ANNEX 15

PUBLICATION GSCHWENDTNER ET AL., 2011

RESEARCH ARTICLE

Effects of genetically modified starch metabolism in potato plants on photosynthate fluxes into the rhizosphere and on microbial degraders of root exudates

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Received 5 August 2010; revised 8 February 2011; accepted 12 February 2011.
Final version published online 17 March 2011.

DOI:10.1111/j.1574-6941.2011.01073.x

Editor: Kornelia Smalla

Keywords
genetically modified potato plants; ^{13}C labelling; phospholipid fatty acid analysis; rhizosphere; microbial biomass; carbon partitioning.

Abstract

A high percentage of photosynthetically assimilated carbon is released into soil via root exudates, which are acknowledged as the most important factor for the development of microbial rhizosphere communities. As quality and quantity of root exudates are dependent on plant genotype, the genetic engineering of plants might also influence carbon partitioning within the plant and thus microbial rhizosphere community structure. In this study, the carbon allocation patterns within the plant-rhizosphere system of a genetically modified amylopectin-accumulating potato line (*Solanum tuberosum* L.) were linked to microbial degraders of root exudates under greenhouse conditions, using ^{13}C-CO_2 pulse-chase labelling in combination with phospholipid fatty acid (PLFA) analysis. In addition, GM plants were compared with the parental cultivar as well as a second potato cultivar obtained by classical breeding. Rhizosphere samples were obtained during young leaf developmental and flowering stages. ^{13}C allocation in above-ground plant biomass, water-extractable organic carbon, microbial biomass carbon and PLFA as well as the microbial community structure in the rhizosphere varied significantly between the natural potato cultivars. However, no differences between the GM line and its parental cultivar were observed. Besides the considerable impact of plant cultivar, the plant developmental stage affected carbon partitioning via the plant into the rhizosphere and, subsequently, microbial communities involved in the transformation of root exudates.

Introduction

As photosynthetically assimilated CO_2 released via rhizodeposition is the primary carbon source in soil during the vegetation period, the composition and the amount of organic compounds in root exudates are very important factors in the development of a typical rhizosphere microbial communities (Lynch & Whipps, 1990). By governing the quality and quantity of root exudates, plants are able to influence beneficial microorganisms which promote plant growth and plant health (Lynch & Whipps, 1990; Raaijmakers *et al.*, 2009). Because of this close interaction between plants and their microbial rhizosphere populations and its importance for plant performance, there is a need to understand whether carbon partitioning in genetically modified (GM) crops is affected by the genetic modification.

It was shown that carbon allocation within plants is highly dependent on plant species, plant genotype and plant vegetation stage (Percival *et al.*, 2001; Carvalho *et al.*, 2006; Shinano *et al.*, 2006). Similar results were obtained for microbial community structure in the rhizosphere (Duineveld *et al.*, 1998, 2001; Smalla *et al.*, 2001; Gyamfi *et al.*, 2002; Marschner *et al.*, 2006). Besides, many studies reported shifts in the diversity pattern of the rhizosphere microbial communities associated with GM plants, although the modification-dependent effects were described as minor

compared with the impact of soil type, plant variety and plant development (Milling *et al.*, 2004; Rasche *et al.*, 2006; Weinert *et al.*, 2009). Nevertheless, so far almost nothing is known about the influence of genetic modification on photosynthate allocation, or if GM-related changes in carbon partitioning in fact lead to shifts in the microbial rhizosphere community.

Phospholipid fatty acid (PLFA) analysis in combination with stable isotope probing (SIP) is a suitable approach to link carbon assimilation within plants directly to the microbial community structure in soil if an atmosphere enriched with ^{13}C-labelled CO_2 is used for plant growth (Lu *et al.*, 2002, 2007; Esperschütz *et al.*, 2009; Wu *et al.*, 2009). Although this method cannot be linked to the species composition of microbial communities because many phospholipids are not related to a single taxonomic group, it offers several advantages compared with nucleic acid-based SIP approaches, such as the possibility of a quantitative evaluation of microbial groups due to the lack of an amplification step and the very sensitive detection of microorganisms metabolizing plant-derived carbon due to analysis by GC-combustion-isotope ratio mass spectrometry. Therefore, potential cross-feeding problems caused by the need for a long incubation time and/or high labelled substrate concentrations that do not reflect environmental conditions can be avoided.

In the present study, a GM potato (*Solanum tuberosum* L.) with modified starch metabolism was used to investigate the influence of genetic modification on photosynthate allocation from plant to soil to microorganisms and on rhizosphere microbial community structure, using ^{13}C pulse-chase labelling combined with PLFA analysis under greenhouse conditions. The GM potato contains an antisense gene fragment of the granule-bound starch synthase gene (*gbss*), which encodes one of the key enzymes for amylose formation. Consequently, transcription leads to an effective inhibition of *gbss* expression and amylose production (Visser *et al.*, 1991; Kuipers *et al.*, 1994). Therefore, tuber starch consists of almost pure amylopectin, which is appropriate for commercial use in the paper, textile and food industries. As a GM starch composition can influence microbial community structure in potato rhizosphere (Milling *et al.*, 2004), we hypothesized that this might be a result of altered carbon allocation in the GM plant. Hence, the objectives of this study were: (1) to assess whether amylopectin-accumulating GM potato line #1332 (Bavarian State Research Center for Agriculture) differs from its nontransgenic parental variety in photosynthate partitioning using ^{13}C pulse-chase labelling, (2) to estimate the effects of GM photosynthate inputs on the microbial rhizosphere community via PLFA–SIP analysis and (3) to relate the potential GM-caused impact to variation among natural potato cultivars and the effect of plant developmental stage.

Materials and methods

Potato cultivars and the GM line

The GM potato line #1332 with increased amylopectin levels in its tubers, its parental cultivar 'Walli', and one additional potato variety used for industrial starch production, 'Ponto', were examined in this study. Both natural cultivars were provided by the Bavarian State Research Center for Agriculture, where the GM line was also developed. The GM line was genetically modified by the insertion of a gene fragment of the granule-bound starch synthase (*gbss*) gene in antisense direction. Consequently, transcription leads to the formation of *gbss* double-stranded RNA and thus inhibition of *gbss* expression and amylose production (Visser *et al.*, 1991; Kuipers *et al.*, 1994). Hence, the tuber starch of the GM line consists of more than 99% amylopectin, whereas the starch granules of 'Walli' and 'Ponto' contain only 75–80% amylopectin (M. Reichmann, pers. commun.). A newly developed method based on PCR screening was used for transformation without antibiotic- or herbicide-resistance selection (http://gmoinfo.jrc.ec.europa.eu/gmp_report.aspx?CurNot=B/DE/03/155), resulting in marker-free GM line #1332.

