ANNEX 6

PUBLICATIONS

© 2013 BASF Plant Science Company GmbH. All Rights Reserved.

This document is protected under copyright law. This document and the information contained herein are confidential and are for use only by the regulatory authority to which it has been submitted by BASF Plant Science Company GmbH ("BPS"), and only in support of actions requested by BPS. Any other use of this document and the information contained herein requires the prior written consent of BPS. The submission of this document by BPS shall not be construed as granting of any rights or licenses.

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

S. E. Hannula[1], H. T. S. Boschker[2], W. de Boer[1] and J. A. van Veen[1,3]

[1]Netherlands Institute of Ecology (NIOO-KNAW), PO Box 50, 6708 PB Wageningen, the Netherlands; [2]Centre for Estuarine and Marine Ecology, Netherlands Institute of Ecology (NIOO-KNAW), PO Box 140, 4400 AC Yerseke, the Netherlands; [3]Insititute of Biology, Leiden University, Leiden, the Netherlands

Author for correspondence:
Emilia Hannula
Tel: +31 317 47 35 07
Email: e.hannula@nioo.knaw.nl

Received: *3 December 2011*
Accepted: *25 January 2012*

New Phytologist (2012) **194**: 784–799
doi: 10.1111/j.1469-8137.2012.04089.x

Key words: arbuscular mycorrhizal fungi (AMF), Ascomycota, Basidiomycota, genetically modified (GM) crops, phospholipid fatty acid analysis with stable isotope probing (PLFA-SIP), RNA-stable isotope probing (SIP), stable isotope.

Summary

- The aim of this study was to gain understanding of the carbon flow from the roots of a genetically modified (GM) amylopectin-accumulating potato (*Solanum tuberosum*) cultivar and its parental isoline to the soil fungal community using stable isotope probing (SIP).
- The microbes receiving ^{13}C from the plant were assessed through RNA/phospholipid fatty acid analysis with stable isotope probing (PLFA-SIP) at three time-points (1, 5 and 12 d after the start of labeling). The communities of Ascomycota, Basidiomycota and Glomeromycota were analysed separately with RT-qPCR and terminal restriction fragment length polymorphism (T-RFLP).
- Ascomycetes and glomeromycetes received carbon from the plant as early as 1 and 5 d after labeling, while basidiomycetes were slower in accumulating the labeled carbon. The rate of carbon allocation in the GM variety differed from that in its parental variety, thereby affecting soil fungal communities.
- We conclude that both saprotrophic and mycorrhizal fungi rapidly metabolize 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 the GM plant and its parental cultivar through effects of differential carbon flow from the plant.

Introduction

It has been estimated that 20–50% of the carbon obtained by plants via photosynthetic assimilation is transferred to the roots and about half of this is further released into the soil (Kuzyakov & Domanski, 2000). This release of exudates strongly affects the soil microbial composition and activity close to the roots, giving rise to the so-called rhizosphere effect (Lynch & Whipps, 1990; Jones *et al.*, 2009). Although the rhizosphere effect has mostly been studied in bacteria, an increasing number of studies indicate 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 (*Solanum tuberosum*) cultivars in intensively managed agricultural soils (Hannula *et al.*, 2010). 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 rhizosphere microbial communities to root exudates (Kuzyakov & Domanski, 2000). One method that has proved to be very useful is the application of different carbon isotopes in tracking ^{13}C 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 analysis in combination with stable isotope probing (PLFA-SIP) has indicated that fungi are a very important group of organotrophic organisms in the rhizosphere and even inside roots receiving considerable amounts 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 the 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 provide information on the identity of the active fungi. It is known that the diversity of fungi in soils is enormous and their functions range from obligate mutualists (Glomeromycota) to saprobes and pathogens (Ascomycota and Basidiomycota), all of which are very important in the rhizosphere (Carlile *et al.*, 2001; Buée *et al.*, 2009). All three fungal phyla are influenced by the

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

plant in one way or another, but the relationships between plants and individual fungal taxa or even species are not known (Christensen, 1989; Broeckling *et al.*, 2008; Buée *et al.*, 2009).

The high variation in rhizodeposition patterns among plant species suggests that genetic modification in plants, especially if the modification targets carbon-related compounds, could result in a change in carbon allocation patterns and thus may give rise to shifts in the abundance of fungal species. It has been reported that carbon allocation within plants is strongly regulated by genotype and stage of development. Several studies (Milling *et al.*, 2004; Götz *et al.*, 2006; Griffiths *et al.*, 2007; O'Callaghan *et al.*, 2008; Weinert *et al.*, 2009; Hannula *et al.*, 2010) have provided information on the effects of transgenic crops on soil bacterial and fungal communities, but only a 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 the genetically modified (GM) potato (*Solanum tuberosum*) cultivar 'Modena', which has 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 targets a biosynthetic pathway, it was hypothesized that this could also result in changes in the 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.

Materials and Methods

Glasshouse experiment and ^{13}C labeling

A glasshouse experiment was performed to compare a GM potato (*Solanum tuberosum* L.) 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 an 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 Hannula *et al.*, 2010) 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, and pH 5.0. The soil was homogenized and sieved (< 2 mm) and transferred to pots (volume 10 l). One tuber of either cultivar was planted per pot and the plants were grown in the glasshouse 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 (Hannula *et al.*, 2010). The day:night period was set at 16 h : 8 h and the maximum daily temperature was *c.* 22°C. Triplicate pots with soil but without plants (bulk soil) were incubated under the same conditions and used as controls to assess the possible accumulation of labeled carbon by fungi without the 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 airtight growth chamber for a total of 30 h. The same number of plants were 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 throughout the experiment. Before 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 h in the light, interrupted by 6 h of nonlabeling 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 h in the light. The total amount of $^{13}CO_2$ added to the chamber was 25 l.

Harvest

After the labeling period, all the pots were removed from the chambers and the rhizosphere soil of three replicate plants per cultivar was immediately harvested from both the $^{13}CO_2$ and $^{12}CO_2$ treatments. The rhizosphere soil was collected by brushing the roots and immediately frozen in liquid nitrogen and kept at −80°C until 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) was kept separate, frozen and freeze-dried for use in the lipid fatty acid analyses.

Shoot, leaves, roots and tubers were collected and weighed, and tuber production was estimated. Representative samples of plant parts were frozen, freeze-dried and kept at −80°C until further analyses were performed.

The same harvesting procedure was repeated 5 and 12 d after the end of the labeling period to monitor the carbon flow. These sampling dates were selected based on the findings of previous studies (Drigo *et al.*, 2010).

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

Freeze-dried plant parts were ground to a mesh size of 0.1 μm. The δ^{13}C value of these samples was analyzed using an elemental analyzer coupled to an isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany) to determine the amount of photosynthates allocated to above- and below-ground parts.

The incorporation of ^{13}C into plants was expressed as the increase in the δ^{13}C value relative to the δ^{13}C values of unlabeled control plants (Δδ^{13}C values). Isotope ratios and atom% of ^{13}C were calculated using the equations described previously (Werner

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

& Brand, 2001). Vienna PeeDee Belamnite (V-PDB) was used as reference material.

PLFAs 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). PLFA 10Me16:0 was used as a specific indicator for actinomycetes (Frostegård *et al.*, 1993). PLFA 18:2ω6.9 was considered an indicator for fungal biomass (Bååth, 2003; Bååth & Anderson, 2003). Unfortunately, the neutral lipid (NLFA) extractions were not successful and we could not relate the NLFA marker to the PLFA marker. Thus, the PLFA 16:1ω5, which is found mainly in arbuscular mycorrhizal fungi (AMF) and which often correlates to 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 into 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.

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 Life Technologies, Carlsbad, CA, USA), inspected for its integrity using the Experion RNA StdSens Analysis System (Experion™; Bio-Rad Laboratories Inc., Hercules, CA, USA) and stored at − 80°C. Total RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Bio-Rad Laboratories Inc.). ^{13}C-enriched RNA was separated from nonlabeled RNA by density-gradient centrifugation and analyzed 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 containing fractions with ^{13}C-enriched RNA and later fractions containing unlabeled ^{12}C RNA. The ^{12}C-labeled plants were used as controls and analyzed in the same way as the ^{13}C-labeled plants.

RT-qPCR and terminal restriction fragment length polymorphism (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 (RevertAid™ First Strand cDNA Synthesis Kit; Fermentas, Burlington, Ontario, Canada). The cDNA produced was further used to quantify the internal transcribed spacer (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.

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 performed using primers and conditions presented in Table 1 and restriction was carried out according to Hannula *et al.* (2010).

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

Table 1 Primers, PCR conditions and enzymes used for restriction analyses

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

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

'heavy' and 'light' fragments were purified with the Qiaqen PCR purification kit (Qiagen, Valencia, CA, USA) 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, Southampton, UK) with a vector:insert ratio of 3 : 1. Approximately 50 successful transformants per time and fragment, that is, 'heavy' and 'light', were selected for amplification, restriction digestion 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) according to manufacturer's instructions and further used for qPCR analyses.

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 statistical analyses were performed on the original nontransformed values.

The quality of T-RFLP data was first visually inspected in GeneMapper software v4.1 (Applied Biosystems, Carlsbad, CA, USA) and then transferred to T-Rex (Culman *et al.*, 2009). True peaks were identified as those in 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 Terminal restriction fragments (TRFs) obtained with different restriction enzymes and labels were correlated (Spearman two-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 a different fraction) was deleted from further analyses. Nonmetric multidimensional scaling (NMDS) with Jaccard as a 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 Analyses of similarity (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 & Dickie, 2007). Three out of four of the enzyme–primer combinations within a 1.5-bp margin had to be found 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 the Shannon–Weaver H' diversity index and a diversity *t*-test was used to compare diversities. All statistical analyses were performed in the statistical program PAST (Hammer *et al.*, 2001).

The PLFA ^{13}C-labeling data were evaluated with principal component analyses (PCAs) and multivariate analysis of variance (MANOVA) was used to determine the overall effects of time and cultivar on mole percentages and $\delta^{13}C$ values of PLFAs compared with the controls.

Results

^{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 (Fig. 1). The ^{13}C values in the control plants were in the normal range (on average

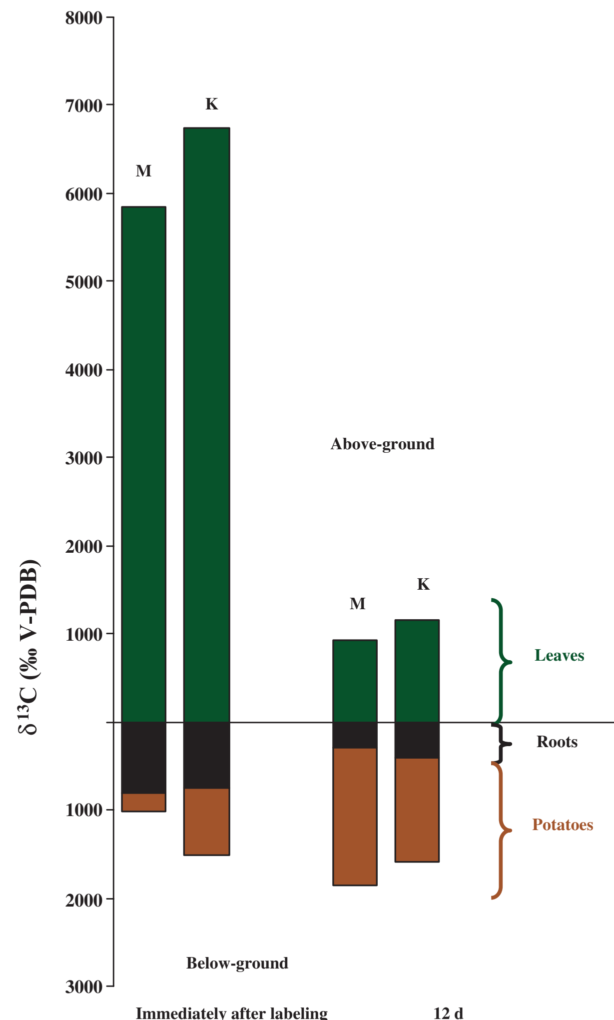

Fig. 1 Distribution of ^{13}C in potato (*Solanum tuberosum*) plants and rhizosphere microbes. The ^{13}C content in different parts of labeled potato plants is expressed as excess compared with nonlabeled control plants harvested at the same time and separated into above-ground parts (leaves and stem combined) and below-ground parts (roots and potatoes). The first columns represent the genetically modified (GM) variety 'Modena' (M) and the second columns its parental isoline 'Karnico' (K). The natural isotopic signatures of the control plants were the same for both cultivars (average $\delta^{13}C$ −28%).

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

δ^{13}C –28%). The amount of labeled carbon in the roots was highest at the first sampling (Fig. 1). This indicates a rapid flux of labeled carbon into the rhizosphere in the very early stages of the experiment. After the first sampling time, the amount of labeled carbon became diluted by ongoing photosynthesis and 12 d after labeling only 35% (significantly less after 12 d than immediately 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 time-point (12 d 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 d than immediately after labeling. After 5 d of labeling, there was a difference between cultivars, but the ^{13}C data for 'Karnico' did not fit into the pattern of other harvests and might thus not be reliable (data not shown).

