

Plant age and genotype affect the bacterial community composition in the tuber rhizosphere of field-grown sweet potato plants

Joana M. Marques¹, Thais F. da Silva¹, Renata E. Vollu¹, Arie F. Blank², Guo-Chun Ding^{3,4}, Lucy Seldin¹ & Kornelia Smalla³

¹Laboratório de Genética Microbiana, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil; ²Departamento de Engenharia Agrônômica, Universidade Federal de Sergipe, Sergipe, SE, Brazil; ³Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Braunschweig, Germany; and ⁴College of Resources & Environmental Sciences, China Agricultural University, Beijing, China

Correspondence: Lucy Seldin, Laboratório de Genética Microbiana, Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes (IMPPG), Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco I, Ilha do Fundão, CEP 21941-590, Rio de Janeiro, Brazil. Tel.: +55 21 2562 6741; fax: +55 21 2560 8344; e-mail: lseldin@micro.ufrj.br

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Introduction

The plant *Ipomoea batatas* L., popularly known as sweet potato, is a dicotyledonous plant that belongs to the *Convolvulaceae* family. Sweet potato plants store starch in their roots and form tuberous roots. They are one of the most important subsistence crops in developing countries and the most important root crop after potato (*Solanum tuberosum*) and cassava (*Manihot esculenta*). Nowadays, China is the world's largest producer of sweet potato supplying 80–85% of the global production. In Brazil, sweet potato is the sixth most consumed crop, and the national production per year averages 11.7 t ha⁻¹. Sweet potatoes are considered one of the main crops in the Northeast Region of Brazil

Abstract

The hypothesis that sweet potato genotypes containing different starch yields in their tuberous roots can affect the bacterial communities present in the rhizosphere (soil adhering to tubers) was tested in this study. Tuberous roots of field-grown sweet potato of genotypes IPB-149 (commercial genotype), IPB-052, and IPB-137 were sampled three and six months after planting and analyzed by denaturing gradient gel electrophoresis (DGGE) and pyrosequencing analysis of 16S rRNA genes PCR-amplified from total community DNA. The statistical analysis of the DGGE fingerprints showed that both plant age and genotypes influenced the bacterial community structure in the tuber rhizosphere. Pyrosequencing analysis showed that the IPB-149 and IPB-052 (both with high starch content) displayed similar bacterial composition in the tuber rhizosphere, while IPB-137 with the lowest starch content was distinct. In comparison with bulk soil, higher 16S rRNA gene copy numbers (qPCR) and numerous genera with significantly increased abundance in the tuber rhizosphere of IPB-137 (*Sphingobium*, *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, *Chryseobacterium*) indicated a stronger rhizosphere effect. The genus *Bacillus* was strongly enriched in the tuber rhizosphere samples of all sweet potato genotypes studied, while other genera showed a plant genotype-dependent abundance. This is the first report on the molecular identification of bacteria being associated with the tuber rhizosphere of different sweet potato genotypes.

where they have a huge social importance (Silveira, 2008; Albuquerque *et al.*, 2012; Wang *et al.*, 2013).

Sweet potato is an easily propagated crop growing well in infertile and nitrogen poor soils, and its storage roots are largely used for food consumption because of their nutritional composition. The sweet potato tuberous roots contain simply fermentable sugars such as glucose, fructose, and sucrose; minimal quantities of fibers and proteins; and they are rich in starch. These nutritional characteristics are also of interest to the alcohol industry; however, sweet potatoes selected for ethanol production must have a higher content of starch and good agricultural yields (Ferrari *et al.*, 2013; Wang *et al.*, 2013). Therefore, national programs of sweet potato genetic

