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^{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,

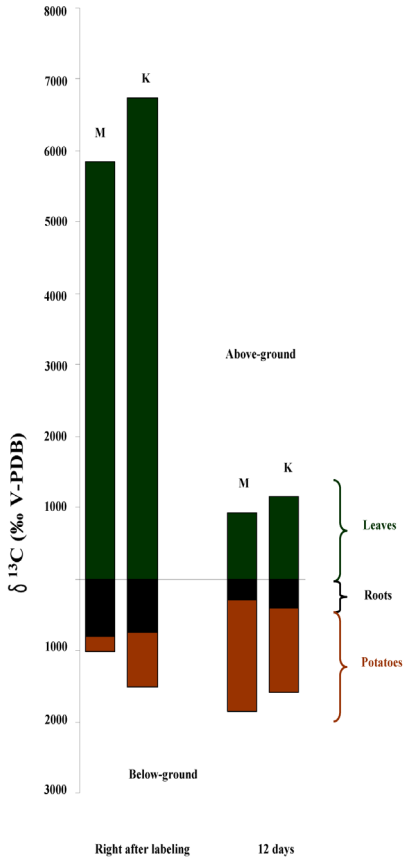

Figure 5.1. Distribution of ^{13}C in potato plants and rhizosphere microbes. The ^{13}C content in different parts of labeled potato plants is expressed as excess compared to non-labeled control plants harvested at the same time and separated in above-ground parts (leaves and stem combined) and below-ground 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 ‰).

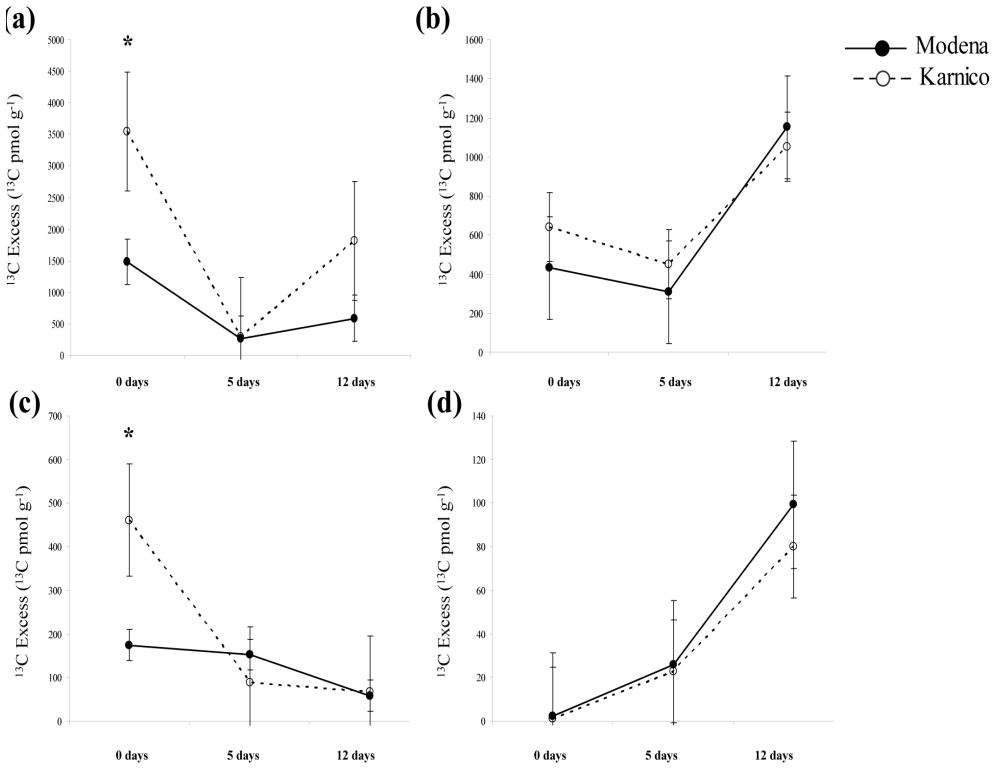

Figure 5.2. The amount of excess ^{13}C in different microbial groups as measured by PLFA analyses. The incorporation of ^{13}C into the markers was calculated for (a) fungi, (b) bacteria, (c) AMF and (d) protozoa based on markers specific to these groups mentioned in text in three time points. The closed 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