Directly after labeling, the ^{13}C content of the GM cultivar and its parental cultivar did not differ significantly, either in their total plant biomass or for any of the plant parts. Analysis of ^{13}C 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. 2). Total ^{13}C in below-ground parts of the plant was positively correlated with the 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 the amount of label in root samples ($r = 0.68$, $P < 0.001$) and in the 16:1ω5 marker ($r = 0.70$, $P < 0.001$). Further, the amounts 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 those in any other markers (Fig. 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 d), fungal PLFAs again contained more ^{13}C than bacterial PLFAs in the rhizosphere of 'Modena' but not in that of 'Karnico' (Fig. 2). The total enrichment of ^{13}C at the first sampling was higher in rhizosphere PLFAs of cultivar 'Karnico' than in those of 'Modena' (Fig. 2). However, this difference appeared

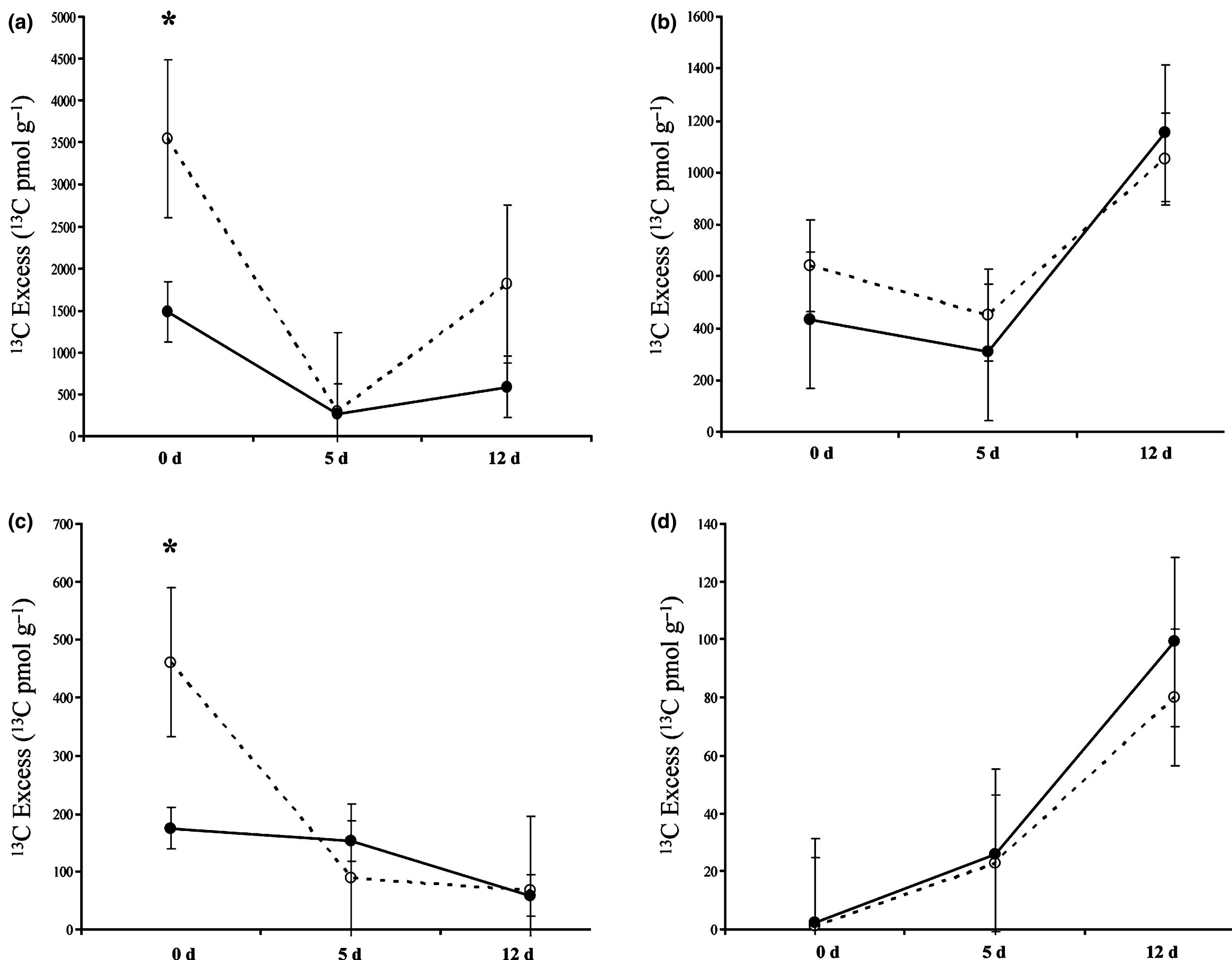

Fig. 2 The amount of excess ^{13}C in different microbial groups as measured by phospholipid fatty acid (PLFA) analyses. The incorporation of ^{13}C into the markers was calculated for (a) fungi, (b) bacteria, (c) arbuscular mycorrhizal fungi (AMF) and (d) protozoa based on markers specific to these groups mentioned in text at three time-points. Closed circles, genetically modified (GM) potato (*Solanum tuberosum*) variety 'Modena'; open circles, its parental cultivar 'Karnico'. PLFAs used as indicators for the different microbial groups are given in the Materials and Methods section. Note that all axes are different and ordered from highest to lowest.

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

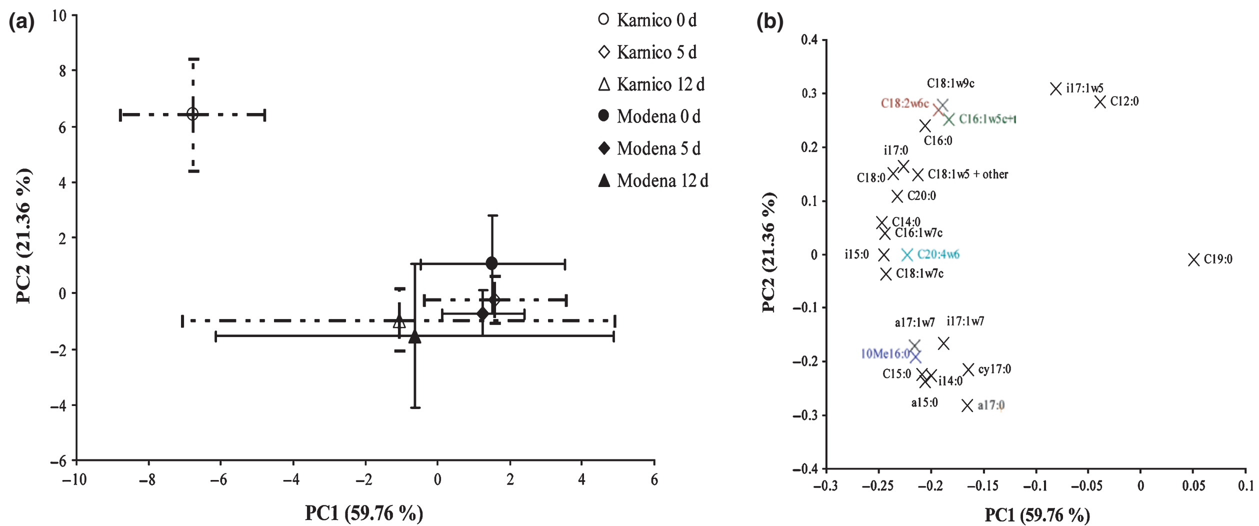

Fig. 3 Principal component analyses (PCAs) of the labeled phospholipid fatty acid (PLFA) (excess ^{13}C pmol g^{-1}) patterns of the rhizospheres of both cultivars at all time-points (a) and PLFAs explaining this pattern (b). Closed symbols and solid variances, the genetically modified (GM) potato (*Solanum tuberosum*) variety 'Modena'; open symbols and dotted line, parental cultivar 'Karnico'. The variance is based on triplicates of each treatment. Black, the bacterial PLFAs explaining the patterns; red, fungal marker; green, arbuscular mycorrhizal fungi (AMF); blue, actinomycetes; gray, nonidentified. For grouping of the PLFAs, see text.

to be caused by a higher accumulation of ^{13}C in fungal PLFAs ($F = 7.098$, $P = 0.04$) but not for any other group. In the rhizospheres of both cultivars, the amounts of ^{13}C in bacterial PLFAs were similar for the first two sampling periods but increased 12 d after labeling (Fig. 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 with '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 varieties (Fig. 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. 3b).

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 d after labeling was ten times higher for Ascomycota than for Basidiomycota. Furthermore, for 'Modena', copy numbers of Ascomycota and Basidiomycota showed opposite temporal patterns (Fig. 4). There were no significant differences in total ('heavy' and 'light' fractions 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. 4a). The decrease in the labeled ITS copy numbers of ascomycetes with a 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% immediately after labeling, 56% and 49% after 5 d and 28% and 27% after 12 d for 'Modena' and 'Karnico, respectively. The ^{13}C-enriched ITS copy numbers of basidiomycetes also did not 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 d) 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 with the total number of copies ranged from 35% to 51%.

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. 5a, Table 2). There were no significant differences

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

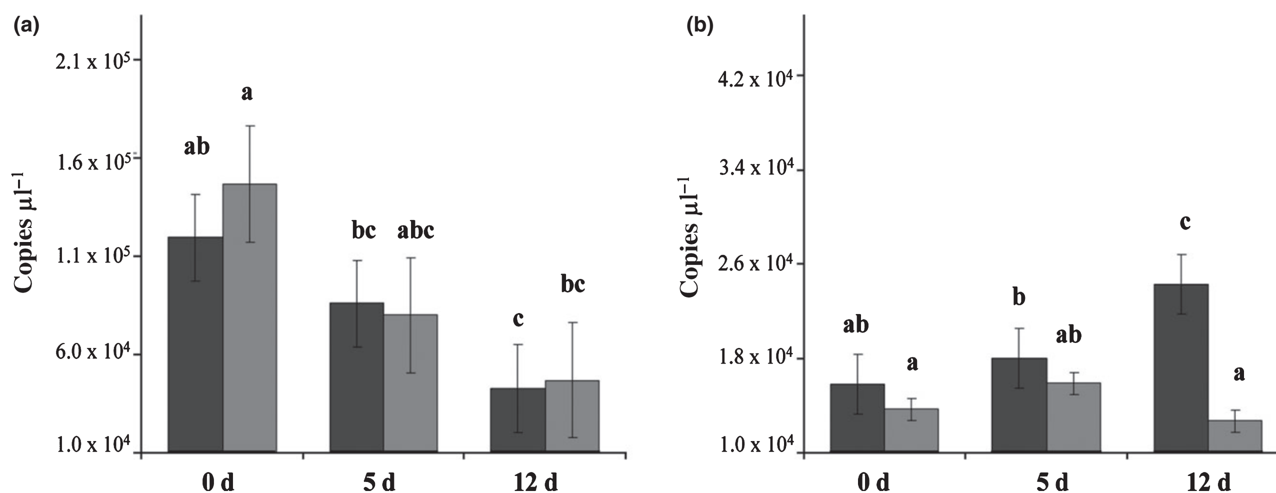

Fig. 4 Internal transcribed spacer (ITS) copy numbers of (a) Ascomycota and (b) Basidiomycota in the heavy fraction at different time-points after labeling. Dark gray bars, genetically modified (GM) potato (*Solanum tuberosum*) cv 'Modena'; light gray bars, its parental cultivar 'Karnico'. Letters above bars indicate significant differences at the level of $P < 0.05$. Note that the axes of (a) and (b) are not the same.

between the cultivars, although the diversity in the ^{13}C fraction was lower in the rhizosphere of 'Modena' 12 d after labeling compared with 'Karnico' ($t = 1.68$, $P = 0.09$). This was mainly a result of a 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 d but not at the earlier sampling dates (Fig. 5b). Sampling time was a strong factor affecting the fungal community structure in the heavy fraction (ANOSIM: $R = 0.977$, $P < 0.001$) (Fig. 5b). All time points were significantly different from each other ($R > 0.92$ and $P < 0.005$).

There were no significant differences between cultivars or harvest times in the total number of ascomycetal TRFs, that is, when light and heavy fractions were combined. The diversity of ascomycetes ranged from 2.71 in the fraction labeled with ^{13}C sampled after 12 d to 3.55 on day 5 (Fig. 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, respectively (Table 3). Five days later 'Modena' and 'Karnico' had 29 and 35 TRFs active in their rhizospheres, 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 different between the time-points (ANOSIM: $R = 0.5$, $P < 0.001$) (Fig. 6d). Although the number of active ascomycetal OTUs did not differ significantly between cultivars,

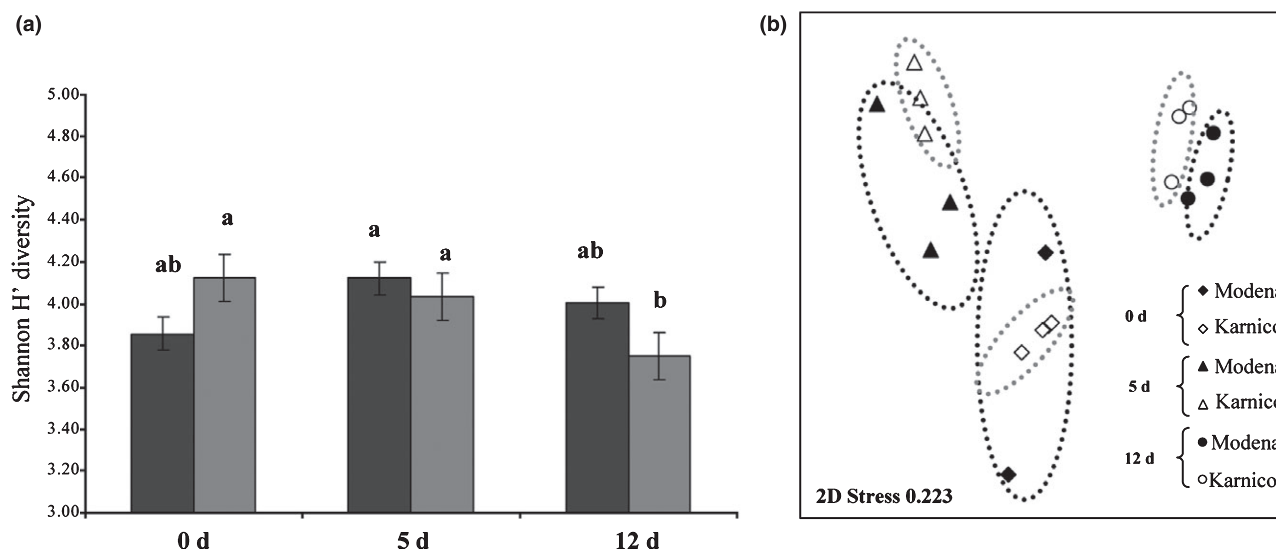

Fig. 5 Diversity (a) and community structure (b) of all active (labeled RNA pool) fungal groups combined. (a) Black bars represent average diversity ($n = 3$) (± SD) of fungi in the rhizosphere of potato (*Solanum tuberosum*) 'Modena', and gray bars average diversity (± SD) in the rhizosphere of 'Karnico' at three different time-points after $^{13}CO_2$ pulse-labeling. Letters above bars indicate significant differences in diversity (diversity *t*-test) at the level of $P < 0.05$. (b) In the nonmetric multidimensional scaling (NMDS) plot: open symbols, the parental variety; closed symbols, the genetically modified (GM) variety. Circles around samples are distinct cultivar and time combinations.