improvement for bioenergy production that seek for varieties with high yields of dry biomass and starch are getting more common in developing countries (Ferrari *et al.*, 2013). Despite the constantly rising number of studies concerning the importance of sweet potato for ethanol production (Srichuwong *et al.*, 2009; Ziska *et al.*, 2009; Lee *et al.*, 2012a; Duvernay *et al.*, 2013), almost nothing can be found in the literature about the bacterial communities present in the soil adhering to tuberous roots (tuber rhizosphere) of sweet potato genotypes with different starch concentrations in their tuberous roots. Considering the importance of the plant-associated microorganisms not only for plant health and growth but also for maintaining soil fertility (Berendsen *et al.*, 2012), insights into the bacterial communities associated with sweet potatoes should be essential components of breeding programs. Previously, Khan & Doty (2009) reported on the endophytic bacterial diversity present in the stems of sweet potato studied by means of culture-dependent methods. In contrast, several studies have already focused on the influence of potato (*Solanum tuberosum*) cultivars with different concentrations of starch on rhizosphere bacterial communities (Millington *et al.*, 2004; Inceoglu *et al.*, 2010, 2011, 2012; Dias *et al.*, 2013). However, in contrast to sweet potato, the tubers of *S. tuberosum* belong to the stem.

To obtain knowledge of the influence of three different sweet potato genotypes (IPB-149, a commercial genotype; and IPB-137 and IPB-052, genotypes tested for ethanol production) on the tuber rhizosphere bacterial communities, the aims of this study were as follows: (1) to investigate the influence of each plant genotype and its starch content in the tuberous roots on the structure of the bacterial community, (2) to evaluate whether there are any effects of plant age on the bacterial community structure, as sweet potato plants were sampled 3 and 6 months after sowing, and (3) to identify the dominant bacteria enriched in the tuber rhizosphere depending on the sweet potato genotype. To achieve these goals, 16S rRNA gene fragments amplified from total community DNA were analyzed by denaturing gradient gel electrophoresis (DGGE), quantitative real-time PCR (qPCR – bacterial 16S rRNA gene copy numbers), and pyrosequencing. Group-specific PCR-DGGEs were also used not only to analyze dominant bacteria but also to allow a better understanding of *Alphaproteobacteria* and *Betaproteobacteria*, *Actinobacteria*, *Pseudomonas*, and *Enterobacteriaceae* which are known as bacterial groups typically associated with roots of other crops and which are assumed to be important for plant health and growth. The molecular approaches used in this study are complementary, and, altogether, they represent powerful tools to analyze bacterial communities as they can provide qualitative and quantitative information.

Materials and methods

Field experiment and soil sampling

In 2011, three different sweet potato genotypes (IPB-149, IPB-137, and IPB-052) were planted in three replicate rows (randomized block design) at the Research Station 'Campus Rural da UFS', in the 'São Cristóvão' municipality (10°55'27"S/37°12'01"W), Sergipe State, Northeast of Brazil. The experimental plot consisted of rows with spaces of 0.8 m between the rows and 0.35 m between plants. The field received 1000 kg of NPK (nitrogen, phosphorous and potassium -6-24-12) per hectare 30 days before planting and every month during the experiment. The experimental farm soil is a red-yellow argisil, classified as sandy loam (sand 74%, silt 21%, and clay 5%) with a slightly acidic pH (5.4). Chemical characteristics of the soil were (per g kg⁻¹): Ca²⁺ – 0.39, Mg²⁺ – 0.43, K⁺ – 21.1, P – 7.0, Al³⁺ – 0.65, Na⁺ – 3.5; and 0.86% of organic carbon.

IPB-149 is a commercial genotype from the State of Sergipe consumed by humans and animals. The IPB-137 and IPB-052 genotypes were obtained from Federal University of Lavras (UFLA), Minas Gerais, Brazil. These genotypes were generated within a governmental program aiming to select potential genotypes for ethanol production. The characteristics of these genotypes are described in Table 1 and were provided by Oliveira Neto (2012). Briefly, the IPB-149 and IPB-052 genotypes had similar starch yield values in the tuberous roots (no significant differences, as determined by Scott–Knott test – $P < 0.05$), and the IPB-137 genotype had the lowest starch yield values (significant difference, as determined by Scott–Knott test – $P < 0.05$). Because of the low starch

Table 1. Characteristics of the tuberous roots of sweet potato genotypes (IPB-149, IPB-137, and IPB-052) sampled 6 months after sowing