Experimental set-up

The soil used in this experiment was taken from the plough layer (0–30 cm) of a field with a potato–winter wheat–maize–winter wheat rotation history from the agroecological research station in Scheyern, approximately 40 km north of Munich, Germany (48°30′N, 11°26′E). It was characterized as sandy Cambisol with 22% clay, 10% silt and 68% sand, pH (0.01 M $CaCl_2$) 5.6, maximum water-holding capacity of 29%, and contained 1.0% total organic carbon and 0.1% total nitrogen. To remove plant residues and gravel, the soil was sieved (< 4 mm), filled into plastic pots (5 L soil per pot) and adjusted to 60% of the water-holding capacity (Linn & Doran, 1984) 1 week before the potato tubers were implanted at the beginning of September 2008. In total, 120 pots were prepared (one plant per pot, 40 pots of each plant cultivar), whereof half were used for pulse-chase labelling and the other half as unlabelled controls. The plants were grown in a greenhouse at 25/15 °C day/night temperature, relative humidity 50% and a photoperiod of 16 h daylight (additional light by sodium vapour discharge lamps, SON-T Agro 400; Philips, the Netherlands). Irrigation was performed by hand with 60–100 mL deionized water every 24 h to keep the water content of the soil between 50% and 60% of the water-holding capacity. When the plants reached the young leaf developmental stage in mid-October, 10 pots of each cultivar were placed into a tent built of transparent plastic foil (ethylene-tetrafluoroethylene, film thickness 80 μm; Koch Membrane GmbH, Aachen, Germany) to separate the plants from the outer greenhouse atmosphere. To estimate the amount of soil autotrophic CO_2 fixation, two

unplanted pots were also placed within the tent. The CO_2 concentration of the tent atmosphere was measured continuously with a photoacoustic CO_2 controller (7MB1300; Siemens, Munich, Germany) and was automatically enriched with ^{13}C-CO_2 (99 atom% ^{13}C, Air Liquide, Düsseldorf, Germany) when it dropped below 300 μmol mol^{-1} in order to keep it within 300–400 μmol mol^{-1}. Using this experimental set-up, an atmosphere with a $^{13}CO_2$ concentration of 45–50% of the total CO_2 concentration was established in the tent. To reduce the CO_2 produced by plant respiration at night, the tent air was pumped through vials containing soda lime (sodium and calcium hydroxide) using a membrane pump (N 0135.3 AN.18; KNF Neuberger, Freiburg, Germany) with a flow of 200 L min^{-1}. The CO_2-depleted air was recycled into the closed tent to ensure a stable CO_2 concentration of approximately 350 μmol mol^{-1} overnight. This labelling occurred for 6 h on two consecutive days.

Afterwards, plant material and rhizosphere soil of 10 labelled and 10 unlabelled control pots were collected by mixing the respective material of two pots to form one composite sample. Rhizosphere soil was defined as the soil still attaching to the roots after vigorous shaking (Yanai *et al.*, 2003) and was sieved through a 2-mm mesh before storing at 4 °C for analysis of water-extractable organic carbon (WEOC) and microbial biomass carbon (C_{mic}), and at −20 °C for PLFA analysis. The soil of the labelled unplanted pots was treated similarly. The plants were cleaned with deionized water and their roots, stems and leaves were dried separately at 65 °C for 48 h for determination of carbon content.

Similar labelling and sampling procedures were repeated upon florescence of the remaining potato plants (end of November 2008).

Carbon analysis of plant material

The dried plant material was ball-milled (Retsch MM2; Retsch GmbH, Haan, Germany), weighed into tin capsules and analysed using an Elemental Analyzer coupled with an Isotopic Ratio Mass Spectrometer (EA-IRMS; Eurovector, Milan, Italy, coupled with a MAT 253, Thermo Electron Corporation, Bremen, Germany) for determination of carbon content and $\delta^{13}C$. The $\delta^{13}C$ values were related to the international Vienna-Pee Dee Bee Belemnite (V-PDB) standard and were calculated as follows (Werner & Brand, 2001):

$$\delta^{13}C\,(‰) = [(R_{Sample}/R_V) - 1] \times 1000 \quad (1)$$

where R_{Sample} and R_V represent the $^{13}C:{}^{12}C$ ratios of sample and international standard V-PDB (0.0111802), respectively.

WEOC and microbial biomass carbon (C_{mic})

Within 3 days after harvest, WEOC of the samples was determined by shaking 7.5 g rhizosphere soil (fresh weight) in 0.01 M $CaCl_2$ solution (1 : 4 w/v) on a rotary shaker for 30 min. After subsequent centrifugation, the supernatant was filtered through 0.45-μm pore-size polycarbonate filters (Whatman Nuclepore Track-Etch Membrane filters). Simultaneously, C_{mic} was determined with the same amount of soil by the chloroform-fumigation method according to Vance *et al.* (1987), using 0.01 M $CaCl_2$ solution (1 : 4 w/v) for extraction (Joergensen, 1995). All extractions were done in triplicate and the extracts were stored at −20 °C until measurement.

Total organic carbon (C_{total}) contents of the extracts were determined on a Total Carbon Analyzer (Shimadzu TOC 5050, Tokyo, Japan) by catalytic high-temperature oxidation. C_{mic} was calculated as the difference between C_{total} in fumigated and nonfumigated samples using a k_{EC}-value of 0.45 (Wu *et al.*, 1990; Joergensen, 1995). Measurement of $\delta^{13}C$ in the extracts was done via on-line coupling of liquid chromatography and stable isotope ratio mass spectrometry (LC-IRMS, Thermo Electron Corporation) according to Krummen *et al.* (2004). The $\delta^{13}C$ in microbial biomass ($\delta^{13}C_{mic}$) was computed as described by Marx *et al.* (2007b):

$$\delta^{13}C_{mic}\,(‰) = [(\delta^{13}C_{fum} \times C_{fum}) - (\delta^{13}C_{n\text{-}fum} \times C_{n\text{-}fum})]/C_{mic} \quad (2)$$

where $\delta^{13}C_{fum}$ and $\delta^{13}C_{n\text{-}fum}$ are $\delta^{13}C$ values of the fumigated (fum) and nonfumigated (n-fum) extracts, respectively, and C_{fum} and $C_{n\text{-}fum}$ are carbon concentrations (in mg L^{-1}) of the fumigated and nonfumigated extracts.