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

Table 2 Diversity of terminal restriction fragments (TRFs) and identified fungal operational taxonomic units (OTUs) (at different taxonomic levels) in the heavy RNA fraction immediately after labeling and 1, 5 and 12 d after labeling and in the light fraction (combined)

		Right after labeling		5 d after labeling		12 d 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	2.42	2.38	2.79	2.85	2.52	2.42	2.67	2.78
	Number of orders	15	16	22	21	15	14	20	22
	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	1.15	1.07	1.67	1.91	1.79	1.65	1.71	1.82
	Number of orders	4	4	7	8	7	6	7	8
	Shannon - H′ classes	0.67	0.69	0.85	0.65	0.45	0.50	0.71	0.68
	Number of classes	2	2	3	3	2	2	3	3
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 closest species match (% identity) was obtained by comparison to known species in GenBank using BlastN. The assignment into orders is based on this similarity.

the community structure did (ANOSIM, $R > 0.5$, $P < 0.05$) at time-points 0 and 12 d after labeling (Fig. 6d).

Not only were there fewer copies of Basidiomycota than of Ascomycota, but the diversity of basidiomycetes in the ^{13}C fraction was also lower, with *c.* 20 TRFs, of which about half could be identified (Table 2). The basidiomycete diversity increased with sampling time (Fig. 6b). The diversity of active basidiomycetes was not significantly different between cultivars 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. 6e) and cultivar. In addition, the cultivar affected the community structure at the last two sampling points ($R = 0.97$, $P < 0.001$).

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 immediately after labeling (Fig. 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 d after labeling. The community fingerprints were, however, not significantly different between the cultivars (Fig. 6f), probably because of the low amount of TRFs and thus the lack of statistical power.

Species composition of active community

The observed differences in community fingerprints and diversities can be partly explained by differences in the species identified (Table 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 h from 'Karnico' and later also from 'Modena', and some OTUs showing the opposite ('Phy1' and 'US3'). Furthermore, some OTUs in the heavy fraction were only detected under one of the

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

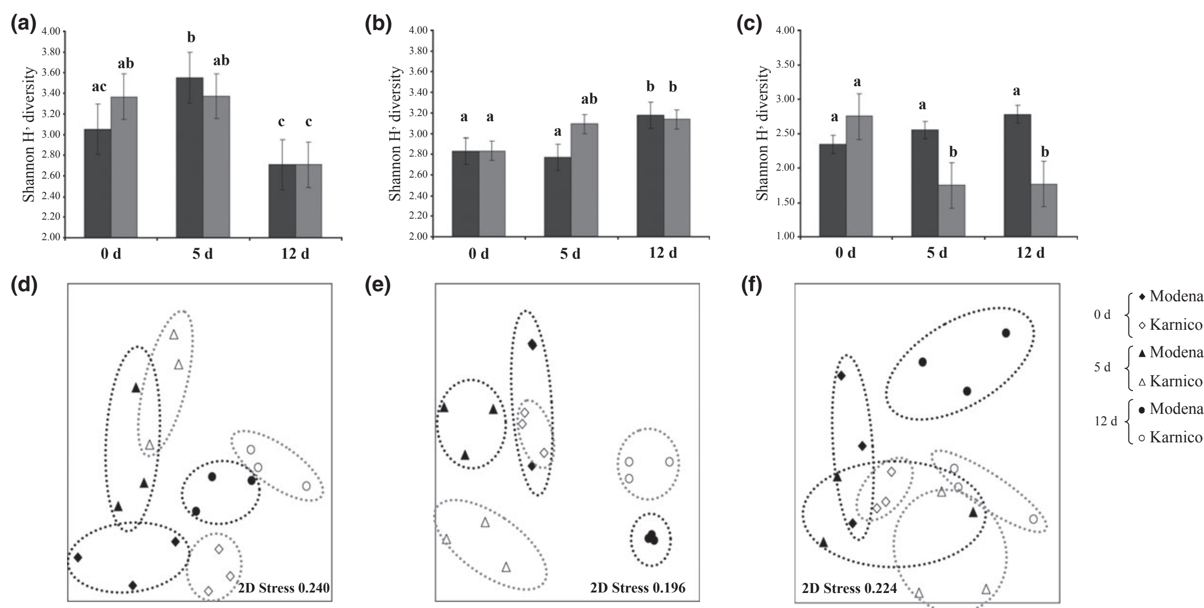

Fig. 6 Diversity (a–c) and community structure (d, e) of Ascomycota (a, d), Basidiomycota (b, e) and Glomeromycota (c, f) in the ^{13}C-enriched fraction of RNA extracted from potato (*Solanum tuberosum*) rhizosphere soil. Bars represent average diversity (n = 3) (± SD) of (a) ascomycetes, (b) basidiomycetes 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 nonmetric multidimensional scaling (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.

cultivars. Nine OTUs were found to receive labeled carbon only in the rhizosphere of 'Karnico' and four OTUs were found only in the fraction with ^{13}C in the rhizosphere of 'Modena' (Table 3). This might explain the observed differences in the community structure when the diversity was similar. The differences in observed OTU composition had only a minor impact at the level of orders (Table 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 at later measuring times Agaricales, Cantharellales and Corticiales dominated 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 5 d after labeling (Fig. 4) can be explained by delayed labeling of a few OTUs ('Cor1', 'Cor3' 'Can1' and 'Hym1') in the rhizosphere of 'Karnico'. All of these OTUs were detected after 5 d in the rhizosphere of 'Modena' but only after 12 d in the rhizosphere of 'Karnico'.

There were in total 13 OTUs of Glomeromycota identified to be active in the rhizosphere during this experiment (Table 3). The differences seen in 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 immediately after the labeling but not from cultivar 'Modena' at the later stages.

Discussion

^{13}C distribution in the plants

Immediately after labeling, a substantial amount of ^{13}C was transferred to the roots (Fig. 1). This is in accordance with earlier findings of quick allocation of carbon to the roots by grassland species (Vandenkoornhuyse *et al.*, 2007). No significant differences were detected in the initial amounts of labeled carbon in roots between the GM cultivar and its parental cultivar. Similar observations were reported in a previous study on earlier phenological stages of potato (Gschwendtner *et al.*, 2011).

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 amounts 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 the 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. 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. Vandenkoornhuyse *et al.* (2007) showed a rapid (within 5 h) incorporation of carbon into the RNA of endophytic bacteria, but studies based on PLFAs have

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

Table 3 Distribution of identified OTUs in the rhizosphere of the genetically modified (GM) variety and its parental isoline at different time-points (immediately after labeling and 5 and 12 d after labeling) in the heavy fraction and the commonly occurring (all time-points) OTUs in the light fraction, indicated as presence-absence

				Right after labeling		5 d after labeling		12 d after labeling		Light Fractions	
Phyla	Name	Order	Closest species (% identity)	Karnico	Modena	Karnico	Modena	Karnico	Modena	Karnico	Modena
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* strain CCM F-11883 (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* strain SH-f13 (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
	Hyp9	Hypocreales	*Nectria* sp. ASIN2 (DQ779785) (100)	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
	Hyp1 2	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
	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

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

Table 3 (*Continued*)

Phyla	Name	Order	Closest species (% identity)	Right after labeling		5 d after labeling		12 d after labeling		Light Fractions	
				Karnico	Modena	Karnico	Modena	Karnico	Modena	Karnico	Modena
	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 miniglutinans* (FJ946483) (94)			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	*Naucoria bohemica* (FJ904179) (97)					X	X	X	X
	Aga2	Agaricales	*Campanella subdendrophora* (AY445118) (95)					X	X	X	X
	Aga3	Agaricales	*Rhodocybe mundula* (DQ089017) (98)					X	X	X	X
	Aga4	Agaricales	*Camarophyllopsis schulzeri* (GU187661) (94)			X	X	X	X	X	X
	Can1	Cantharellales	*Rhizoctonia solani* (EU730860) (97)	X		X	X	X	X	X	X
	Can2	Cantharellales	*Uthatobasidium fusisporum* (AF518593) (96)						X	X	X
	Can3	Cantharellales	*Thanatephorus cucumeris* (HM625913) (98)				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
	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

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

Table 3 (*Continued*)

Phyla	Name	Order	Closest species (% identity)	Right after labeling		5 d after labeling		12 d after labeling		Light Fractions	
				Karnico	Modena	Karnico	Modena	Karnico	Modena	Karnico	Modena
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

Total fungi are calculated by combining the three phyla. The letters behind numbers in level of TRFs and OTUs indicate significance at $P < 0.05$. The OTUs are assigned to orders as presented in Table 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).

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

detected slower incorporation of the carbon into lipids (Rinnan & Bååth, 2009). In our study, 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 an indicator for fungi. The use of this marker as an indicator of fungal biomass is often debated, but, as we found a highly significant positive correlation between PLFA 18:2ω6.9 and active fungal ITS copy number, we conclude that this marker is a useful indicator of fungal biomass in the rhizosphere, despite the presence of living roots (Frostegård *et al.*, 2011). Earlier studies also showed that fungi quickly incorporate carbon from plants into their phospholipids (Lu *et al.*, 2007; Wu *et al.*, 2009; Drigo *et al.*, 2010; Gschwendtner *et al.*, 2011). Another large part (*c.* 9% of the total in the first sampling) of the total ^{13}C was detected in PLFA 16:1ω5, mainly representing AMF (Olsson & 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 such as intensively managed agricultural soils (Cesaro *et al.*, 2008; Cheeke *et al.*, 2011). Yet, results obtained for earlier developmental stages of potato were similar, 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. 4, table 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 of rhizodepositions. In addition to the fast accumulators, we detected another group of fungi benefiting from plant-derived carbon at later time-points after labeling and probably 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 the findings of 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 as a consequence of their inability to gain access to the interior of the root. The PLFA marker for protozoa (20:4ω6), which is not known to be able to use plant-derived carbon readily, revealed a delayed response to the ^{13}C addition, possibly because they were feeding on labeled bacteria or fungi.

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 the fungal community changed in favor of (basidiomycetal and ascomycetal) species probably better adapted to a 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, for example, 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 3). These OTUs are expected to be good competitors for simple root exudates but not for more complicated carbon sources and thus active only immediately after labeling. Orders typically receiving carbon immediately from the plant were the basidiomycetal Tremellales and the ascomycetal Capnodiales while basidiomycetal orders Agaricales, Cantharellales, Sordariales, 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 immediately labeling, while the other OTUs assigned to the same order received heavy carbon only 5 or 12 d later. These observed differences between individual OTUs within orders indicate differences between closely related species with respect to carbon resource utilization. The high number of OTUs closely related to known decomposer species can partly be attributed to the late phenological stage at which the labeling was performed. Although no senescent leaves were allowed to drop onto the soil, we could detect sequences from orders with many known decomposer species receiving labeled carbon, especially 5 and 12 d after labeling (Table 3), indicating that, in addition to root exudates, there might be another pathway by which the fungi receive carbon, probably via decomposing dead root material. (Dennis *et al.*, 2010).

Effect of GM trait on active soil microbial communities

PLFA analyses showed no overall effect of cultivar (GM vs 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. 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 carried out on plants with the same genetic modification (although in a different soil) using PLFA markers revealed no significant differences between the cultivar with the GM trait and its parental isoline in fungal biomass or plant exudation patterns (Gschwendtner *et al.*, 2011). However, that study was carried out in the earlier growth stages EC30 and EC60, while

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

our study focused on the senescent stage EC90. This can explain the differences in the results, as it has been shown that the amount of carbon allocated to the roots increases with increasing age of the plant and initiation of carbon storage structures (i.e. tubers in potato) (Timlin *et al.*, 2006), making any differences more obvious in later growth stages. These age-dependent exudation patterns might explain the differences in the outcomes of earlier studies conducted on GM plants, as they were carried out on plants at different growth stages (Rossi *et al.*, 2007; Wu *et al.*, 2009; Gschwendtner *et al.*, 2011), thus confirming the importance of considering the plant phenological state when designing experiments (van Overbeek & van Elsas, 2008; Weinert *et al.*, 2010). Indeed, it was previously shown that differences between this GM cultivar and its parental variety in carbon allocation below-ground and microbial communities in the field could be seen at the stage of senescence (Hannula *et al.*, 2010).

