not bear any risk to fungal decomposer community and despite initial differences in decomposition, the total decomposition rate of the GM-variety is similar to its parental variety. This finding was confirmed in the field experiments as no long term effect of GM-variety could be detected in the fields in the following years.

In summary, detailed greenhouse studies revealed differences between GM- and its parental variety. The field studies, however, confirmed that these differences are transient in field conditions and that fungi in the potato rhizosphere are affected more by soil type, sampling year, plant growth stage and cultivar type than the genetic modification.

Summary

Samenvatting

Samenvatting

Transgene technieken zijn tegenwoordig een acceptabele manier om gewassen te verbeteren. Ondanks de toenemende hoeveelheid landbouwgrond die gebruikt wordt voor het verbouwen van genetisch gemodificeerde (GG) gewassen, zijn de effecten hiervan op de schimmelgemeenschap in de bodem grotendeels onbekend. Het doel van dit proefschrift was daarom om de effecten van GG aardappelen, die een andere zetmeelsamenstelling hebben, op de schimmel gemeenschap in de bodem te bepalen door te kijken naar veranderingen in wortel afscheiding en weefselsamenstelling in de planten. Daarbij zijn vergelijkingen gemaakt tussen de GG- (genaamd "Modena") en de moederplant (genaamd "Karnico"), in ogenschouw nemend dat natuurlijke variaties (jaar, klimaat, veldlocatie, groei stadium van de plant, verschillende aardappel soorten) al optreden bij gebruik van niet GG aardappelen. Allereerst zijn de methodes ontwikkeld die nodig zijn om deze evaluaties uit te voeren. Vervolgens zijn deze methodes gebruikt op 2 veldlocaties in een 3-jarig experiment om de natuurlijke variatie die verschillende aardappelsoorten hebben op de bodemschimmels te bepalen en in 2 kasexperimenten om de effecten op wortel uitscheiding en plantweefsel compositie te bestuderen.

Hoofdstuk 3 beschrijft de ontwikkeling van de methodes die het mogelijk maakten om de effecten van de GG-gewassen op de bodemschimmel samenstelling en functionaliteit te bestuderen die in de rest van het proefschrift gebruikt zijn. Om aan te tonen dat de methodes goed functioneren is in eerste instantie in een 1-jaars veld experiment gekeken naar de dynamiek van schimmels in velden waarop GG-aardappelen, de originele aardappelsoort en vier andere aardappelsoorten groeiden. Hieruit bleek onder andere dat de schimmelgemeenschapsamenstelling -en functionaliteit nagenoeg identiek was voor de GG-aardappel in vergelijking met de originele aardappel variëteit.

In hoofdstuk 4 is de normale variatie van landbouw gerelateerde activiteiten in het hierboven beschreven veldexperiment geëvalueerd. De groeifase van de plant bleek het grootste effect te hebben op de schimmel gemeenschap in de rhizosfeer. De grootste schimmelbiomassa werd gevonden in de fase waarin de aardappelen al verouderd waren, hoewel verschillende schimmelgroepen verschillende patronen in hun dominantie lieten zien. Ook werden er meer ascomyceten dan basidiomyceten en schimmels in het algemeen gezien in 2010 op beide proefvelden in vergelijking met 2008. Dit is een indicatie dat ook de veranderde bemestingsstrategie een invloed kan hebben op de schimmelgemeenschap. Ondanks het grote verschil in organisch materiaal in de grond van de twee proefvelden, bleek de veldlocatie verrassend genoeg geen invloed te hebben op de diversiteit en functionaliteit van de gemeenschap van hogere schimmels en de resultaten van de twee velden konden gecombineerd worden om de normale variatie te bepalen. Ondanks dat er verschillen werden gemeten in schimmel-gerelateerde parameters in de vergelijking van de verschillende aardappelsoorten, waren er geen direct gerelateerde effecten en waren de meeste verschillen niet consistent tussen de velden en de verschillende jaren. Verder werden er geen significante verschillen gemeten tussen de GG-soort "Modena" en de originele soort "Karnico", waarmee bewezen is dat deze twee aardappelsoorten een zeer vergelijkbaar effect hebben op de schimmel gemeenschap

en functionaliteit. Het enige langere termijn verschil was het verschil in aantallen schimmels in de rhizosfeer in één veld in de verouderingsgroeifase van de aardappelen in 2008, 2009 en 2010. Dit fenomeen werd verder onderzocht in hoofdstuk 5.