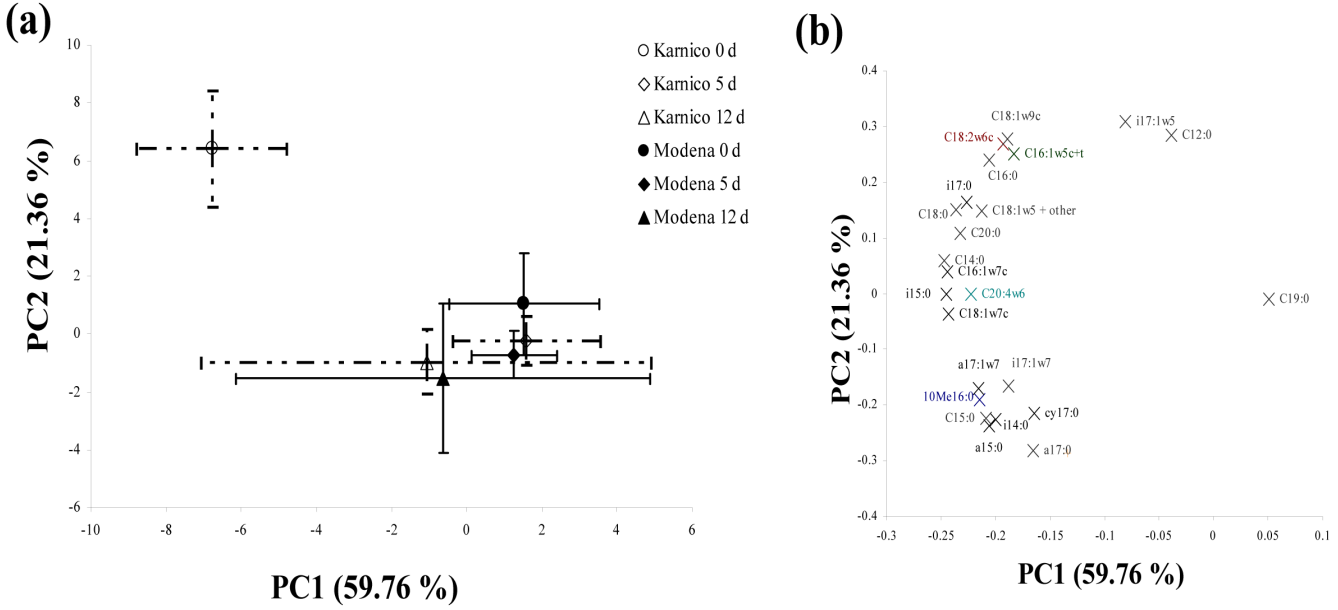

Figure 5.3. PCA analyses of the labeled PLFA (excess ^{13}C pmol g^{-1}) patterns of the rhizosphere of both cultivars at all time points (a) and PLFAs explaining this pattern (b). The closed symbols and solid variances represent the GM-variety 'Modena' and open symbols and dotted line parental cultivar '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'

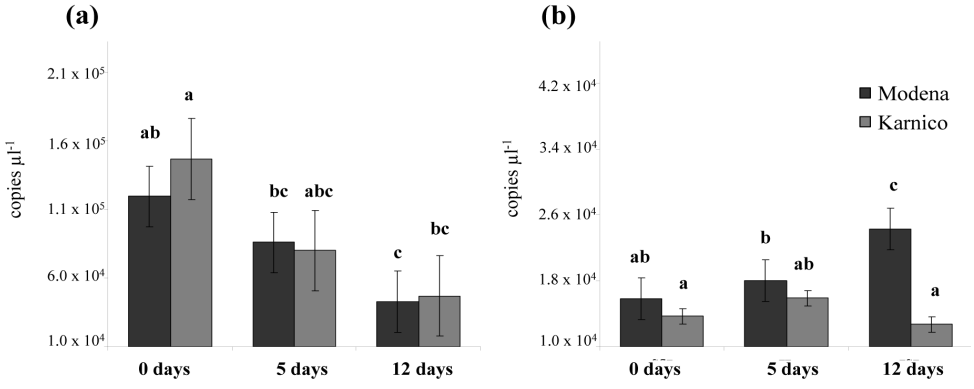

Figure 5.4. ITS copy numbers of (A) *Ascomycota* and (B) *Basidiomycota* in the heavy fraction at different time points after labeling. The first bars represent the genetically modified cultivar 'Modena' and second bar its parental cultivar 'Karnico'. Letters above bars indicate significant differences 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 %.