PLFA analysis

PLFA were extracted and analysed as described in detail by Zelles *et al.* (1995) and Esperschütz *et al.* (2009). Briefly, lipids of 25 g rhizosphere soil (dry weight) were extracted by a two-phase extraction procedure with chloroform/methanol and water. The total lipid extract was subdivided into neutral, glycol- and phospholipids on a silica-bonded phase column (SPE-SI 2 g/12 mL; Bond Elut, Agilent Technologies, Waldbronn, Germany). After mild alkaline hydrolysis of the phospholipid fraction, the unsubstituted fatty acid methyl esters (FAME) were extracted with hexane/dichloromethane on an aminopropyl column. FAME were further separated into saturated (SATFA), monounsaturated (MUFA) and polyunsaturated fatty acids using an Ag^+-impregnated SCX column (Bond Elut, Agilent). Afterwards, the fractions were stored at 4 °C until measurement.

Analysis was performed on a gas chromatograph/mass spectrometry system (5973MSD GC/MS; Agilent Technologies, Palo Alto, CA) linked to an isotope ratio mass spectrometer (GC/MS-C-IRMS, Delta Plus^{Advantage}; Thermo Electron Corporation) via a combustion unit. FAME were separated and detected via GC/MS, while the isotopic composition of fatty acids was analysed after combustion (GC Combustion III; Thermo Electron Corporation) in the IRMS. The separation of the FAME was done as described by Esperschütz *et al.* (2009).

MUFA were measured underivatized to obtain the correct isotopic signature. Subsequently, disulphide derivatization and a second measurement were carried out to determine the double-bond position (Zelles *et al.*, 1995). Sample analysis was done in duplicate and repeated when the variation of $\delta^{13}C_{V\text{-}PDB}$ in the internal standard exceeded 0.5‰. According to Werner & Brand (2001), the $\delta^{13}C$ ratios of the individual FAME were corrected in relation to the measurement difference between the $\delta^{13}C_{V\text{-}PDB}$ ratio of the internal standard in the sample and the known $\delta^{13}C_{V\text{-}PDB}$ ratio of the standard (nonadecanoic acid methyl ester, $\delta^{13}C_{V\text{-}PDB} = -30.5$‰ and myristic acid methyl ester, $\delta^{13}C_{V\text{-}PDB} = -28.7$‰). To obtain the actual PLFA ratio ($\delta^{13}C_{PLFA}$), the carbon atom in the methyl group added into the carbon isotope ratios of the FAME ($\delta^{13}C_{FAME}$) during derivatization was computed:

$$\delta^{13}C_{PLFA} = [(n+1) \times \delta^{13}C_{FAME} - (1 \times \delta^{13}C_{MeOH})]/n \quad (3)$$

where n is the number of carbon atoms in the PLFA and $\delta^{13}C_{MeOH}$ is the $\delta^{13}C_{V\text{-}PDB}$ ratio of methanol used for derivatization (-38.5‰, determined by LC-IRMS).

The percentage of carbon newly incorporated into the individual PLFA biomarker relative to the labelling atmosphere was calculated according to Farquhar *et al.* (1989) using a fractionation factor α:

$$\alpha = [(\delta^{13}C_{CO_2}/1000) + 1]/[\delta^{13}C_{plant}/1000) + 1] \quad (4)$$

where $\delta^{13}C_{CO_2}$ is $\delta^{13}C_{V\text{-}PDB}$ of the regular atmosphere (-11‰) and $\delta^{13}C_{plant}$ is the computed $\delta^{13}C_{PLFA}$ ratio of the nonlabelled plants.

Using α, the newly incorporated carbon (C_{new}) was calculated as follows:

$$\delta^{13}C_{max} = \{[(\delta^{13}C_{tent}/1000) + 1] \times \alpha - 1\} \times 1000 \quad (5)$$

$$C_{new} = (\delta^{13}C_{new} - \delta^{13}C_{plant})/(\delta^{13}C_{max} - \delta^{13}C_{plant}) \times 100 \quad (6)$$

where $\delta^{13}C_{max}$ is the maximum possible label incorporation with regard to the individual α and the $\delta^{13}C$ ratio of the tent atmosphere ($\delta^{13}C_{tent}$), and $\delta^{13}C_{new}$ is the δ value of the individual PLFA measured under $\delta^{13}C_{tent}$, respectively.

The mass spectra of the individual FAME were identified by comparison with established fatty acid libraries (Solvit; Lucerne, Switzerland), using MSD CHEMSTATION (version D.02.00.237). The following PLFA nomenclature was used, based on Frostegård *et al.* (1993): fatty acids are designated as the total number of carbon atoms : number of double bonds, followed by the position of the double bond from the methyl end of the molecule (ω). The prefixes 'cy', 'i' and 'a' indicate cyclopropyl groups, iso- and anteiso-branching, respectively. '10Me' denotes a methyl branching at the tenth carbon atom from the carboxyl end of the molecule, and 'br' indicates an unknown methyl-branching position.

According to Zelles (1999), iso- and anteiso-branched fatty acids were used as indicators for Gram-positive bacteria, and monounsaturated and cyclopropyl PLFA as indicators of Gram-negative bacteria. '10Me' fatty acids could be taken as a marker for actinomycetes (Frostegård & Bååth, 1996). PLFA 18:2ω6.9 indicates fungal biomass (Bååth, 2003). PLFA 16:1ω5 can be found mainly in arbuscular mycorrhizal fungi (AMF) (Olsson *et al.*, 1999; Joergensen & Wichern, 2008). The ratios of Gram-positive to Gram-negative bacteria, Gram-positive bacteria to actinomycetes and fungi to bacteria were used to show shifts in microbial community structure (Frostegård & Bååth, 1996; Fierer *et al.*, 2003).

Statistical analysis

Data were analysed by ANOVA at the significance level $P < 0.05$ using SPSS 11.5 (SPSS Inc.). The normal distribution of the data was checked by the Kolmogorov–Smirnov test and histograms. If necessary, the data were log transformed before analysis. The homogeneity of the variances was checked by the Levene test. For the pairwise comparison of means with the ANOVA, either the Tukey test or, if the homogeneity of the variances was not given, the Games–Howell test was used.