Characteristics*	Genotypes		
Average yield	IPB-149	IPB-137	IPB-052
Number of tuberous roots (t ha ⁻¹)	17.85b	10.72b	36.05a
Tuberous roots dry mass (%)	40.12a	35.07b	34.91b
Insects susceptibility (rate: 1–5 [†])	1.24b	2.25a	1.20b
Starch yield (t ha ⁻¹)	4.70a	2.28b	5.97a
Ethanol production (L ha ⁻¹)	5105.24a	2478.36b	6492.12a

Different online letters indicate significant differences ($P < 0.05$) as determined using the Scott–Knott test.

*Data from Oliveira Neto (2012).

[†]Rate 1–5 according to Silveira (1993), where rate 1: root free of insect damage; rate 2: roots slightly damaged; rate 3: roots with observable damage; rate 4: damages covering most of roots surface; and rate 5: roots showing damages on the entire surface.

content, this genotype was not further considered for ethanol production.

Sweet potato sampling was carried out 3 (t_1) and 6 (t_2) months after planting. At each sampling time, five plants per sweet potato genotype were randomly harvested from the three replicate rows and the roots were shaken to remove the loosely attached soil. The soil still adhering to the tuberous roots was brushed off from all tuberous roots per plant, and this soil was considered as tuber rhizosphere. A total of five plants (individual plants were considered as replicate) were analyzed per plant genotype. The plants of the different genotypes displayed comparable plant growth developmental stages. In addition, five bulk soil samples (Falcon tube cores; each core representing a single sample) were taken from the same field (distances between the sampling points *c.* 1 m) 6 (t_2) months after planting. The samples were homogenized by sieving. Samples were kept at $-20\text{ }^\circ\text{C}$ before total community DNA (TC-DNA) extraction.

TC-DNA extraction and DGGE analysis of 16S rRNA gene amplicons

TC-DNA was extracted from the tuber rhizosphere of five replicate samples per genotype and five bulk soil samples (0.5 g per sample) using the Fast DNA Spin Kit for soil (Qbiogene, BIO 101 Systems) according to the manufacturer's instructions. Bacterial 16S rRNA gene fragments were amplified from TC-DNA with the primers F984-GC and R1378 as described by Heuer *et al.* (1997). In addition, a nested-PCR approach was applied for the amplification of 16S rRNA genes of *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*, *Pseudomonas*, and *Enterobacteriaceae*, followed by PCR with primers F984-GC and R1378 as previously described (Heuer *et al.*, 1997; Gomes *et al.*, 2001; Costa *et al.*, 2007; Binh *et al.*, 2010). DGGE analyses of the 16S rRNA gene amplicons were performed according to Gomes *et al.* (2005). The DGGE gels were silver-stained according to Heuer *et al.* (2001). The software package GELCOMPAR 4.5 was used for cluster analysis of DGGE profiles. Dendrograms were constructed based on the Pearson correlation index for each pair of lanes within a gel using unweighted pair group method with arithmetic mean averages (UPGMA). The pairwise Pearson correlation indices were used to test for significant effects by application of the previously described permutation test (Kropf *et al.*, 2004).

Determination of 16S rRNA gene copy numbers

TC-DNA from the tuber rhizosphere of the three sweet potato genotypes and bulk soil samples was used as template in a quantitative real-time PCR (qPCR). The

primers and the TaqMan probe used to quantify bacterial 16S rRNA genes by qPCR were described by Takai & Horikoshi (2000). PCR amplifications were performed in 50 μL of reaction mixture containing 1.25 U TrueStart polymerase (Fermentas, St. Leon-Rot, Germany), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 2.5 mM MgCl_2 , and 0.25 μM primers and probe. Thermocycles were 10 min at $94\text{ }^\circ\text{C}$ and 40 cycles consisting of 15 s at $95\text{ }^\circ\text{C}$, 15 s at $50\text{ }^\circ\text{C}$, and 60 s at $60\text{ }^\circ\text{C}$. Templates to generate standard curves were prepared by serial dilutions of gel-purified PCR products from *Escherichia coli* 16S rRNA gene fragments. Significant differences between samples were tested in pairwise comparisons using Tukey test ($P < 0.05$; SAS 9.3; SAS Institute Inc., Cary, NC).