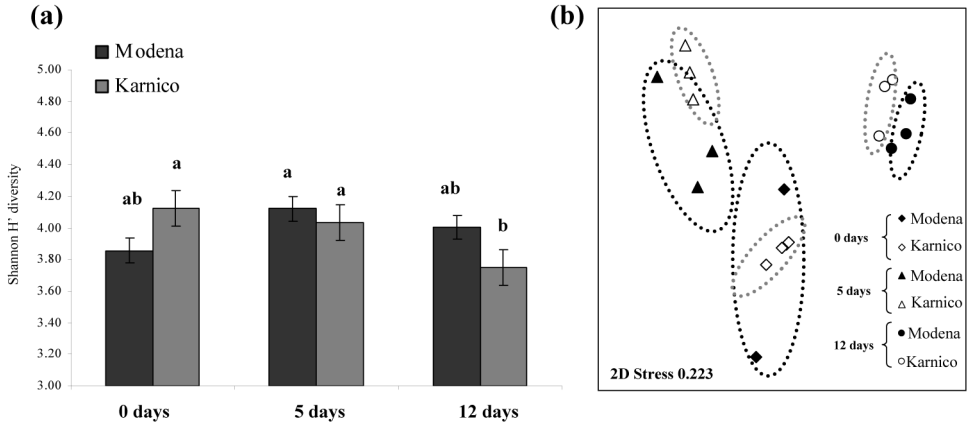

Figure 5.5. Diversity (a) and community structure (b) of all active (labeled RNA pool) fungal groups combined. (a) Black bars represent average diversity (n=3) (±SD) of fungi in the rhizosphere of 'Modena' and gray bars average diversity (±SD) in the rhizosphere of 'Karnico' at three different time points 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

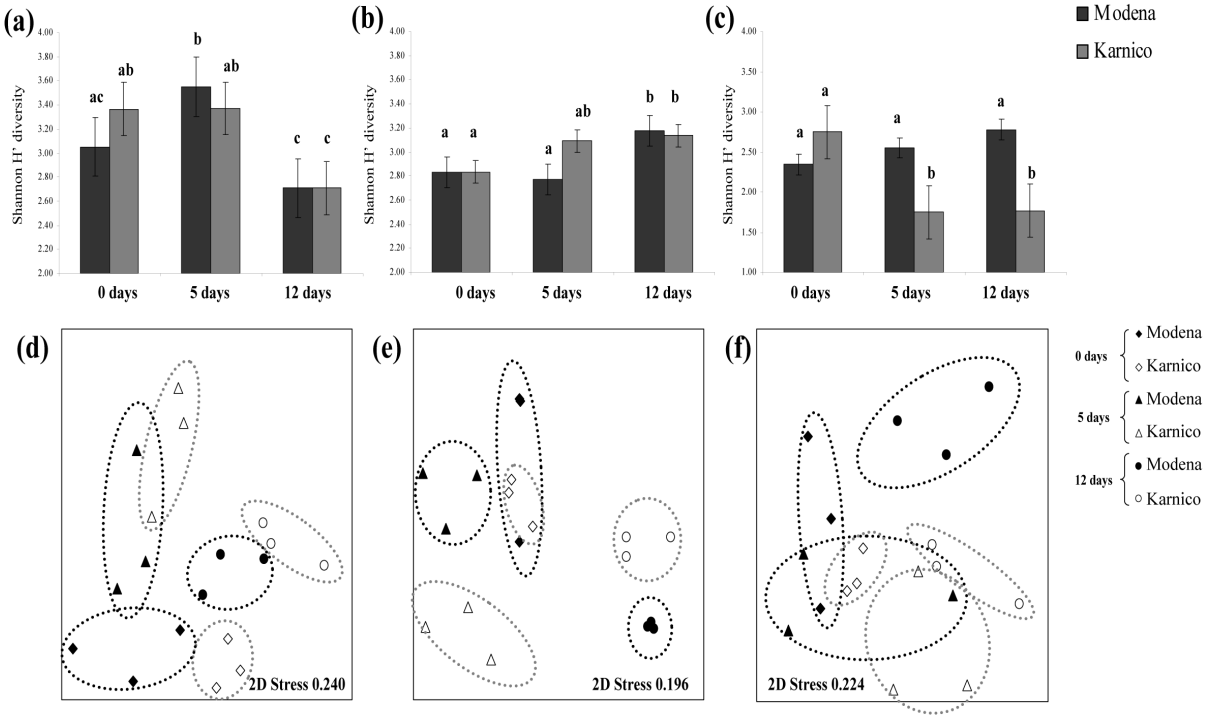

Figure 5.6. Diversity (a-c) and community structure (d-e) of *Ascomycota* (a & d), *Basidiomycota* (b & e) and *Glomeromycota* (c & f) in the ^{13}C-enriched fraction of RNA extracted from potato rhizosphere soil. Bars represent average diversity (n=3) (±SD) of (a) ascomycetes, (b) basidiomycetes and (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