Results

Incorporation of ^{13}C-labelled carbon into plant biomass

The majority of the assimilated ^{13}C was retained in the aboveground plant biomass (Fig. 1). Interestingly, the amount of aboveground incorporated ^{13}C decreased with increasing plant age, ranging from a maximum of 3.6×10^3‰ V-PDB at the young leaf developmental stage to a minimum of 1.7×10^3‰ V-PDB at the flowering stage, while the aboveground plant biomass increased (Supporting Information, Fig. S1). Whereas for the GM line #1332 and its nontransgenic parental cultivar 'Walli', significantly higher ^{13}C incorporation into leaves than into stem was observed at both sampling times (2.1×10^3 vs. 1.3×10^3‰ V-PDB at EC_{30}; 1.0×10^3 vs. 7.2×10^2‰ V-PDB at EC_{60}), no such partitioning was found for the second natural cultivar 'Ponto'. Furthermore, the ^{13}C amount in 'Ponto' stems was considerably higher than that in the other cultivars at the flowering stage. In contrast, neither temporal nor cultivar differences were found for the ^{13}C incorporation into roots.

Incorporation of ^{13}C-labelled carbon into WEOC and C_{mic}

To assess cultivar differences related to the carbon flux via plants into soil, the amounts of ^{13}C of WEOC and C_{mic}, respectively, was determined (Fig. 2). As microbial biomass of labelled and unlabelled unplanted soil samples showed no

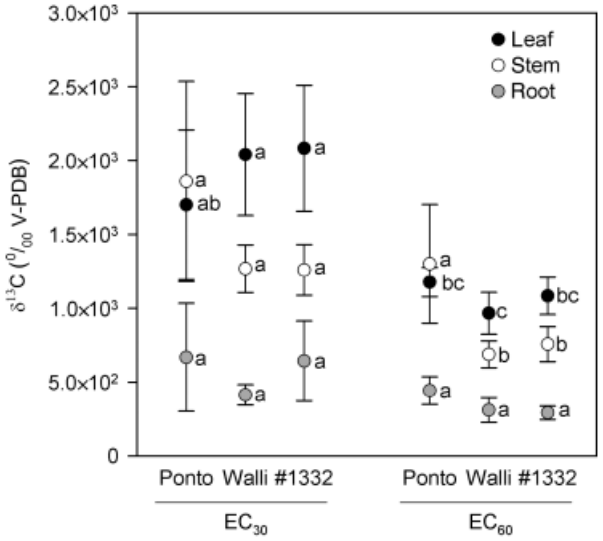

Fig. 1. ^{13}C incorporation ($\delta^{13}C$‰ V-PDB) into total carbon of potato plant parts ($n = 5$, error bars represent SDs) at different plant developmental stages (EC_{30}, young leaf development; EC_{60}, flowering) for different potato cultivars ('Ponto', 'Walli' and #1332). Significance ($P < 0.05$) is indicated by different letters.

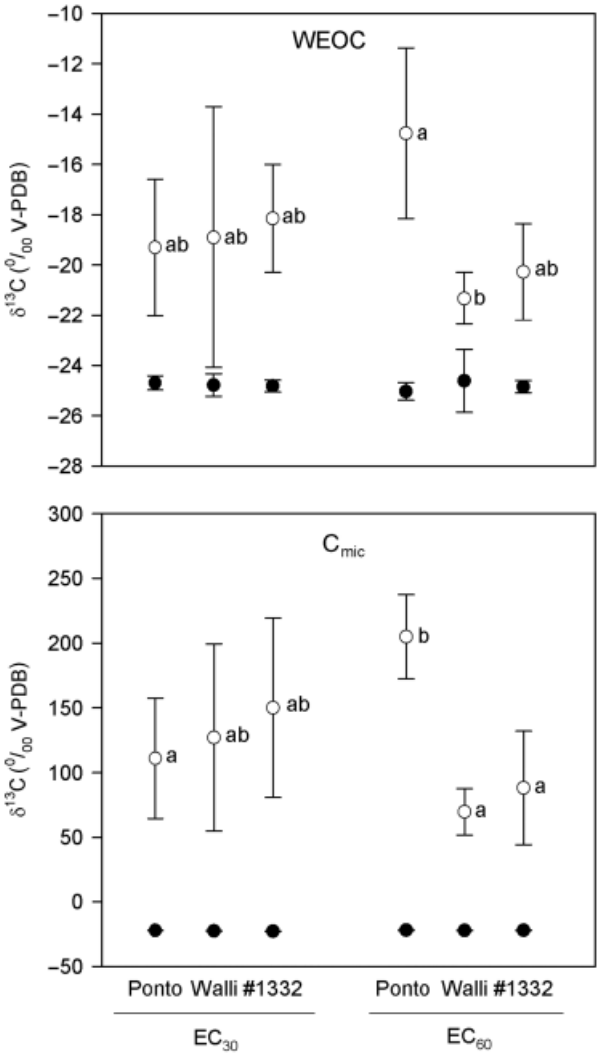

Fig. 2. ^{13}C incorporation ($\delta^{13}C$‰ V-PDB) into WEOC and microbial biomass (C_{mic}) of unlabelled (•) and labelled (○) plants ($n = 5$, error bars represent SDs) at different plant developmental stages (EC_{30}, young leaf development; EC_{60}, flowering) for different potato cultivars ('Ponto', 'Walli' and #1332). Significant differences ($P < 0.05$) among the labelled plants are indicated by different letters.

difference in ^{13}C enrichment (data not shown), microbial autotrophic CO_2 fixation could be neglected. Although the fraction of WEOC showed a significant increase in ^{13}C incorporation compared with the nonlabelled control pots (Fig. 2), its amount of ^{13}C was obviously lower than that of rhizosphere soil microbial biomass (average −18.8 vs. 125.2‰ V-PDB). Furthermore, no temporal differences were found for ^{13}C enrichment of WEOC. In contrast, the ^{13}C incorporation into C_{mic} among the cultivar 'Ponto' doubled with increasing plant age up to 205‰ V-PDB at florescence. Consequently, 'Ponto' showed the highest ^{13}C amount at EC_{60}.