While some studies reported effects of modified crops on soil bacterial numbers (Siciliano & Germida, 1999; Dunfield & 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 has detected significant cultivar-dependent differences (Milling *et al.*, 2004; Götz *et al.*, 2006; Hart *et al.*, 2009). The approach of using RNA-SIP on fungal communities as a tool to investigate the side effects of GM plants is very promising, as it allows differences between a GM cultivar and the parental variety to be detected. Previously, Rasche *et al.* (2009) investigated differences in shoot endophytic bacteria between two cultivars of potato using DNA-SIP and found a cultivar-related shift in bacterial communities after 4 d of labeling, very similar to the differences that we observed here for soil fungal communities. In this study, we showed that a potato cultivar 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. In contrast, using PLFA-SIP as an indicator of microbial communities under *Bacillus thuringiensis* (Bt) rice (*Oryza sativa*) and its parental isoline, Wu *et al.* (2009) did not find differences in ^{13}C distribution in roots or the 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. 6). Previously, Vandenkoornhuyse *et al.* (2007) observed differences in active glomeromycete communities between plant species and explained this 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. Previously, some studies carried out on Bt maize (*Zea mays*) isolines expressing Cry1Ab protein 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 rhizospheres of the two cultivars could be explained by the 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 glasshouse conditions between the GM crop and its parental isoline, caution in extrapolating these results to the field scale is warranted. Earlier field observations did not reveal significant differences in bacterial or fungal communities between this GM cultivar and its parental cultivar (Hannula *et al.*, 2010; Inceoglu *et al.*, 2010), although differences between the two were greatest at the stage of senescence, probably as a result of differences in rhizodeposition (Weinert *et al.*, 2009). Moreover, comparing the GM cultivar only with its parental variety and neglecting intraspecific variation in carbon distribution can cause false significant results, especially when evaluating potential risks of GM crops (Hannula *et al.*, 2010). Differences between cultivars in their carbon allocation patterns should be investigated to strengthen the results presented here.

Conclusions

We conclude that both saprotrophic and mycorrhizal fungi rapidly metabolize 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 attributable to different compositions of rhizodeposits. The differences in carbon allocation and microbial communities assimilating carbon between the GM cultivar and its parental variety, although plausible, may not reflect long-term effects in natural systems. However, the current study was carried out especially 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.

Acknowledgements

The authors would like to thank Nadia Marttin, Agata Pijl and Henk Duyts for technical assistance and Peter Bruinenberg from AVEBE/AVERIS seeds for providing the tubers and soil material. This research was financed by ERGO grant number 838.06.052 of the Netherlands Organization for Scientific Research. This is publication 5211 of the Netherlands Institute of Ecology (NIOO-KNAW).

References

Abdo Z, Schuette UME, Bent SJ, Williams CJ, Forney LJ, Joyce P. 2006. Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. *Environmental Microbiology* **8**: 929–938.

Bååth E. 2003. The use of neutral lipid fatty acids to indicate the physiological conditions of soil fungi. *Microbial Ecology* **45**: 373–383.

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

Bååth E, Anderson TH. 2003. Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. *Soil Biology & Biochemistry* **35**: 955–963.
Boschker HTS. 2004. Linking microbial community structure and functioning: stable isotope (^{13}C) labeling in combination with PLFA analysis. In: Kowalchuk GA, de Bruijn FJ, Head IM, Akkermans AD, van Elsas JD, ed. *Microbial ecology*. Dordrecht, the Netherlands: Kluwer Academic Publishers, 1673–1688.
Boschker JT, Nold SC, Wellsbury P, Bos D, de Graaf W, Pel R, Parkes RJ, Cappenberg TE. 1998. Direct linking of microbial populations to specific biogeochemical processes by ^{13}C-labelling of biomarkers. *Nature* **392**: 801–805.
Botha A. 2011. The importance and ecology of yeasts in soil. *Soil Biology & Biochemistry* **43**: 1–8.
Broeckling CD, Broz AK, Bergelson J, Manter DK, Vivanco JM. 2008. Root exudates regulate soil fungal community composition and diversity. *Applied and Environmental Microbiology* **74**: 738–744.
Buée M, De Boer W, Martin F, van Overbeek L, Jurkevitch E. 2009. The rhizosphere zoo: an overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant and Soil* **321**: 189–212.
Butler JL, Williams MA, Bottomley PJ, Myrold DD. 2003. Microbial community dynamics associated with rhizosphere carbon flow. *Applied and Environmental Microbiology* **69**: 6793–6800.
Carlile MJ, Watkinson SC, Gooday GW. 2001. *The fungi*. San Diego, CA, USA: Academic Press.
Cesaro P, van Tuinen D, Copetta A, Chatagnier O, Berta G, Gianinazzi S, Lingua G. 2008. Preferential colonization of *Solanum tuberosum* L. roots by the fungus *Glomus intraradices* in arable soil of a potato farming area. *Applied and Environmental Microbiology* **74**: 5776–5783.
Cheeke TE, Pace BA, Rosenstiel TN, Cruzan MB. 2011. The influence of fertilizer level and spore density on arbuscular mycorrhizal colonization of transgenic Bt 11 maize (*Zea mays*) in experimental microcosms. *FEMS Microbiology Ecology* **75**: 304–312.
Christensen M. 1989. A view of fungal ecology. *Mycologia* **81**: 1–19.
Culman S, Bukowski R, Gauch H, Cadillo-Quiroz H, Buckley D. 2009. T-REX: software for the processing and analysis of T-RFLP data. *BMC Bioinformatics* **10**: 171.
De Graaff MA, Classen AT, Castro HF, Schadt CW. 2010. Labile soil carbon inputs mediate the soil microbial community composition and plant residue decomposition rates. *New Phytologist* **188**: 1055–1064.
Denef K, Bubenheim H, Lenhart K, Vermeulen J, van Cleemput O, Boeckx P, Müller C. 2007. Community shifts and carbon translocation within metabolically-active rhizosphere microorganisms in grasslands under elevated CO_2. *Biogeosciences* **4**: 769–779.
Dennis PG, Miller AJ, Hirsch PR. 2010. Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? *FEMS Microbiology Ecology* **72**: 313–327.
Drigo B, Pijl AS, Duyts H, Kielak A, Gamper HA, Houtekamer MJ, Boschker HTS, Bodelier PLE, Whiteley AS, van Veen JA *et al.* 2010. Shifting carbon flow from roots into associated microbial communities in response to elevated atmospheric CO_2. *Proceedings of the National Academy of Sciences, USA* **107**: 10938–10942.
Dunfield KE, Germida JJ. 2001. Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica napus*. *FEMS Microbiology Ecology* **38**: 1–9.
Fitzjohn RG, Dickie IA. 2007. TRAMPR: an R package for analysis and matching of terminal-restriction fragment length polymorphism (TRFLP) profiles. *Molecular Ecology Notes* **7**: 583–587.
Frostegård A, Tunlid A, Bååth E. 1993. Phospholipid fatty-acid composition, biomass, and activity of microbial communities from 2 soil types experimentally exposed to different heavy-metals. *Applied and Environmental Microbiology* **59**: 3605–3617.
Frostegård Å, Tunlid A, Bååth E. 2011. Use and misuse of PLFA measurements in soils. *Soil Biology & Biochemistry* **43**: 1621–1625.
Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for Basidiomycetes – Application to the identification of Mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.
Girlanda M, Bianciotto V, Cappellazzo GA, Casieri L, Bergero R, Martino E, Luppi AM, Perotto S. 2008. Interactions between engineered tomato plants expressing antifungal enzymes and nontarget fungi in the rhizosphere and phyllosphere. *FEMS Microbiology Letters* **288**: 9–18.
Gollotte A, van Tuinen D, Atkinson D. 2004. Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaris* and *Lolium perenne* in a field experiment. *Mycorrhiza* **14**: 111–117.
Götz M, Nirenberg H, Krause S, Wolters H, Draeger S, Buchner A, Lottmann J, Berg G, Smalla K. 2006. Fungal endophytes in potato roots studied by traditional isolation and cultivation-independent DNA-based methods. *FEMS Microbiology Ecology* **58**: 404–413.
Griffiths BS, Heckmann LH, Caul S, Thompson J, Scrimgeour C, Krogh PH. 2007. Varietal effects of eight paired lines of transgenic Bt maize and near-isogenic non-Bt maize on soil microbial and nematode community structure. *Plant Biotechnology Journal* **5**: 60–68.
Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ. 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environmental Microbiology* **66**: 5488–5491.
Gschwendtner S, Esperschütz J, Buegger F, Reichmann M, Müller M, Munch JC, Schloter M. 2011. Effects of genetically modified starch metabolism in potato plants on photosynthate fluxes into the rhizosphere and on microbial degraders of root exudates. *FEMS Microbiology Ecology* **76**: 564–575.
Hack H, Gal H, Klemke T, Klose R, Meier U, Strauss R, Witzenberger A. 2001. The BBCH scale for phenological growth stages of potato (*Solanum tuberosum* L.). In: Meier U, ed. *Growth stages of mono- and dicotyledonous plants, BBCH Monograph*. Berlin, Braunchweig, Germany: Federal Biological Research Centre for Agriculture and Forestry, 7–16.
Hammer Ø, Harper DAT, Ryan PD. 2001. PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4**: 9.
Hannula SE, de Boer W, van Veen JA. 2010. *In situ* dynamics of soil fungal communities under different genotypes of potato, including a genetically modified cultivar. *Soil Biology & Biochemistry* **42**: 2211–2223.
Harman GE, Howell CR, Viterbo A, Chet I, Lorito M. 2004. *Trichoderma* species–opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology* **2**: 43–56.
Hart MM, Powell JR, Gulden RH, Dunfield KE, Pauls KP, Swanton CJ, Klironomos JN, Antunes PM, Koch AM, Trevors JT. 2009. Separating the effect of crop from herbicide on soil microbial communities in glyphosate-resistant corn. *Pedobiologia* **52**: 253–262.
Inceoglu O, Salles JF, van Overbeek L, van Elsas JD. 2010. Effects of plant genotype and growth stage on the betaproteobacterial communities associated with different potato cultivars in two fields. *Applied and Environmental Microbiology* **76**: 3675–3684.
Jones DL, Nguyen C, Finlay RD. 2009. Carbon flow in the rhizosphere: carbon trading at the soil–root interface. *Plant and Soil* **321**: 5–33.
Kowalchuk GA, Bruinsma M, van Veen JA. 2003. Assessing responses of soil microorganisms to GM plants. *Trends in Ecology & Evolution* **18**: 403–410.
Kuzyakov Y, Domanski G. 2000. Carbon input by plants into the soil. Review. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* **163**: 421–431.
Larena I, Salazar O, Gonzalez V, Julian MC, Rubio V. 1999. Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. *Journal of Biotechnology* **75**: 187–194.
Lu YH, Abraham WR, Conrad R. 2007. Spatial variation of active microbiota in the rice rhizosphere revealed by *in situ* stable isotope probing of phospholipid fatty acids. *Environmental Microbiology* **9**: 474–481.
Lu Y, Murase J, Watanabe A, Sugimoto A, Kimura M. 2004. Linking microbial community dynamics to rhizosphere carbon flow in a wetland rice soil. *FEMS Microbiology Ecology* **48**: 179–186.

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

Lynch JM, Whipps JM. 1990. Substrate flow in the rhizosphere. *Plant and Soil* **129**: 1–10.

Manefield M, Whiteley AS, Griffiths RI, Bailey MJ. 2002. RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Applied and Environmental Microbiology* **68**: 5367–5373.

Mauclaire L, Pelz O, Thullner M, Abraham WR, Zeyer J. 2003. Assimilation of toluene carbon along a bacteria-protist food chain determined by ^{13}C-enrichment of biomarker fatty acids. *Journal of Microbiological Methods* **55**: 635–649.

Mestre CM, Rosa CA, Safar SVB, Libkind D, Fontenla SB. 2011. Yeast communities associated with the bulk-soil, rhizosphere and ectomycorrhizosphere of a *Nothofagus pumilio* forest in Northwestern Patagonia, Argentina. *FEMS Microbiology Ecology* **78**: 531–541.

Milling A, Smalla K, Maidl FX, Schloter M, Munch JC. 2004. Effects of transgenic potatoes with an altered starch composition on the diversity of soil and rhizosphere bacteria and fungi. *Plant and Soil* **266**: 23–39.

O'Callaghan M, Gerard EM, Bell NL, Waipara NW, Aalders LT, David BB, Conner AJ. 2008. Microbial and nematode communities associated with potatoes genetically modified to express the antimicrobial peptide magainin and unmodified potato cultivars. *Soil Biology & Biochemistry* **40**: 1446–1459.

Olsson PA, Bååth E, Jakobsen I, Sölderström B. 1995. The use of phospholipid and neutral lipid fatty-acids to estimate biomass of arbuscular mycorrhizal fungi in soil. *Mycological Research* **99**: 623–629.