Het doel van hoofdstuk 5 was om inzicht te krijgen in de koolstof distributie van de wortels van GG- en de originele aardappel naar de schimmel gemeenschap in de grond wat onderzocht werd met "stable isotope probing" (SIP). Dit experiment werd uitgevoerd met grond verkregen van één van de velden beschreven in hoofdstuk 3 & 4 en de verouderde groeifase werd onderzocht om de geobserveerde verschillen in het veldexperiment te bevestigen. De micro-organismen die 13C van de plant ontvingen, werden bepaald met RNA/PLFA-SIP op 3 tijdstippen en de schimmelgemeenschappen Ascomycota, Basidiomycota en Glomeromycota werden gescheiden geanalyseerd. Ascomyceten en glomeromyceten ontvingen al koolstof van de plant respectievelijk 1 en 5 dagen na de labeling, terwijl basidiomyceten langzamer waren in het opnemen van gelabeld koolstof. De snelheid van koolstofafscheiding van de GG-aardappelen was anders dan van de originele variant waardoor de schimmel gemeenschap werd beïnvloed. Er kon ook geconcludeerd worden dat saprotropische en mycorrhizale schimmels snel organische substraten kunnen metaboliseren die van de wortel naar de rhizosfeer gaan. Verder waren er grote verschillen in het gebruik van componenten afkomstig van de wortel op een lager fylogenetisch niveau binnen de onderzochte fyla. De actieve gemeenschap in de rhizosfeer is verschillend voor GG-planten en de originele planten wat wordt veroorzaakt door de effecten van verschillende koolstof bewegingen vanaf de planten.

Het effect van potentieel onbedoelde veranderingen in plant compositie, die mogelijk schimmels binnen het bodem voedselweb beïnvloeden, werd onderzocht in hoofdstuk 6. De aardappelplanten die voor hoofdstuk 5 in een kas waren gegroeid werden gebruikt en de bladeren en (poot)aardappelen werden in zakken gedaan met grond afkomstig van de veldlocaties. Het gewichtsverlies van zowel de composterende bladeren als pootaardappelen werd geanalyseerd na 1, 3 en 6 maanden incubatie in de bodemmonsters en de data werd gecombineerd met metingen van extracellulaire enzym-activiteiten van de schimmels (laccase, Mn-peroxidase en cellulase) en moleculaire analyse van de schimmel gemeenschap functionaliteit en fylogenetische diversiteit. Deze experimenten toonden aan dat de initiële decompositie van de bladeren en pootaardappelen sneller verliep met de originele aardappelsoort, wat ook leidde tot een andere schimmel gemeenschap samenstelling. Na dit initiële verschil werden geen verschillen meer gemeten na 3 en 6 maanden decompositie wat aangeeft dat het initiële verschil niet tot langdurige effecten leidt. Daarom kan geconcludeerd worden dat zetmeel-gemodificeerde aardappelen geen risico vormen voor de schimmel gedomineerdevoedselketen in de bodem en dat ondanks het initieel gemeten verschil de overall decompositie snelheid van de GG-aardappelsoort vergelijkbaar is met die van de originele aardappelsoort. Deze bevinding werd bevestigd in de veldexperimenten.