Microbial community structure in the rhizosphere

In total, 29 individual PLFA were identified (Fig. 3). The most common fatty acids were br16:0, 18:1ω9, 18:1ω7 and 18:2ω6.9, representing 43% of the total PLFA. When considering the fatty acids separately, only differences related to plant vegetation stage were found (br16:0, 18:1ω9, n20:0, 20:3ω6.9.12, 20:4ω6.9.12.15). However, by grouping the individual PLFA into markers selective for Gram-positive bacteria (br14:0, i15:0, a15:0, br15:0, i16:0, br16:0, 10Me17:0, i17:0, a17:0, 10Me18:0, i18:0, br19:0), Gram-negative bacteria (16:1ω9, 16:1ω7, cy17:0, 18:1ω7, 18:1ω5, cy19:0), actinomycetes (10Me17:0, 10Me18:0) and fungi (18:2ω6.9), respectively, cultivar-dependent shifts in microbial community structure appeared. The ratio of Gram-positive to Gram-negative bacteria, total Gram-positive bacteria to actinomycetes and fungi to bacteria is illustrated in Fig. 4. In the rhizosphere of 'Walli' at the early leaf developmental stage, the ratio of Gram-positive to Gram-negative bacteria was 1.6, twice as high as that of the second natural cultivar 'Ponto'. Because the ratio declined significantly towards EC_{60}, no shifts among the cultivars were found at the flowering stage. On the contrary, the Gram-positive bacteria to actinomycetes ratio at the first sampling time point was significantly lower in the rhizosphere of 'Walli' and #1332 (7.3 and 7.6, respectively) compared with 'Ponto' (9.1), but increased significantly with increasing plant age. Consequently, again no cultivar-dependent

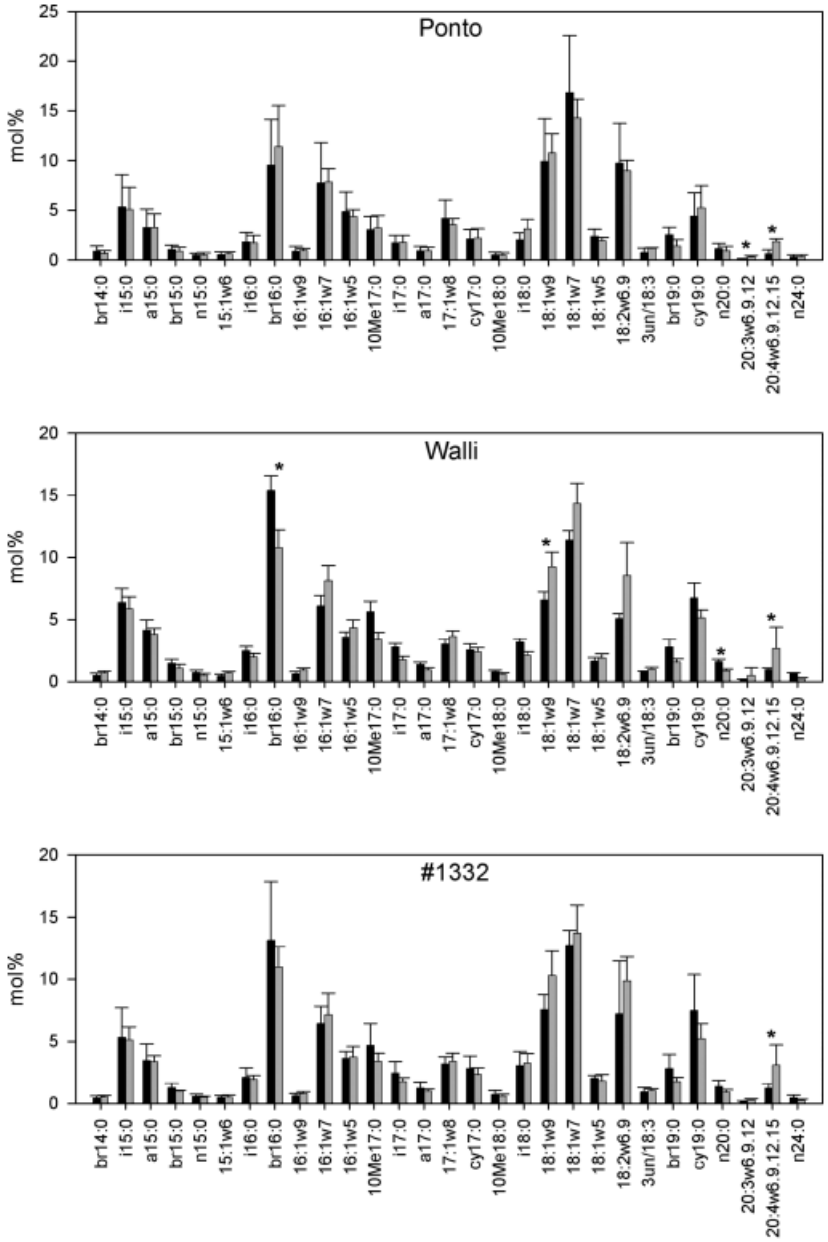

Fig. 3. Relative abundance of PLFA (mol%) in rhizosphere soil ($n = 5$, error bars represent SDs) for different potato cultivars ('Ponto', 'Walli' and #1332) at different plant developmental stages (black bars, EC_{30}; grey bars, EC_{60}). Significance ($P < 0.05$) is indicated by asterisks.

differences were observed at the flowering stage. Similar shifts were found when relating fungal to bacterial PLFA: the ratio in the rhizosphere of 'Ponto' was in the range of 0.1 and hence twice as high as that of 'Walli'.

Incorporation of ^{13}C-labelled carbon into rhizosphere microorganisms

The relative increase of the ^{13}C label in the individual PLFA normalized to the unlabelled control plants is listed in Table 1. All of the detected fatty acids, except the two cyclopropyl SATFA (cy17:0 and cy19:0), showed significant ^{13}C enrichment for at least one cultivar. Interestingly, ^{13}C incorporation into PLFA br15:0 was significant for rhizosphere microorganisms of 'Walli' and #1332 at the young leaf developmental stage, whereas at EC_{60}, enrichment was observed only for 'Ponto'. However, the maximum relative increase of ^{13}C in br15:0 was 0.02% (which represents 0.4% of the total ^{13}C labelling) and therefore very low. The PLFA 20:3ω6.9.12, 18:3, 18:2ω6.9 and br16:0, indicating protozoa, fungi and Gram-positive bacteria, respectively, showed the highest relative ^{13}C incorporation, ranging from 0.2% to 1.7% and contained altogether 63% of the total ^{13}C incorporated into the fatty acids. Furthermore, PLFA 16:1ω5,