Olsson PA, Johnson NC. 2005. Tracking carbon from the atmosphere to the rhizosphere. *Ecology Letters* **8**: 1264–1270.

van Overbeek L, van Elsas JD. 2008. Effects of plant genotype and growth stage on the structure of bacterial communities associated with potato (*Solanum tuberosum* L.). *FEMS Microbiology Ecology* **64**: 283–296.

Radajewski S, Ineson P, Parekh NR, Murrell JC. 2000. Stable-isotope probing as a tool in microbial ecology. *Nature* **403**: 646–649.

Rangel-Castro JI, Killham K, Ostle N, Nicol GW, Anderson IC, Scrimgeour CM, Ineson P, Meharg A, Prosser JI. 2005. Stable isotope probing analysis of the influence of liming on root exudate utilization by soil microorganisms. *Environmental Microbiology* **7**: 828–838.

Rasche F, Lueders T, Schloter M, Schaefer S, Buegger F, Gattinger A, Hood-Nowotny RC, Sessitsch A. 2009. DNA-based stable isotope probing enables the identification of active bacterial endophytes in potatoes. *New Phytologist* **181**: 802–807.

Rinnan R, Bååth E. 2009. Differential utilization of carbon substrates by bacteria and fungi in tundra soil. *Applied and Environmental Microbiology* **75**: 3611–3620.

Rossi L, Costantini ML, Brilli M. 2007. Does stable isotope analysis separate transgenic and traditional corn (*Zea mays* L.) detritus and their consumers? *Applied Soil Ecology* **35**: 449–453.

Siciliano SD, Germida JJ. 1999. Taxonomic diversity of bacteria associated with the roots of field-grown transgenic *Brassica napus* cv. *Quest*, compared to the non-transgenic *B-napus* cv. *Excel* and *B. rapa* cv. *Parkland*. *FEMS Microbiology Ecology* **29**: 263–272.

Timlin D, Rahman SML, Baker J, Reddy VR, Fleisher D, Quebedeaux B. 2006. Whole plant photosynthesis, development, and carbon partitioning in potato as a function of temperature. *Agronomy Journal* **98**: 1195–1203.

Turrini A, Sbrana C, Nuti MP, Pietrangeli BM, Giovannetti M. 2004. Development of a model system to assess the impact of genetically modified corn and aubergine plants on arbuscular mycorrhizal fungi. *Plant and Soil* **266**: 69–75.

Van der Wal A, Van Veen JA, Smant W, Boschker HTS, Bloem J, Kardol P, Van der Putten WH, De Boer W. 2006. Fungal biomass development in a chronosequence of land abandonment. *Soil Biology & Biochemistry* **38**: 51–60.

Vandenkoornhuyse P, Mahe S, Ineson P, Staddon P, Ostle N, Cliquet JB, Francez AJ, Fitter AH, Young JPW. 2007. Active root-inhabiting microbes identified by rapid incorporation of plant-derived carbon into RNA. *Proceedings of the National Academy of Sciences, USA* **104**: 16970–16975.

de Vetten N, Wolters AM, Raemakers K, van der Meer I, ter Stege R, Heeres E, Heeres P, Visser R. 2003. A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. *Nature Biotechnology* **21**: 439–442.

Weinert N, Meincke R, Gottwald C, Heuer H, Gomes NCM, Schloter M, Berg G, Smalla K. 2009. Rhizosphere communities of genetically modified zeaxanthin-accumulating potato plants and their parent cultivar differ less than those of different potato cultivars. *Applied and Environmental Microbiology* **75**: 3859–3865.

Weinert N, Meincke R, Gottwald C, Heuer H, Schloter M, Berg G, Smalla K. 2010. Bacterial diversity on the surface of potato tubers in soil and the influence of the plant genotype. *FEMS Microbiology Ecology* **74**: 114–123.

Werner RA, Brand WA. 2001. Referencing strategies and techniques in stable isotope ratio analysis. *Rapid Communications in Mass Spectrometry* **15**: 501–519.

Wu WX, Liu W, Lu HH, Chen YX, Devare M, Thies J. 2009. Use of ^{13}C labeling to assess carbon partitioning in transgenic and nontransgenic (parental) rice and their rhizosphere soil microbial communities. *FEMS Microbiology Ecology* **67**: 93–102.

© 2012 The Authors
New Phytologist © 2012 New Phytologist Trust

A 3-Year Study Reveals That Plant Growth Stage, Season and Field Site Affect Soil Fungal Communities while Cultivar and GM-Trait Have Minor Effects

Silja Emilia Hannula[1]*, Wietse de Boer[1], Johannes van Veen[1,2]

1 Netherlands Institute of Ecology (NIOO-KNAW), Department of Microbial Ecology, Wageningen, The Netherlands, **2** Insititute of Biology, Leiden University, Leiden, The Netherlands

Abstract

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 are 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. To compare the effects, one GM cultivar, the parental isoline, and four non-related cultivars were planted in the fields and analysed 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 studied 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.

Citation: Hannula SE, de Boer W, van Veen J (2012) A 3-Year Study Reveals That Plant Growth Stage, Season and Field Site Affect Soil Fungal Communities while Cultivar and GM-Trait Have Minor Effects. PLoS ONE 7(4): e33819. doi:10.1371/journal.pone.0033819

Editor: Kevin McCluskey, University of Missouri, United States of America

Received December 6, 2011; **Accepted** February 17, 2012; **Published** April 17, 2012

Copyright: © 2012 Hannula et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This research was financed by ERGO grant number 838.06.052 of the Netherlands Organization for Scientific Research. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: e.hannula@nioo.knaw.nl

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) [1]. 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. These impacts may 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 [2].

The rhizosphere is a hot-spot of microbial abundance and metabolic activity due to the resources released by plants [3,4]. 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 [5,6].

The structure and functioning of soil microbial communities is affected by soil type [7–9], plant growth stage [7,10–13], and other abiotic and biotic factors such as agricultural management [14,15]. 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 [7,13,16–22], and only three studies [23–25] 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 [26]. 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.

Materials and Methods

Field Set-up and Sampling

Two agricultural sites VMD and BUI were selected for this experiment [19]. 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 S1. 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 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 P_2O_5 and 145–200 kg ha^{-1} potassium (K) as K_2O or K_2SO_4 in 2008 and 2009. In 2010 organic fertilizer in form of pig manure (14 ton ha^{-1}in field VMD and 25 ton ha^{-1} in field BUI, respectively) was added together with inorganic fertilizers (table S1). 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 [27]. 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) [28]. 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 $-80^{\circ}C$ for molecular analyses, another part was kept at $-20^{\circ}C$ prior to enzymatic analyses and ergosterol measurements and another part was used for immediate analyses of soil water content and pH (table S1). Soil water content was determined from fresh material as weight loss after overnight drying at $105^{\circ}C$.

Enzymatic Analyses

Quantification of ergosterol, via the alkaline extraction method, was used as an estimate of fungal biomass [29]. 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.* [30].

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 Hannula *et al.* [19]. Appropriate dilutions based on test runs of terminal restriction fragments (TRFs) were analyzed with an ABI 3130 sequencer using GeneScan^{TM} −500 LIZ (Applied Biosystems) and used as a size standard.

Clone libraries were constructed as described in Hannula *et al.* [19] 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) [31] 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.

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 [32]. 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 [33]. 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 [34]. In short, ANOSIM is a non-parametric test of significant differences between groups by comparing distances between groups to distances within groups. We used Jaccard as a distance index and 10000 permutations. Pairwise

Table 1. ANOVA comparisons of several fungal-related parameters between fields, years, growth stages, cultivars and GM-trait and the interaction effects of the cultivar.

	Field (df. 1)		Year (df.2)		Growth stage (df. 3)		Cultivar (df. 5)		GM- parent (df. 1)		Year×cultivar		Field×cultivar		Growth stage×cultivar		Year×Growth stage×field×cultivar	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Ergosterol (mg/g)	0.13	0.72	48.17	**<0.001**	19.38	**<0.001**	1.47	0.20	0.12	0.73	1.40	0.18	1.00	0.42	0.97	0.49	1.72	0.071
Laccases (µmol/g)	0.63	0.43	14.39	**<0.001**	21.19	**<0.001**	1.05	0.39	0.36	0.55	1.84	0.052	1.27	0.28	2.39	**0.004**	1.72	0.052
Mn-Peroxidases (µmol/g)	1.06	0.10	1.96	0.14	9.81	**<0.001**	3.31	0.06	0.67	0.42	1.02	0.43	1.07	0.38	1.86	**0.031**	1.69	**0.043**
Cellulases (µmol/g)	17.74	**<0.001**	23.94	**<0.001**	19.01	**<0.001**	1.08	0.37	0.04	0.83	4.03	**<0.001**	3.96	**0.002**	3.12	**<0.001**	1.29	0.35
# of Ascomycetes	0.41	0.52	6.28	**<0.001**	25.15	**<0.001**	1.51	0.19	2.73	0.11	0.72	0.69	0.48	0.79	2.67	**0.001**	1.38	0.16
# of Basidiomycetes	1.65	0.20	51.60	**<0.001**	20.14	**<0.001**	0.72	0.61	0.16	0.69	0.52	0.88	0.08	1.00	1.12	0.34	0.39	0.97
# of AMF	0.61	0.44	15.29	**<0.001**	6.09	**<0.001**	0.66	0.65	0.35	0.55	0.49	0.88	0.89	0.49	0.34	0.98	0.50	0.91

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.
doi:10.1371/journal.pone.0033819.t001

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.

Results

Soil Enzymatic Analyses, Fungal Biomass and Fungal Richness

Fungal-related parameters in plots cropped with the GM-variety seemed to fall within normal variation among potato cultivars observed in time (table 1). The largest explaining factor for most of the measured parameters was the plant phenological growth stage, followed by year and the soil type (table 1). Ergosterol analyses indicated that soil fungal biomass strongly dependent on plant growth stage and varied from year to year (table 1, Fig. 1). Although growth stage was affecting the fungal biomass, there were no significant differences between pre- and post-cropping situations or in bulk soils ($F = 1.31$, $p = 0.25$). 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' ($F = 4.131$ 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 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.1). The GM cultivar 'Modena' was not significantly different from the other cultivars or the parental variety (table 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 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 ($n = 702$, R^2 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 (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 ($R^2 = .11$ 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 $P < 0.005$).

The measured extracellular enzymes (laccases, Mn-peroxidases and cellulases) were all affected by plant growth stage; highest activities were measured during senescence (table 1, Fig. 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.

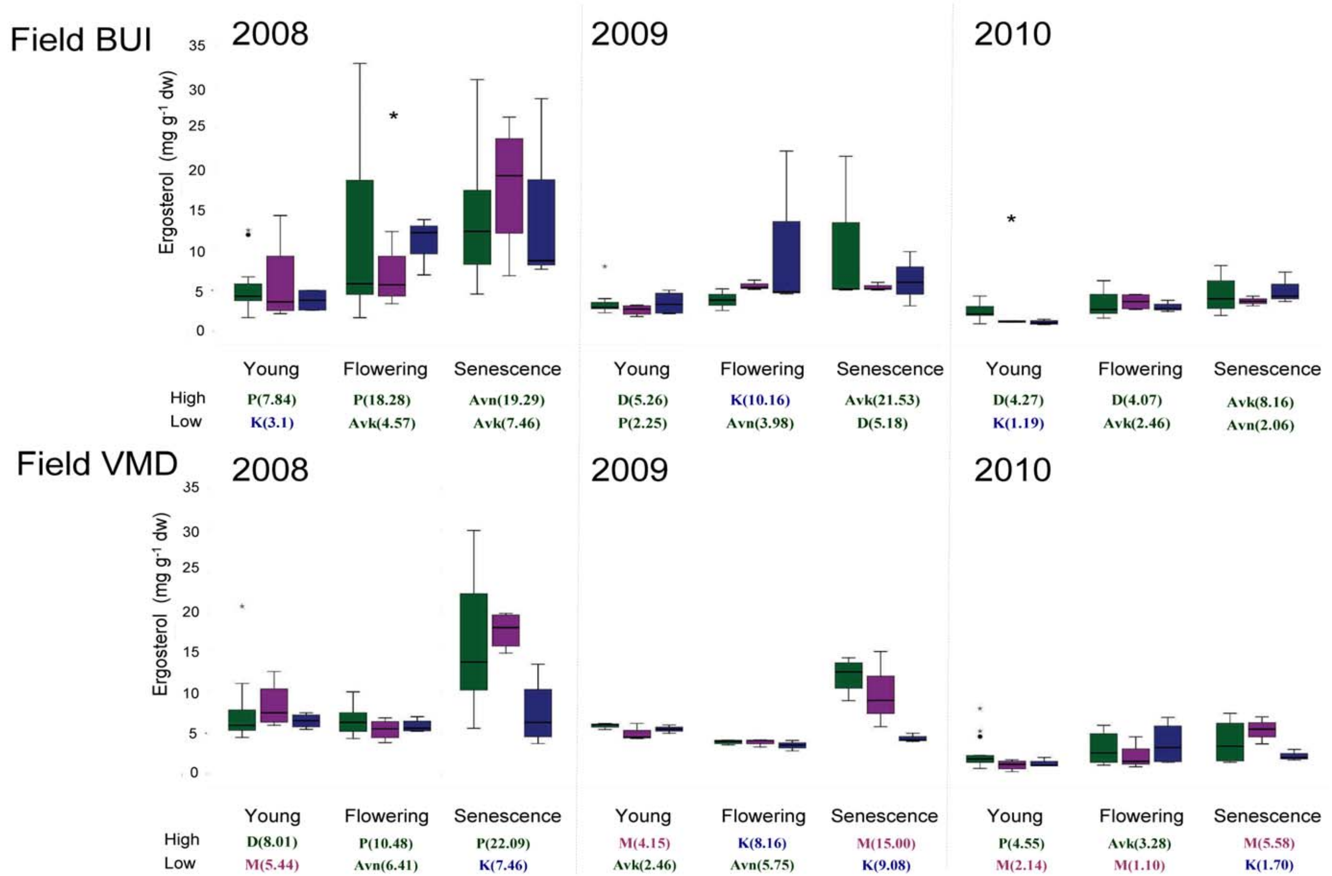

Figure 1. Change in fungal biomass. Boxplots of fungal biomass in the rhizosphere as measured by ergosterol concentrations during 3 years in different growth stages and in both field locations. The baseline (all other cultivars combined, n = 16) is marked with green boxplots, the GM-variety (n = 4) with 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).
doi:10.1371/journal.pone.0033819.g001

When looking at individual time points and fields the ascomycete, basidiomycete and glomeromycete richness was only once significantly different between cultivars (table 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 occassion different between GM- and parental cultivar (table 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 the growth stage was the strongest explanatory factor of differences in the community function (Fig. 2). The stage senescence clearly separated from the other stages along PC1 (ANOVA; $F = 9.57–13.74$, $p<0.001$) which was explained with higher ergosterol and enzymatic activities during senescence. The PC2 was explained by the 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. S1). 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. 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. 3, Table 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 however, 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 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. 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

Table 2. ANOVA analysis of effects of cultivar (including all cultivars) and GM-cultivar 'Modena' versus parental cultivar 'Karnico'on fungal biomass, enzymatic activities and fungal richness in the rhizosphere in different fields, years and growth stages.