Samenvatting

Tiivistelmä

Viime vuosina geenimuuntelusta on tullut hyväksyttävä keino muunnella kasvien perimää. Vaikka maailmanlaajuisesti maa-ala, jolla näitä muuntogeenisiä kasveja kasvatetaan, on kasvanut vuosittain, muuntogeenisten kasvien vaikutusta maaperän sieniyhteisöihin ei tunneta. Tämän väitöskirjan tavoitteena on arvioida geenimuunnellun perunan vaikutuksia maaperän sieniyhteisöihin. Mahdollisia eroja perinteisen jalostuksen kautta luotuihin perunalajikkeisiin ovat muutokset juurien maahan erittämissä hiiliyhdisteissä sekä itse kasvin koostumuksessa, mikä voi vuorostaan vaikuttaa maatumiseen. Tavoitteena on verrata geenimuuntelulla luotua lajiketta sen muuntelemattomaan alkuperäislajikkeeseen sekä neljään muuhun perinteiseen lajikkeeseen. Vertailu toteutettiin sekä kolmen vuoden kenttäviljelykokeilla, joiden aikana perinteisten lajikkeiden vaikutuksia maaperän sieniin tutkittiin ja verrattiin geenimuunneltuun lajikkeeseen sekä kahdessa kasvihuonekokeessa, joissa vaikutuksia juurieritykseen ja kasvin osien maatumiseen tutkittiin tarkemmin. Jotta geenimuunnellun vaikutuksia sieniyhteisöihin voitaisiin arvioida, on ensin arvioitava itse perunan, eri perunalajikkeiden, perunan kasvuvaiheen sekä sään, maalajin ja ilmaston vaikutuksia maaperän mikrobeihin. Lisäksi on kehitettävä luotettavia metodeja arvioida vaikutuksia.

Kappaleessa 3 esitellään metodologia, jota myös myöhemmissä kappaleissa käytetään arvioimaan sieniyhteisön koostumusta ja toiminnallisuutta. Tässä kappaleessa myös arvioidaan geenimuunnellun ja viiden muun lajikkeen vaikutuksia eri sieniyhteisöihin yhden kasvukauden koneessa. Yhteisön koostumusta tutkittiin molekylaarisilla sormenjälkitekniikoilla ja yhdistettiin perinteisempiin funktionaalisuuta mittaaviin entsymaattisiin mittauksiin. Sieniyhteisö geenimuunnellun lajikkeen juuristossa oli hyvin samankaltainen kuin sen alkuperäislajikkeen juuristossa.

Kappaleessa 4 arvioitiin maatalouskäytäntöjen sekä maaperätyypin ja sään vaikutuksia sieniyhteisöihin. Kolmen vuoden kenttäkokeessa kasvin kasvuvaiheella oli suurin vaikutus kaikista tutkituista tekijöistä juuriston sieniyhteisöihin. Eniten sienirihmastoa havaittiin ränsistymisvaiheessa (EC90). Toinen sieniyhteisöön vaikuttava tekijä oli vuosi ja siten sää ja maatalouskäytännöt kuten lannoitus ja kastelu. Lannoitus vaikutti erityisesti kantasieniä vähentävästi ja kotelosieniä lisäävästi.

Yllättäen, vaikka maaperän orgaanisen aineen koostumuksenon aikaisemmissa tutkimuksissa todettu vaikuttavan maaperän mikrobiyhteisöihin, tässä tutkimuksessa ei eroja kahden hyvin erilaisen maaperän, ja hyvin erilaisen orgaanisen aineen pitoisuuden välillä havaittu. Vaikka joissain mitatuissa muutujissa havaittiin eroja eri perunalajikkeiden välillä, erot sieniyhteisöissä olivat pieniä ja sattumanvaraisia. Geenimuunneltu lajike (Modena) muistutti lähinnä sen ei-muunneltua vastaavaa lajiketta (Karnico) eikä niiden välillä ollut suuria merkittäviä eroja. Ainoastaan ränsistymisvaiheessa olevien perunoiden juuristossa havaittiin eroja sienten määrässä kaikkina tutkimusvuosina toisella pelloista. Tätä ilmiötä tarkastellaan lähemmin kasvihuonetesteissä.

Kappaleen 5 tavoitteena oli tutkia juurieritteiden ja niistä hyötyvien sienien eroja GM-perunan ja sen alkuperäis lajikkeen välillä. Tämä toteutettin stabiilien isotooppien avulla eli antamalla hiilen luonnostaan painavampaa isotooppia (^{13}C) kasveille hiilidioksidin muodossa ($^{13}CO_2$). Tutkimusta varten maaperä kerättiin