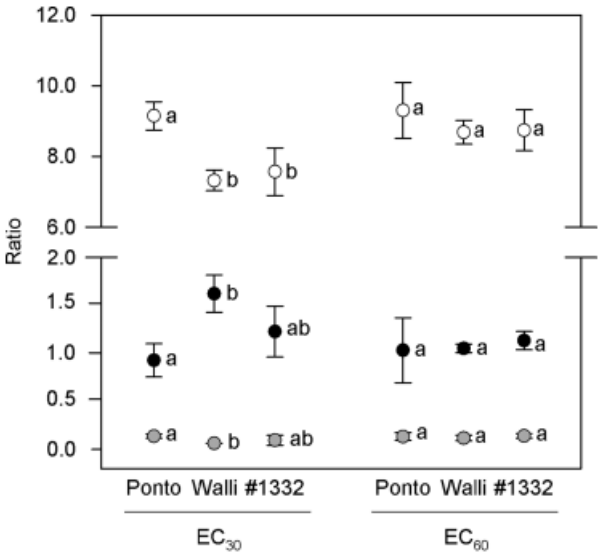

Fig. 4. Ratio of the relative abundance of Gram-positive to Gram-negative bacteria (●), Gram-positive bacteria to actinomycetes (○) and fungi to bacteria (◎) ($n = 5$, error bars represent SDs) at different plant developmental stages (EC_{30}, young leaf development; EC_{60}, flowering) for different potato cultivars ('Ponto', 'Walli' and #1332). Significance ($P < 0.05$) is indicated by different letters.

mainly representing AMF, showed a major ^{13}C sink in the rhizosphere, with 6.3% of the total ^{13}C included in fatty acids. Considering the fatty acids separately, cultivar-dependent differences in ^{13}C enrichment were observed. At the flowering stage, for Gram-positive bacterial (i15:0, i17:0), Gram-negative bacterial (16:1ω7, 18:1ω7, 18:1ω5), fungal (18:2ω6.9), AMF (16:1ω5) and protozoal (20:3ω6.9.12) fatty acids, a significantly higher incorporation of ^{13}C was found in the rhizosphere of 'Ponto' compared with the other two potato varieties. Furthermore, temporal shifts in ^{13}C enrichment occurred for PLFA representing actinomycetes (10Me18:1), Gram-negative bacteria (16:1ω7, 17:1ω8), AMF (16:1ω5) and protozoa (18:3, 20:3ω6.9.12).

Discussion

^{13}C distribution in plant compartments

The analysis of ^{13}C incorporation into the potato plants showed that the majority of assimilated ^{13}C was retained in the aboveground biomass. Surprisingly, despite increasing leaf and stem biomass during plant development (Fig. S1), the appropriate $^{13}C:^{12}C$ ratios decreased from the young leaf developmental stage to florescence. In contrast, the relative ^{13}C enrichment of roots remained constant during sampling, suggesting an enhanced translocation of photoassimilates to roots with increasing plant age, which might be due to the initiation of tuber development shortly before flowering (Dwelle & Love, 1993; Fernie & Willmitzer, 2001). In agreement, several other studies observed a rising ^{13}C enrichment in roots during plant growth when examining beech trees (Esperschütz *et al.*, 2009), oats (Yevdokimov *et al.*, 2006) and maize or wheat (Marx *et al.*, 2007a), showing roots to be a highly active plant compartment requiring large amounts of carbon for growth. In agreement with Lu *et al.* (2002), our results revealed different carbon allocation among the cultivars, as the ^{13}C amount of leaves was significantly higher than that of stems for both the GM line #1332 and its nontransgenic parental cultivar 'Walli', whereas no such partitioning was found for the third cultivar 'Ponto'. Although we did not observe a GM-related effect on the ^{13}C incorporation into the individual plant parts, other studies investigating different transgenic plants expressing the *Bacillus thuringiensis* (Bt) toxin did observe differences related to carbon assimilation between the GM plant and its parental cultivar (Hebbar *et al.*, 2007; Rossi *et al.*, 2007; Wu *et al.*, 2009). In contrast to 'Bt plants', the GM line investigated in the present study did not contain any foreign gene and expressed only potato-derived *gbss* sequences, which might explain the lack of transformation effects on carbon partitioning.

^{13}C distribution in WEOC and C_{mic}

Plants release a major part of their photosynthetically fixed carbon as root exudates into the soil (Soerensen, 1997), where it contributes mainly to the pool of WEOC (Hütsch *et al.*, 2002). This root-derived carbon is an important source of readily available carbon for microorganisms (Paterson *et al.*, 2007) and is thus rapidly metabolized. Following the ^{13}C flux from plants into soil, we observed much lower $\delta^{13}C$ V-PDB values in WEOC than in C_{mic}. This is in accordance with previous reports (Yevdokimov *et al.*, 2006; Marx *et al.*, 2007a; Esperschütz *et al.*, 2009) and is probably due to the considerably small fraction of labelled rhizodeposits remaining in WEOC after microbial uptake (Yevdokimov *et al.*, 2006; Marx *et al.*, 2007b). Our results revealed a strong correlation between the ^{13}C amounts of WEOC and C_{mic} ($R = 0.8$, $P < 0.01$), reflecting the close relationship and the importance of root exudates for the initialization of food webs in soil. According to the carbon partitioning in the plant itself, differences in ^{13}C incorporation between the natural cultivars were observed but not GM-related differences. Similarly, Wu *et al.* (2009) did not monitor changes in the amount of ^{13}C in microbial biomass between Bt rice and the nontransgenic rice cultivar.