				Ergosterol (mg/g)		Laccases (μmol/g)		Mn-Peroxidases (μmol/g)		Cellulases (μmol/g)		# of Ascomycetes		# of Basidiomycetes		# of AMF	
				Cultivar	**GM-Parent**	**Cultivar**	**GM-Parent**	**Cultivar**	**GM-Parent**	**Cultivar**	**GM-Parent**	**Cultivar**	**GM-Parent**	**Cultivar**	**GM-Parent**	**Cultivar**	**GM-Parent**
			df.	**5**	**1**	**5**	**1**	**5**	**1**	**5**	**1**	**5**	**1**	**5**	**1**	**5**	**1**
Field BUI	**2008**	Young	*F*	0.86	0.24	0.89	2.99	1.02	1.32	0.48	0.92	0.22	0.06	0.90	0.05	1.09	0.10
			P	0.53	0.65	0.51	0.15	0.44	0.30	0.79	0.38	0.95	0.82	0.50	0.83	0.42	0.78
		Flowering	*F*	**12.64**	2.13	1.00	0.59	0.76	0.42	0.79	0.00	0.38	0.25	0.48	0.02	1.89	0.47
			P	**<0.001**	0.20	0.44	0.47	0.59	0.84	0.57	0.96	0.86	0.63	0.79	0.89	0.15	0.53
		Senescence	*F*	2.07	0.15	2.61	0.20	**9.19**	0.85	1.25	0.51	**3.67**	**39.06**	0.66	0.38	1.93	0.28
			P	0.12	0.72	0.06	0.67	**<0.001**	0.40	0.33	0.50	**0.018**	**0.025**	0.66	0.57	0.15	0.62
	2009	Young	*F*	1.22	1.13	1.24	0.50	0.73	0.61	0.94	0.61	0.13	0.07	0.47	0.19	1.12	2.42
			P	0.36	0.33	0.35	0.51	0.59	0.46	0.47	0.46	0.97	0.80	0.75	0.69	0.40	0.17
		Flowering	*F*	1.04	0.72	0.45	2.63	1.00	0.34	0.69	5.53	0.17	3.00	0.87	0.00	0.04	5.44
			P	0.41	0.45	0.77	0.16	0.45	0.58	0.62	0.06	0.85	0.33	0.49	0.96	0.96	0.26
		Senescence	*F*	1.32	0.20	1.68	1.32	1.42	1.36	1.80	0.71	0.32	1.93	1.60	2.10	1.24	1.15
			P	0.32	0.68	0.26	0.33	0.33	0.33	0.23	0.46	0.81	0.21	0.27	0.24	0.37	0.40
	2010	Young	*F*	**9.49**	0.01	1.09	0.11	0.11	0.26	1.32	0.49	0.92	1.94	0.84	1.26	0.34	0.35
			P	**0.001**	0.94	0.41	0.75	0.99	0.63	0.31	0.52	0.49	0.21	0.54	0.31	0.88	0.58
		Flowering	*F*	0.85	0.77	1.06	0.64	0.84	0.69	0.47	0.13	0.19	0.02	2.52	0.06	0.34	1.08
			P	0.54	0.42	0.41	0.46	0.54	0.80	0.79	0.73	0.96	0.89	0.31	0.81	0.88	0.49
		Senescence	*F*	4.73	1.42	0.65	0.22	1.89	0.30	1.55	1.51	0.33	1.04	0.68	0.00	1.53	**6.42**
			P	0.11	0.30	0.63	0.66	0.17	0.61	0.24	0.27	0.86	0.38	0.62	0.95	0.24	**0.044**
Field VMD	**2008**	Young	*F*	1.96	1.49	1.20	1.21	0.76	0.09	1.03	0.34	2.13	2.50	0.34	0.10	0.55	1.54
			P	0.13	0.27	0.35	0.31	0.59	0.78	0.43	0.58	0.11	0.17	0.89	0.77	0.74	0.26
		Flowering	*F*	0.67	0.33	1.43	1.19	0.73	0.65	1.23	1.93	0.56	0.33	0.46	1.47	2.10	1.03
			P	0.65	0.59	0.27	0.32	0.61	0.46	0.35	0.21	0.73	0.59	0.72	0.44	0.12	0.35
		Senescence	*F*	1.43	**17.50**	1.24	0.51	2.43	0.18	0.81	0.10	0.66	1.93	0.56	0.26	0.66	0.93
			P	0.26	**0.006**	0.33	0.50	0.08	0.69	0.56	0.76	0.66	0.21	0.73	0.65	0.66	0.38
	2009	Young	*F*	2.91	0.54	0.66	0.22	0.27	0.06	0.32	0.15	0.57	0.24	0.58	0.36	1.06	0.62
			P	0.17	0.50	0.59	0.66	0.85	0.82	0.81	0.72	0.65	0.64	0.64	0.57	0.40	0.46
		Flowering	*F*	7.03	0.62	0.98	0.18	**4.24**	1.18	1.22	2.83	0.67	2.00	0.25	0.85	1.58	0.29
			P	0.23	0.47	0.44	0.69	**0.03**	0.32	0.35	0.14	0.54	0.22	0.90	0.41	0.29	0.63
		Senescence	*F*	0.77	**14.16**	1.57	1.18	1.35	1.24	0.36	0.01	1.35	1.04	**6.00**	4.09	2.38	5.89
			P	0.68	**0.011**	0.23	0.32	0.30	0.31	0.84	0.92	0.36	0.38	**0.03**	0.11	0.13	0.06

Table 2. Cont.

			Ergosterol (mg/g)		Laccases (μmol/g)		Mn-Peroxidases (μmol/g)		Cellulases (μmol/g)		# of Ascomycetes		# of Basidiomycetes		# of AMF	
			Cultivar	GM-Parent	Cultivar	GM-Parent	Cultivar	GM-Parent	Cultivar	GM-Parent	Cultivar	GM-Parent	Cultivar	GM-Parent	Cultivar	GM-Parent
2010	Young	*F*	0.86	0.30	0.83	1.05	0.65	2.97	0.68	0.93	0.53	0.18	0.83	1.67	**4.01**	4.17
		P	0.53	0.61	0.55	0.34	0.66	0.14	0.65	0.37	0.75	0.68	0.55	0.24	**0.03**	0.11
	Flowering	*F*	0.75	0.99	1.76	1.00	2.13	1.00	0.78	0.86	1.08	0.74	0.25	0.09	2.02	8.17
		P	0.60	0.36	0.17	0.36	0.11	3.56	0.58	0.39	0.41	0.42	0.90	0.78	0.18	0.07
	Senescence	*F*	0.27	**13.59**	1.54	1.17	0.18	0.13	0.30	0.06	0.95	0.46	0.47	1.02	0.12	0.52
		P	0.84	**0.014**	0.30	0.54	0.90	0.74	0.82	0.82	0.45	0.53	0.71	0.35	0.95	0.51

Significant P-values are marked with bold.
doi:10.1371/journal.pone.0033819.t002

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). Growth stage, had a strong significant effect on ascomycete and basidiomycete diversities (Fig. 4, table 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). 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, table 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 5). Basidiomycete diversity was different in rhizospheres between cultivars 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).

Legacy of GM-crops

The fields were sampled after the growth seasons 2008 and 2009 and, in addition, rhizosphere of barley was sampled in June 2009 in the field where potatoes were grown in 2008. There were no significant differences in ergosterol content, enzymatic activities, fungal richness or fungal diversity between soils where 'Modena' and 'Karnico' had been grown (table 6). In the rhizosphere of barley there was no effect of previous genotype detected at all. Furthermore, no effect could be detected of different potato genotypes on the fungal community fingerprints in post-harvest samples and in the rhizosphere of barley (Fig. 5, table 6).

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 the soil type. In addition, 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) [19].

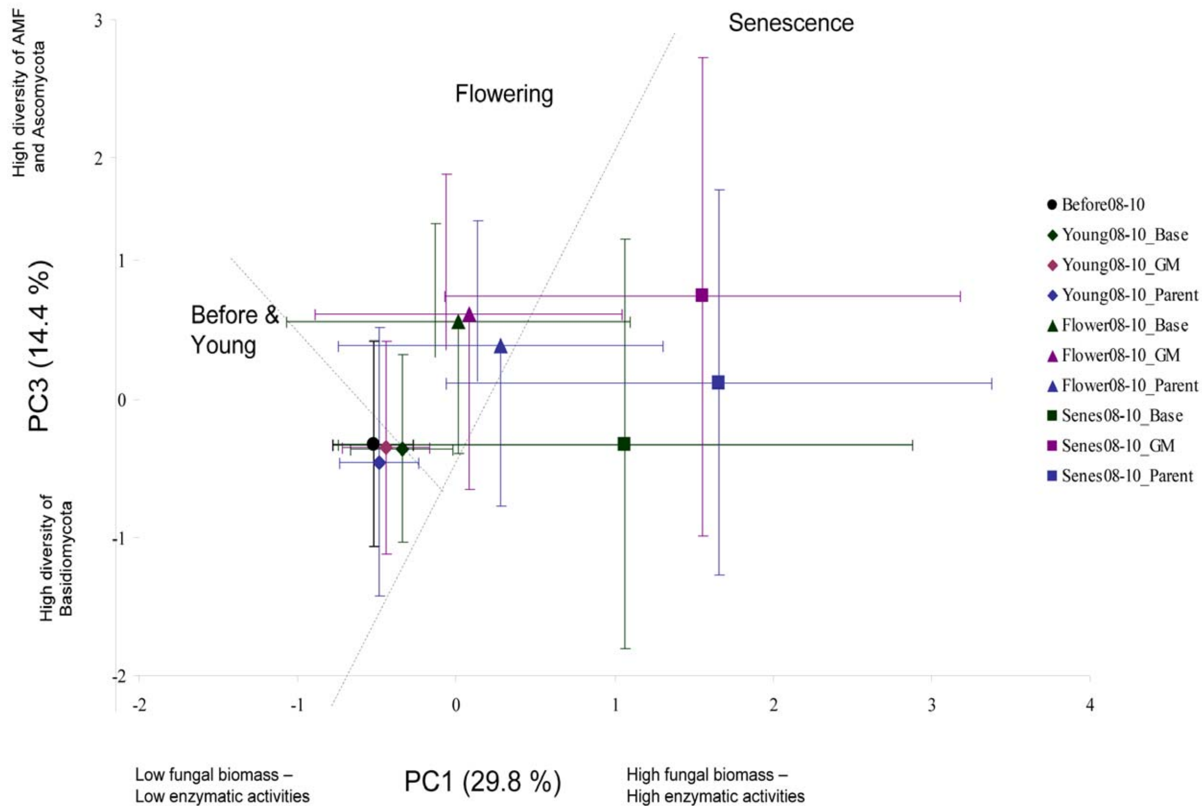

Figure 2. Principal component analysis of functioning and diversity of fungal communities in plots cropped with different potato cultivars. For clarity, the years and field sites are combined. Pre-cropping samples are represented by black circles, young plant stage samples with diamonds, flowering plants 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.
doi:10.1371/journal.pone.0033819.g002

The succession of microbial communities during plant growing season can be explained by two possible mechanisms [7]. The first one is related 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 temperature and moisture. The second, more likely, mechanism is the changes in quality and quantity of root exudates and rhizodeposits with growth stage [35,36] 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 [8,37]. Earlier studies indicated that bacterial and fungal communities in the rhizosphere would either decrease [7,13,20,38] or increase [10,11,39–41] 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 decomposable material (dead roots and leaves) is already available while root exudation still continues thereby broadening the spectrum of substrate availability [42]. Yet, the increase and magnitude of the fungal biomass and its activity in the rhizosphere at that stage is surprising. Until now, the prevailing belief was that the fungal biomass is low in soils under intensive agricultural management. Earlier results with the same cultivars under controlled conditions confirm our observations [43].