kappaleissa 3 & 4 käytetyltä pellolta ja perunakasvit kasvatettiin ränsistymisvaiheeseen kasvihuoneessa, jonka jälkeen painavaa hiilidioksidia lisättiin. Tämän kulkua lehdistä juuriin ja edelleen maaperän mikrobeille seurattiin molekulaarisilla metodeilla eri ajankohtina. Tutkimuksessa havaittiin että kotelosienet ja arbuskulaarinen mykorritsa olivat ensimmäisiä juuresta saadun hiilen saajia. Kantasienet hyötyivät hiilestä hitaammin, mahdollisesti välikäsien kautta. Hiilen kierron nopeus ja osuus hiilestä joka päätyi maahan oli erilainen GM-perunan ja sen alkuperäislajikkeen välillä samalla vaikuttaen myös maaperän sieniyhteisöihin. Sekä hajottajasienet että mykorritsasienet kykenivät nopeasti käyttämään juuresta tihkuvia hiiliyhdisteitä, mutta eri sienilajeilla oli erilaisia strategioita ja nopeuksia näiden yhdisteiden käyttämiseen.

Kappaleessa 6 arvioitiin perunan tärkkelysmuuntelun vaikutuksia maaperän hajoittajayhteisöihin sekä sadonkorjuun jälkeen pelloille jätetyiden kasvien osien hajoamiseen. Tässä tutkimuksessa käytettiin samoja kasveja kuin kappaleessa 5. Kasveista kerättiin mukulat ja lehdet, mitkä haudattiin karikepussissa samaan maahan missä kasvit olivat kasvaneet. Sekä mukuloiden että lehtien painon vähenemistä seurattiin yhteensä kuusi kuukautta, ja sekä karikkeesta että mullasta tutkittiin sienten entsyymiaktiivisuutta ja sieniyhdeisöiden koostumusta ja toimintaa. Tutkimuksessa havaittiin että ensimmäisen kuukauden aikana sekä mukulat että lehden, jotka olivat peräisin geenimuunnellusta lajikkeesta menettivät painoaan hitaammin kuin alkuperäislajikkeen vastaavat kasvinosat. Tällä oli yhteys myös sieniyhteisöön. Myöhemmissä mittaukissa eroja hajoamisnopeudessa tai sieniyhteisöissä ei enää havaittu, mikä kertoo alun havaittujen erojen olevan ohimeneviä. Myöskään peltokokeissa ei havaittu seuraavina vuosina minkäänlaisia vaikutuksia edellisvuoden geenimuunnelluistya perunoista.

Tiivistettynä, kasvihuonekokeissa havaittiin joitakin eroja geenimuunnellun lajikkeen ja sen alkuperäis lajikkeen välillä, mutta kenttäkokeissa havaittiin että muut tekijät kuten sää, maaperätyyppi ja perunan kasvuvaihe vaikuttavat juuriston sieniyhteisöihin paljon enemmän kuin yksi muunneltu geeni. Myös normaalien lajikkeiden välillä havaittiin eroja, jotka olivat suurempia kuin geenimuuntelun aiheuttamat.

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Curriculum vitae

Emilia Hannula was born on 4th of November 1983 in Helsinki, Finland. She received her secondary education in Ressun lukio in Helsinki. In 2003 she started her University education at the University of Helsinki, at the department of Ecology and Environmental Science. During summers and breaks from university she worked as an assistant in a familiar breast cancer research group in Biomedicum. In 2005, after showing interest in microbes and selecting Microbial Ecology as her major topic, she started her Master project on the effect of diesel and its removal on the Baltic Sea bacteria under supervision of Prof. Dr. M. Romantschuk. For this she received a grant from University of Helsinki. During summer of 2005 she had an internship alongside with master thesis related experiments on quantification of bacteria in different stages of composting at Lahden tutkimuslaboratoriot. She finished her Master thesis and obtained her master degree in 2007. The same year she started her PhD project described in this thesis at the Center of Terrestrial Ecology of the Netherlands Institute of Ecology (NIOO-KNAW), under the supervision of Prof. Dr. J.A. van Veen and Prof. Dr. W. de Boer. This project was funded by NWO project ERGO on Ecology regarding genetically modified organisms. In January 2012 she continued work in NIOO as post-doc in an EU funded EcoFINDERS project. She is currently focused on soil fungal community functioning in European soils.

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