Influence of different potato cultivars and plant developmental stage on microbial community structure

The two most abundant fatty acids were 18:1ω7 and br16:0, representing 14% and 12%, respectively, of the total PLFA

Table 1. Relative increase of the ^{13}C label in individual PLFA in rhizosphere soil related to control plants ($n = 5 \pm$ SD) at different plant developmental stages (EC_{30}: young leaf development, EC_{60}: flowering) for different potato cultivars ('Ponto', 'Walli' and #1332)

	EC_{30}						EC_{60}					
Increase %	Ponto	±	Walli	±	#1332	±	Ponto	±	Walli	±	#1332	±
br14:0	0.027 ab	0.016	0.022 b	0.010	0.035 ab	0.011	0.043 a	0.006	0.028 ab	0.006	0.030 ab	0.010
i15:0	0.022 ab	0.010	0.011 ab	0.005	0.022 ab	0.010	0.016 a	0.003	0.008 b	0.002	0.011 ab	0.005
a15:0	0.013 a	0.005	0.007 a	0.005	0.011 a	0.004	0.013 a	0.003	0.007 a	0.002	0.008 a	0.002
br15:0	0.011 ab	0.013	0.014 a	0.005	0.017 ab	0.015	0.021 ab	0.014	0.000 ab	0.007	0.002 b	0.003
n15:0	0.027 ab	0.014	0.023 ab	0.010	0.027 ab	0.009	0.039 a	0.007	0.023 ab	0.004	0.019 b	0.004
15:1ω6	0.015 a	0.009	0.006 a	0.002	0.009 a	0.003	0.011 a	0.005	0.005 a	0.001	0.005 a	0.001
i16:0	0.027 a	0.009	0.023 a	0.009	0.029 a	0.009	0.027 a	0.003	0.016 a	0.004	0.016 a	0.002
br16:0	0.265 ab	0.141	0.271 ab	0.140	0.237 a	0.042	0.623 b	0.179	0.343 ab	0.056	0.326 ab	0.048
16:1ω9	0.134 a	0.113	0.036 a	0.017	0.042 a	0.013	0.070 a	0.013	0.042 a	0.025	0.049 a	0.042
16:1ω7	0.117 a	0.056	0.145 ab	0.073	0.103 a	0.019	0.287 b	0.066	0.133 a	0.017	0.126 a	0.015
16:1ω5	0.127 a	0.091	0.205 ac	0.084	0.115 a	0.052	0.512 b	0.199	0.293 bc	0.084	0.277 c	0.075
10Me17:0	0.026 a	0.007	0.039 a	0.009	0.038 a	0.007	0.081 a	0.034	0.033 a	0.004	0.034 a	0.005
i17:0	0.119 ab	0.078	0.061 ab	0.019	0.142 ab	0.058	0.102 a	0.023	0.045 b	0.006	0.041 b	0.004
a17:0	0.032 a	0.010	0.026 a	0.008	0.037 a	0.010	0.061 a	0.022	0.026 a	0.007	0.027 a	0.004
17:1ω8	0.011 a	0.004	0.005 ac	0.002	0.008 a	0.002	0.003 ab	0.004	0.001 b	0.002	0.003 bc	0.002
cy17:0	0.000 a	0.002	0.001 a	0.002	0.002 a	0.003	0.002 a	0.003	0.000 a	0.000	0.000 a	0.001
10Me18:0	0.002 a	0.002	−0.002 ab	0.002	0.001 ab	0.003	0.007 b	0.005	0.002 ab	0.003	0.002 ab	0.001
i18:0	0.092 a	0.052	0.073 a	0.034	0.081 a	0.026	0.099 a	0.037	0.064 a	0.009	0.046 a	0.008
18:1ω9	0.106 ab	0.062	0.076 ab	0.037	0.065 a	0.007	0.155 b	0.041	0.086 ab	0.011	0.070 ab	0.008
18:1ω7	0.165 ab	0.066	0.148 ab	0.059	0.119 a	0.021	0.286 b	0.080	0.104 a	0.021	0.103 a	0.018
18:1ω5	0.123 ab	0.060	0.102 ab	0.036	0.093 a	0.021	0.227 b	0.063	0.101 a	0.025	0.095 a	0.017
18:2ω6.9	0.295 ab	0.156	0.810 ab	0.440	0.859 ab	0.428	0.733 a	0.232	0.360 ab	0.056	0.239 b	0.112
3un/18:3	0.237 a	0.093	1.214 ab	0.700	1.021 ab	0.826	0.511 b	0.072	0.253 ab	0.135	0.578 ab	0.801
br19:0	0.004 a	0.003	0.005 a	0.002	0.008 a	0.004	0.009 a	0.003	0.004 a	0.001	0.004 a	0.001
cy19:0	0.002 a	0.001	0.000 ab	0.000	0.000 bc	0.001	−0.001 bc	0.000	−0.001 c	0.000	0.000 bc	0.001
n20:0	0.030 a	0.024	0.015 a	0.009	0.027 a	0.012	0.042 a	0.015	0.015 a	0.003	0.013 a	0.004
20:3ω6.9.12	0.893 a	0.521	1.120 ac	0.412	0.527 a	0.349	1.727 b	0.341	0.788 a	0.382	0.981 ab	0.235
20:4ω6.9.12.15	0.117 a	0.054	0.108 a	0.046	0.069 a	0.036	0.115 a	0.055	0.070 a	0.014	0.124 a	0.171
n24:0	0.214 a	0.233	0.090 a	0.057	0.176 a	0.084	0.199 a	0.067	0.089 a	0.019	0.104 a	0.038

Significance ($P < 0.05$) among rows is indicated by different letters.

on average. This indicates a high proportion of Gram-negative and Gram-positive bacteria in the rhizosphere of potato plants, which is in the line with previous reports (Frostegård *et al.*, 1993; Esperschütz *et al.*, 2009; Wu *et al.*, 2009). Furthermore, by comparing the abundance of bacterial subpopulations in the rhizosphere, our results revealed significant differences in the ratios of Gram-positive to Gram-negative bacteria as well as Gram-positive bacteria to actinomycetes between the potato cultivars. This might be due to plant genotype-specific root exudation pattern, as it is known that root exudates clearly influence microbial community structure (Marschner *et al.*, 2001; Söderberg *et al.*, 2002). No difference in community composition between the GM line and its nontransgenic parent cultivar was observed. Although PLFA analysis cannot give the species composition of microbial communities, this finding coincides with previous studies using nucleic acid-based methods, where GM plant-related impacts on the community structure of microorganisms were also not found (Saxena & Stotzky, 2001; Schmalenberger & Tebbe, 2002; Gschwendtner *et al.*, 2010). Therefore, it could be assumed that the genetic modification did not affect microbial diversity in the rhizosphere.

PLFA 18:2ω6.9 is often used as a biomarker for fungi (Frostegård & Bååth, 1996; Fierer *et al.*, 2003; Esperschütz *et al.*, 2009; Wu *et al.*, 2009). Although there has been some discussion in the past about its validity to quantify fungal biomass, as this fatty acid also occurs in plant cells (Fierer *et al.*, 2003). Kaiser *et al.* (2010) proved in a recent article that the contribution of root-derived PLFA to soil-borne fungal biomarker 18:2ω6.9 can be neglected. Furthermore, oleic acid 18:1ω9 is also an important component of fungal membranes, but can be present in Gram-negative bacteria, too (Zelles, 1999). Thus, only linoleic acid 18:2ω6.9 was used in this study for calculation of fungal phospholipids. By relating 18:2ω6.9 to bacterial PLFA, we observed different abundance ratios between the natural cultivars, but again no shift in microbial community between the GM line

and its nontransgenic parental cultivar. In accordance, other authors reported that GM plants had no significant impact on the fungal rhizosphere community (Milling *et al.*, 2004; Wu *et al.*, 2009; Gschwendtner *et al.*, 2010).