Surprisingly, despite the strong differences in soil organic matter content, 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 [7–9,12,18] affecting soil microbial communities. Bacterial communities appeared to differ strongly between the two fields used in this study, both for bulk soil and rhizosphere [44]. 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 [14].

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 [45]. Notably, in our study we detected more ascomycetes and less basidiomycetes and fungi in general in 2010 compared to 2008 in both fields (Fig. 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 soil fungi are important determinants of key soil ecosystems functions such as decomposition of organic matter. Indeed, we could detect a correlation

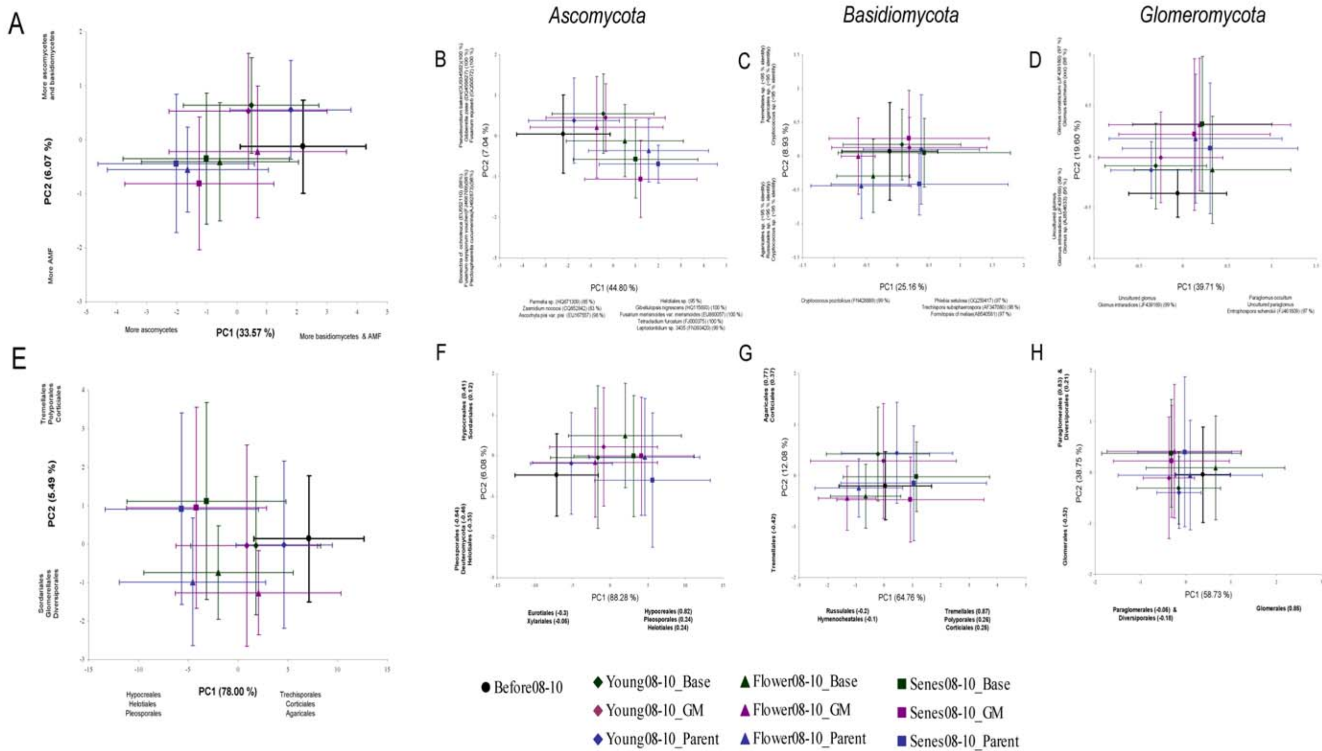

Figure 3. Principal component analysis of community structure of identified fungi. The PCA analysis was done both at the level of individual OTUs and of orders for total fungi (A & E), *Ascomycota* (B & F), *Basidiomycota* (C & G) and *Glomeromycota* (D & H). Figures A–D depict the identified fungal OTUs whereas figures E–H indicate the levels of orders. Orders together with identified OTUs are given in table S1. 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.
doi:10.1371/journal.pone.0033819.g003

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 primarily by basidiomycetes as well as some ascomycetal groups [46]. However, not much is known of the ecology. AM fungi are strongly affected by agricultural practices and changes in soil characteristics [47–49] 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.* [14] 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 [13,18,47] while another one [20] found no cultivar dependent alterations in the fungal communities. We found some indications of cultivar dependence, for instance 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.

Table 3. ANOSIM comparisons between the fields, years, growth stages, cultivars and GM-trait for *Ascomycota*, *Basidiomycota* and *Glomeromycota*.

	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.
Significant P-values are marked with bold.
doi:10.1371/journal.pone.0033819.t003

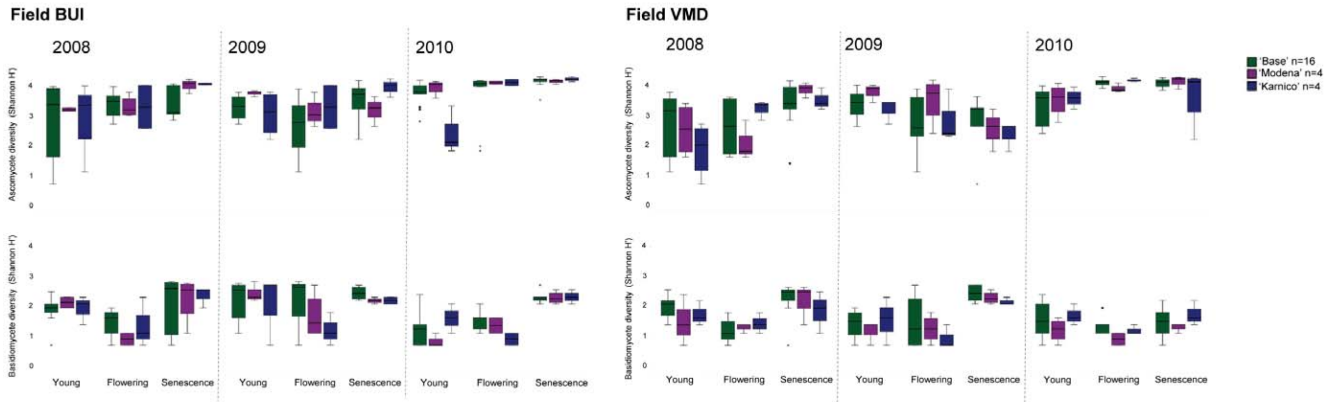

Figure 4. Effect of cultivar, year, growth stage and field on fungal diversity. Boxplots of changes in diversity of *Ascomycota* and *Basidiomycota* between years, growth stages, fields and between baseline, GM and its parental variety. The baseline (all other cultivars combined, n = 16) is marked with 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 5.
doi:10.1371/journal.pone.0033819.g004

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. [18].

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 significant effect was the difference in the amount of fungi in the rhizosphere of the two cultivars in the field VMD during senescence, in all years of the study. 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 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 [19,33,40,50,51]. The soil micro-organisms have an important role in soil ecosystem functioning such as decomposition of plant residues and nutrient cycling [52]. Thus 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

Table 4. The effect of field site, year, growth stage and cultivar on soil ascomycete, basidiomycete and glomeromycete diversity for different taxonomic levels.

		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.
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.
doi:10.1371/journal.pone.0033819.t004

Table 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.

				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		0.21	0.60		0.38	0.38	
			P	0.89	0.75	n.s.	0.95	0.48	n.s.	0.86	0.85	n.s.
		Flowering	*F*	0.92	0.00		0.79	0.56		0.62	2.34	
			P	0.91	1.00	n.s.	0.58	0.50	n.s.	0.69	0.22	n.s.
		Senescence	*F*	2.93	0.05		0.99	0.30		0.93	0.60	
			P	0.11	0.83	n.s.	0.47	0.61	n.s.	0.50	0.50	n.s.
	2009	Young	*F*	0.88	1.22		0.23	0.40		0.99	0.88	
			P	0.51	0.52	n.s.	0.92	0.55	n.s.	0.46	0.38	n.s.
		Flowering	*F*	2.86	1.14		3.28	0.21		0.64	0.71	
			P	0.14	0.35	n.s.	0.10	0.63	n.s.	0.56	0.44	n.s.
		Senescence	*F*	1.35	3.29		4.88	1.19		nd	nd	
			P	0.35	0.21	n.s.	0.77	0.29	n.s.	nd	nd	n.s.
	2010	Young	*F*	**6.25**	**13.80**	**3.83/11.27**	0.79	5.98		0.54	0.60	
			P	**0.03**	**0.01**	**0.02/0.001**	0.58	0.07	n.s.	0.74	0.50	n.s.
		Flowering	*F*	0.46	0.00		2.86	2.24		0.43	0.13	
			P	0.80	0.98	n.s.	0.14	0.38	n.s.	0.82	0.74	n.s.
		Senescence	*F*	1.37	1.14		0.31	0.29		4.41	0.82	
			P	0.30	0.35	n.s.	0.86	0.82	n.s.	0.99	0.40	n.s.
Field VMD	**2008**	Young	*F*	1.73	1.89		0.45	0.19		**4.77**	**38.37**	**2.38/40.38**
			P	0.19	0.49	n.s.	0.80	0.69	n.s.	**0.01**	**0.00**	**0.02/0.001**
		Flowering	*F*	0.85	4.35		0.43	0.37		2.23	0.41	
			P	0.52	0.17	n.s.	0.79	0.58	n.s.	0.15	0.59	n.s.
		Senescence	*F*	0.99	0.04		0.57	0.34		0.13	0.32	
			P	0.48	0.86	n.s.	0.73	0.59	n.s.	0.93	0.62	n.s.
	2009	Young	*F*	0.64	1.79		0.45	0.26		0.51	0.20	
			P	0.62	0.41	n.s.	0.73	0.64	n.s.	0.69	0.67	n.s.
		Flowering	*F*	0.64	1.08		**4.95**	0.90		0.47	0.71	
			P	0.61	0.41	n.s.	**0.04**	0.39	n.s.	0.76	0.44	n.s.
		Senescence	*F*	0.35	1.21		**5.54**	0.93	**4.37**/1.61	2.31	23.78	
			P	0.84	0.44	n.s.	**0.02**	0.38	**0.04**/0.26	0.15	0.16	n.s.

Table 5. Cont.

			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	
2010	Young	*F*	0.49	0.42		0.62	2.92		1.12	1.89	
		P	0.78	0.84	n.s.	0.66	0.15	n.s.	0.56	0.49	n.s.
	Flowering	*F*	2.66	**7.84**	1.43/**1.17**	2.72	2.34		0.46	0.33	
		P	0.08	**0.05**	0.28/**0.03**	0.13	0.22	n.s.	0.77	0.67	n.s.
	Senescence	*F*	0.84	0.91		0.54	2.87		0.49	0.42	
		P	0.50	0.38	n.s.	0.67	0.17	n.s.	0.75	0.86	n.s.

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.

doi:10.1371/journal.pone.0033819.t005

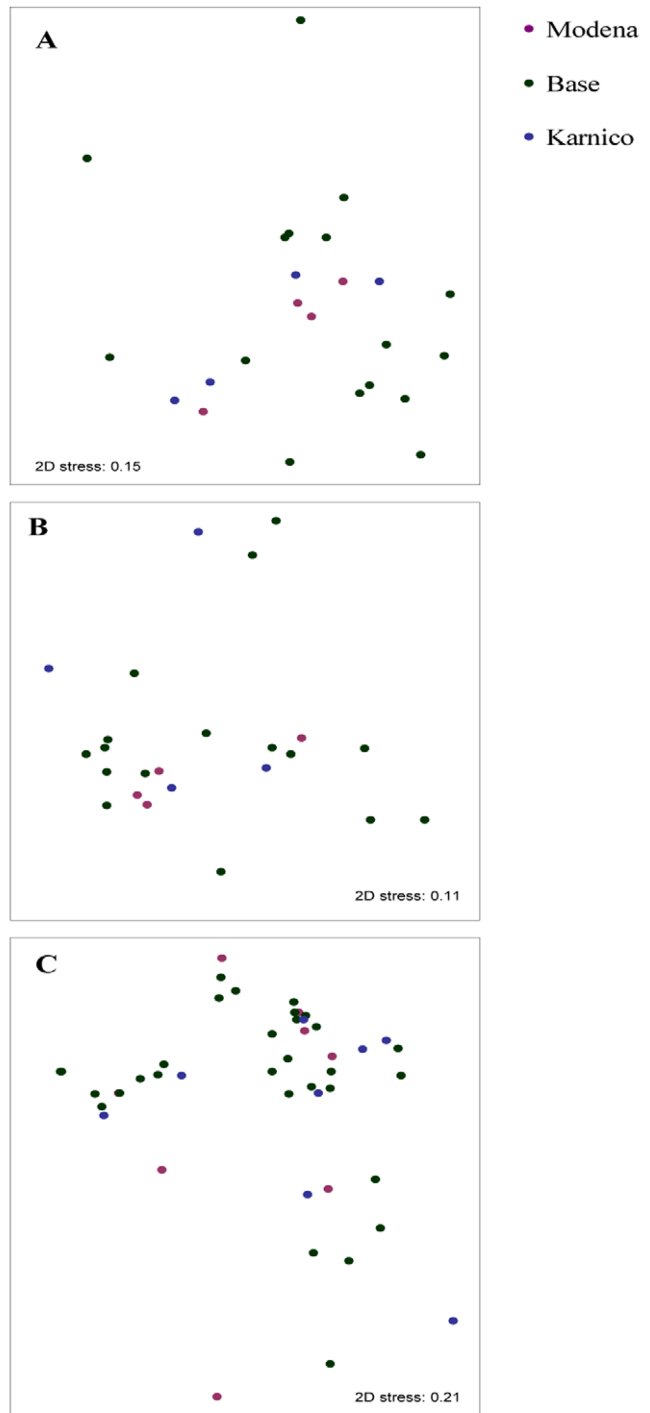

Figure 5. Long term effect of GM-trait on fungal community. NMDS of effects of GM-variety in the next crop (barley) rhizosphere in field BUI on ascomycetes (A), basidiomycetes (B) and in fields BUI and VMD on glomeromycetes (C). The GM-variety 'Modena' is marked with purple markers, the parental cultivar 'Karnico' with blue markers, and baseline (all other cultivars combined) green markers. Details on statistical analysis are given in table 6.
doi:10.1371/journal.pone.0033819.g005

similar observation was made for bacteria after cropping of transgenic canola [53].

In conclusion, plant growth stage, year and field site were the factors contributing most to variation in the potato-associated

Table 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.

		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

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.
doi:10.1371/journal.pone.0033819.t006

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. We hypothesized that this modification would have no direct but rather indirect unintended effects of the modification on the plant physiology through production of different exudates. 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.

Supporting Information

Figure S1 Principal component analysis of function and diversity of fungal communities in between growth stages, fields and years. Field BUI is marked with closed symbols and solid lines while field VMD with open symbols and dotted lines. Year 2008 is marked with black markers, year 2009 with red markers and 2010 with blue marker. The explanatory parameters are mentioned next to the axis.
(TIF)

Table S1 Soil characteristics and fertilizers added to the fields. In the fertilizer treatments CAN = Calcium Ammonium Nitrate, NP = nitrogen as ammonium sulphate and phosphorous as P_2O_5 and ORG = organic fertilizer = pig manure.
(XLS)

Acknowledgments

The authors would like to thank Wiecher Smant, Özgül Ineoglu and Nadia Marttin for technical assistance and collaboration during field sampling and Peter Bruinenberg and Paul Heeres from AVEBE/AVERIS seeds for valuable assistance in the field experiment. This is publication 5227 of the Netherlands Institute of Ecology (NIOO-KNAW).

Author Contributions

Conceived and designed the experiments: SEH WdB JvV. Performed the experiments: SEH. Analyzed the data: SEH WdB JvV. Wrote the paper: SEH WdB JvV.

References

1. Wolfenbarger LL, Phifer PR (2000) Biotechnology and ecology - The ecological risks and benefits of genetically engineered plants. Science 290: 2088–2093.
2. Birch ANE, Griffiths BS, Caul S, Thompson J, Heckmann LH, et al. (2007) The role of laboratory, glasshouse and field scale experiments in understanding the interactions between genetically modified crops and soil ecosystems: A review of the ECOGEN project. Pedobiologia 51: 251–260.
3. Lynch JM, Whipps JM (1990) Substrate flow in the rhizosphere. Plant and Soil 129: 1–10.
4. Raaijmakers J, Paulitz T, Steinberg C, Alabouvette C, Moënne-Loccoz Y (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant and soil 321: 341–361.
5. Carlile MJ, Watkinson SC, Gooday GW (2001) The Fungi. San Diego, CA, USA: Academic Press.
6. Buée M, De Boer W, Martin F, van Overbeek L, Jurkevitch E (2009) The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. Plant and Soil 321: 189–212.

7. Wang GH, Xu YX, Jin J, Liu JD, Zhang QY, et al. (2009) Effect of soil type and soybean genotype on fungal community in soybean rhizosphere during reproductive growth stages. Plant and Soil 317: 135–144.
8. Garbeva P, van Veen JA, van Elsas JD (2004) Microbial diversity in soil: Selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annual Review of Phytopathology 42: 243–270.
9. Berg G, Smalla K (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiology Ecology 68: 1–13.
10. Gomes NCM, Heuer H, Schönfeld J, Costa R, Hagler-Mendonca L, et al. (2001) Bacterial diversity of the rhizosphere of maize (Zea mays) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant and Soil 232: 167–180.
11. Gomes NCM, Fagbola O, Costa R, Rumjanek NG, Buchner A, et al. (2003) Dynamics of fungal communities in bulk and maize rhizosphere soil in the tropics. Applied and Environmental Microbiology 69: 3758–3766.
12. Buyer JS, Roberts DP, Russek-Cohen E (2002) Soil and plant effects on microbial community structure. Canadian Journal of Microbiology 48: 955–964.
13. Gschwendtner S, Reichmann M, Muller M, Radl V, Munch JC, et al. (2010) Effects of genetically modified amylopectin-accumulating potato plants on the abundance of beneficial and pathogenic microorganisms in the rhizosphere. Plant and Soil 335: 413–422.
14. Verbruggen E, Röling WFM, Gamper HA, Kowalchuk GA, Verhoef HA, et al. (2010) Positive effects of organic farming on below-ground mutualists: large-scale comparison of mycorrhizal fungal communities in agricultural soils. New Phytologist 186: 968–979.
15. Griffiths BS, Caul S, Thompson J, Birch ANE, Cortet J, et al. (2007) Microbial and microfaunal community structure in cropping systems with genetically modified plants. Pedobiologia 51: 195–206.
16. Donegan KK, Schaller DL, Stone JK, Ganio LM, Reed G, et al. (1996) Microbial populations, fungal species diversity and plant pathogen levels in field plots of potato plants expressing the Bacillus thuringiensis var tenebrionis endotoxin. Transgenic Research 5: 25–35.
17. Donegan KK, Seidler RJ, Doyle JD, Porteous LA, Digiovanni G, et al. (1999) A field study with genetically engineered alfalfa inoculated with recombinant Sinorhizobium meliloti: effects on the soil ecosystem. Journal of Applied Ecology 36: 920–936.
18. Weinert N, Meincke R, Gottwald C, Heuer H, Gomes NCM, et al. (2009) Rhizosphere communities of genetically modified zeaxanthin-accumulating potato plants and their parent cultivar differ less than those of different potato cultivars. Applied and Environmental Microbiology 75: 3859–3865.
19. Hannula SE, de Boer W, van Veen JA (2010) In situ dynamics of soil fungal communities under different genotypes of potato, including a genetically modified cultivar. Soil Biology & Biochemistry 42: 2211–2223.
20. Milling A, Smalla K, Maidl FX, Schloter M, Munch JC (2004) Effects of transgenic potatoes with an altered starch composition on the diversity of soil and rhizosphere bacteria and fungi. Plant and Soil 266: 23–39.
21. Hart MM, Powell JR, Gulden RH, Dunfield KE, Pauls KP, et al. (2009) Separating the effect of crop from herbicide on soil microbial communities in glyphosate-resistant corn. Pedobiologia 52: 253–262.
22. Lee S-H, Kim C-G, Kang H (2011) Temporal Dynamics of Bacterial and Fungal Communities in a Genetically Modified (GM) Rice Ecosystem. Microbial Ecology 61: 646–659.
23. Donegan KK, Palm CJ, Fieland VJ, Porteous LA, Ganio LM, et al. (1995) Changes in levels, species and DNA fingerprints of soil-microorganisms associated with cotton expressing the *Bacillus-Thuringiensis* var *Kurstaki* endotoxin. Applied Soil Ecology 2: 111–124.
24. Götz M, Nirenberg H, Krause S, Wolters H, Draeger S, et al. (2006) Fungal endophytes in potato roots studied by traditional isolation and cultivation-independent DNA-based methods. FEMS Microbiology Ecology 58: 404–413.
25. Wei XD, Zou HL, Chu LM, Liao B, Ye CM, et al. (2006) Field released transgenic papaya affects microbial communities and enzyme activities in soil. Plant and Soil 285: 347–358.
26. van Overbeek L, van Elsas JD (2008) Effects of plant genotype and growth stage on the structure of bacterial communities associated with potato (*Solanum tuberosum L.). FEMS Microbiology Ecology* 64: 283–296.
27. de Vetten N, Wolters AM, Raemakers K, van der Meer I, ter Stege R, et al. (2003) A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. Nature Biotechnology 21: 439–442.
28. Hack H, Gal H, Klemke T, Klose R, Meier U, et al. (2001) The BBCH scale for phenological growth stages of potato (*Solanum tuberosum* L.). In: Meier U, ed. Growth Stages of Mono- and Dicotyledonous Plants, BBCH Monograph: Federal Biological Research Centre for Agriculture and Forestry.
29. de Ridder-Duine AS, Smant W, van der Wal A, van Veen JA, de Boer W (2006) Evaluation of a simple, non-alkaline extraction protocol to quantify soil ergosterol. Pedobiologia 50: 293–300.
30. van der Wal A, van Veen JA, Pijl AS, Summerbell RC, de Boer W (2006) Constraints on development of fungal biomass and decomposition processes during restoration of arable sandy soils. Soil Biology & Biochemistry 38: 2890–2902.
31. Fitzjohn RG, Dickie IA (2007) TRAMPR: an R package for analysis and matching of terminal-restriction fragment length polymorphism (TRFLP) profiles. Molecular Ecology Notes 7: 583–587.
32. Culman SW, Bukowski R, Gauch HG, Caliddo-Quiroz H, Buckley DH (2009) T-REX: Software for the processing and analysis of T-RFLP data.BMC Bioinformatics 10: 171.
33. Abdo Z, Schuette UME, Bent SJ, Williams CJ, Forney LJ, et al. (2006) Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. Environmental Microbiology 8: 929–938.
34. Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4: 9 pp.
35. Jones DL, Hodge A, Kuzyakov Y (2004) Plant and mycorrhizal regulation of rhizodeposition. New Phytologist 163: 459–480.
36. Marschner P, Neumann G, Kania A, Weiskopf L, Lieberei R (2002) Spatial and temporal dynamics of the microbial community structure in the rhizosphere of cluster roots of white lupin (*Lupinus albus*; L.). Plant and Soil 246: 167–174.
37. Duineveld BM, Kowalchuk GA, Keijzer A, van Elsas JD, van Veen JA (2001) Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. Applied and Environmental Microbiology 67: 172–178.
38. Acosta-Martínez V, Dowd S, Sun Y, Allen V (2008) Tag-encoded pyrosequencing analysis of bacterial diversity in a single soil type as affected by management and land use. Soil Biology & Biochemistry 40: 2762–2770.
39. Sessitsch A, Gyamfi S, Tscherko D, Gerzabek MH, Kandeler E (2004) Activity of microorganisms in the rhizosphere of herbicide treated and untreated transgenic glufosinate-tolerant and wildtype oilseed rape grown in containment. Plant and Soil 266: 105–116.
40. Lottmann J, Heuer H, de Vries J, Mahn A, During K, et al. (2000) Establishment of introduced antagonistic bacteria in the rhizosphere of transgenic potatoes and their effect on the bacterial community. FEMS Microbiology Ecology 33: 41–49.
41. Smalla K, Wieland G, Buchner A, Zock A, Parzy J, et al. (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Applied and Environmental Microbiology 67: 4742–4751.
42. Broeckling CD, Broz AK, Bergelson J, Manter DK, Vivanco JM (2008) Root exudates regulate soil fungal community composition and diversity. Applied and Environmental Microbiology 74: 738–744.
43. Hannula SE, Boschker HTS, de Boer W, Van Veen JA (2012) ^{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. New Phytologist accepted: xx- xx.
44. Inceoglu O, Salles JF, van Overbeek L, van Elsas JD (2010) Effects of plant genotype and growth stage on the betaproteobacterial communities associated with different potato cultivars in two fields. Applied and Environmental Microbiology 76: 3675–3684.
45. Marschner P, Kandeler E, Marschner B (2003) Structure and function of the soil microbial community in a long-term fertilizer experiment. Soil Biology & Biochemistry 35: 453–461.
46. Bödeker ITM, Nygren CMR, Taylor AFS, Olson A, Lindahl BD (2009) ClassII peroxidase-encoding genes are present in a phylogenetically wide range of ectomycorrhizal fungi. Isme Journal 3: 1387–1395.
47. Turrini A, Sbrana C, Nuti MP, Pietrangeli BM, Giovannetti M (2004) Development of a model system to assess the impact of genetically modified corn and aubergine plants on arbuscular mycorrhizal fungi. Plant and Soil 266: 69–75.
48. Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? Nature 394: 431–431.
49. Giovannetti M, Sbrana C, Turrini A (2005) The impact of genetically modified crops on soil microbial communities. Rivista Di Biologia-Biology Forum 98: 393–417.
50. Lukow T, Dunfield PF, Liesack W (2000) Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiology Ecology 32: 241–247.
51. Lottmann J, Heuer H, Smalla K, Berg G (1999) Influence of transgenic T4-lysozyme-producing potato plants on potentially beneficial plant-associated bacteria. FEMS Microbiology Ecology 29: 365–377.
52. Kennedy AC (1999) Bacterial diversity in agroecosystems. Agriculture Ecosystems & Environment 74: 65–76.
53. Dunfield KE, Germida JJ (2003) Seasonal changes in the rhizosphere microbial communities associated with field-grown genetically modified canola (Brassica napus). Applied and Environmental Microbiology 69: 7310–7318.