Besides the genotype-specific influence on microbial community composition in the rhizosphere, our results also revealed a clear effect of the plant developmental stage. This may be due to the change of the amount and the chemical composition of root exudates during plant growth (Jones *et al.*, 2004). Whereas at EC_{30}, the microbial rhizosphere community was considerably cultivar specific, no differences between the cultivars were found at the flowering stage, suggesting that quantity and quality of rhizodeposits converge between the different cultivars from EC_{30} to EC_{60}. In agreement, Weinert *et al.* (2009) observed the highest similarity in DGGE pattern of microbial rhizosphere communities at EC_{60} when investigating GM potatoes over the growing season.

^{13}C incorporation into the rhizosphere microbial community

All PLFA detected were significantly enriched with ^{13}C compared with those in the rhizosphere of the unlabelled control plants, except the Gram-negative biomarkers cy17:0 and cy19:0. These fatty acids are derivates of 16:1ω7 and 18:1ω7, respectively, and are known to be formed under environmental stress conditions such as starvation to stabilize cell membrane (Frostegård *et al.*, 1993). The lack of ^{13}C incorporation suggests that these stress PLFA were not actively formed in the microbial community structure throughout the experiment. In concordance, Esperschütz *et al.* (2009) observed a decreasing abundance of cy17:0 and cy19:0 over time when investigating rhizosphere microorganisms of beech trees after dormancy, indicating that growth conditions for Gram-negative bacteria improve during plant development.

Although the relative abundance of individual PLFA revealed only temporal shifts in the microbial community pattern, the rhizosphere microbial populations strongly differed in metabolizing root-derived carbon between the cultivars. Commonly, ^{13}C incorporation into the PLFA was significantly higher in the rhizosphere of 'Ponto' than of 'Walli' or #1332, particularly at the flowering stage. This correlates with the increased ^{13}C label detected in the total rhizosphere microbial biomass of 'Ponto' and might be the result of more effective turnover of root exudates and/or an enhanced $^{13}C:{}^{12}C$ ratio of root exudates. The latter is indicated by the raised ^{13}C amount of aboveground plant biomass compared with the other cultivars and reflects different carbon partitioning in and via the plant cultivars. In concordance, when considering rhizosphere PLFA of different natural rice lines, variations in ^{13}C enrichment were found and suggested to be a result of a genotype-induced difference in the quality and/or quantity of root exudates and/or isotope discrimination (Lu *et al.*, 2004). Although ^{13}C incorporation differed between 'Ponto' and the other cultivars, the genetic modification seemed to have no effect on carbon partitioning or activity of microbial populations, as no change in the ^{13}C amount of the PLFA was observed between 'Walli' and #1332.

Besides a considerable incorporation of the ^{13}C label into fungal biomarker 18:2ω6.9, which reflects a strong interaction between plant and fungi, as previously described by other authors (Lu *et al.*, 2004; Esperschütz *et al.*, 2009; Wu *et al.*, 2009), the largest ^{13}C incorporation was detected in PLFA 18:3 and 20:3ω6.9.12, which have been described as markers for Gram-negative bacteria as well as for protozoa. It can be postulated that the high labelling was linked mainly to Gram-negative bacteria, which are known to be the most active in assimilating root-derived carbon (Wu *et al.*, 2009). Although protozoa are not directly influenced by root exudates, they graze on bacteria (Bonkowski *et al.*, 2000) and thus incorporate labelled carbon as secondary consumers.

Furthermore, a major part of total ^{13}C was detected in PLFA 16:1ω5, mainly representing AMF (Olsson *et al.*, 1999; Joergensen & Wichern, 2008). Interestingly, despite the importance of mycorrhizal associations for nutrient uptake, Cesaro *et al.* (2008) observed only low AMF root colonization for potatoes, suggesting that this is a result of high nutrient concentrations in the soil and therefore a lack of need for mycorrhizal symbiosis. In agreement, in the present study low root mycorrhization was observed microscopically. This was surprising because PLFA 16:1ω5 contributed substantially to the total amount of fatty acids in the rhizosphere and also showed high ^{13}C incorporation. However, due to the restriction of microscopic approaches for quantification mycorrhizal biomass, further analyses are necessary to evaluate whether the considerable ^{13}C incorporation into 16:1ω5 represents active AMF or a high turnover rate of root-derived carbon by Gram-negative bacteria, which can also form this PLFA (Zelles, 1999).

Conclusions

PLFA analysis in combination with ^{13}C labelling could be a useful tool in risk-assessment studies to analyse the immediate influence of plant genome transformation on carbon-partitioning characteristics within the plant and, subsequently, into the rhizosphere microbial community. Although our results revealed no differences between the GM line and its nontransgenic parental cultivar concerning photosynthate partitioning (objective 1) and total living community of microbial degraders of root exudates (objective 2), the influence of plant genotype and plant developmental stage could be shown (objective 3). However, our

data do not allow a generalization due to risk assessment, as on the one hand, PLFA analysis provides only limited insight into microbial population structure, and on the other, this experiment was performed as a greenhouse study under optimal growth conditions. Whether the GM plant could be more affected by abiotic and biotic stressors in the field, resulting in changes in carbon allocation compared with the wild type, needs to be addressed in further experiments.

Acknowledgements

This study was supported by grant 772e-U8793-2006/10-2 from the Bayerisches Staatsministerium für Umwelt, Gesundheit und Verbraucherschutz (StMUGV). The plant material was provided by the seed collection of the Bavarian State Research Center for Agriculture. We thank Peter Kary for optimizing the greenhouse facilities for this experiment. Furthermore, Christine Kollerbaur is gratefully acknowledged for her assistance in PLFA extraction.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Biomass of potato plants ($n = 5$, error bars represent SDs) at different plant developmental stages (EC_{30}, young leaf development; EC_{60}, flowering) for different potato cultivars ('Ponto', 'Walli' and #1332).

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