Pyrosequencing and sequence analysis

TC-DNA extracted from three replicates of the tuber rhizosphere per sweet potato genotype and three bulk soil t_2 samples was used by the Biotechnology Innovation Center (BIOCANT, Aveiro, Portugal) for pyrosequencing. The replicates were selected for pyrosequencing according to their TC-DNA quality and concentration ($\text{ng } \mu\text{L}^{-1}$ of TC-DNA). The hypervariable V3–V4 regions of 16S rRNA gene fragments were amplified with bacterial primers 338F and 802R (RDP's Pyrosequencing Pipeline: <http://pyro.cme.msu.edu/pyro/help.jsp>) with the 454 A or 454 B adaptors. The PCRs were performed with the Fast Start polymerase (Roche, Penzberg, Germany) in reaction mixes containing 3 mM MgCl_2 , 6% DMSO, 0.2 μM of each primer, and 200 mM dNTP. Denaturation was carried out at $94\text{ }^\circ\text{C}$ for 3 min, followed by 35 cycles of $94\text{ }^\circ\text{C}$ for 30 s, $44\text{ }^\circ\text{C}$ for 45 s, and $72\text{ }^\circ\text{C}$ for 60 s, and a final elongation step at $72\text{ }^\circ\text{C}$ for 2 min. Sequencing was performed on a 454 Genome Sequencer FLX platform according to standard 454 protocols (Roche – 454 Life Sciences, Branford, CT).

Pyrosequencing data were evaluated according to Ding *et al.* (2012). Sequences matching the barcode and primer were selected, and the unpaired regions of selected sequences were truncated based on BLASTN analysis against a bacterial database (Pruesse *et al.*, 2007). Only those sequences with a length above 200 bp were included in the further analyses. Multiple alignments and operational taxonomic unit (OTU > 97% sequence identity) assignment were performed with the software package MOTHUR (v1.14.0; Schloss *et al.*, 2009). The RDP MultiClassifier was used for taxonomical assignment of each sequence at > 80% confidence (Wang *et al.*, 2007). Both results were integrated into a taxonomic OTU report with each row representing one OTU, its taxonomic position and the number of sequences for each sample. To compare the bacterial community structure in the tuber rhizosphere

and bulk soil samples based on OTUs, cluster analyses were performed according to the pairwise Pearson correlation that was also used for DGGE analyses. The reliability of the clusters was tested by 500 times bootstrap analysis. Additionally, weighted UniFrac distance (Crawford *et al.*, 2009) using consensus sequence for each OTU was calculated for cluster and principal coordination analysis (PcoA). ANOSIM (R Vegan) and permutation tests based on Pearson correlation indices and UniFrac distance were applied to analyze the significant difference in community compositions among samples. Discriminative taxonomic groups between tuber rhizosphere and bulk soil samples were identified by Tukey's honest significance tests under a generalized linear model via a logistic function for binomial data with the package MULTCOMP (Hothorn *et al.*, 2008). All statistical analyses as well as the plots of rarefaction curves were carried out with software R (2.12) with/without add-on packages.

Results

Bacterial 16S rRNA gene copy numbers in TC-DNA from tuber rhizosphere and bulk soil samples

Bacterial 16S rRNA gene copy numbers in TC-DNA from tuber rhizosphere samples from IPB-149, IPB-137, and IPB-052 genotypes (t_1 and t_2) and bulk soil (t_2) were determined by qPCR (Fig. 1) and tested for significant differences between samples using Tukey test ($P < 0.05$). The bacterial 16S rRNA gene copy numbers per gram of soil ranged between 10^8 and 10^9 for t_1 samples and 10^7 and 10^9 for t_2 samples (Fig. 1). The genotypes IPB-149,

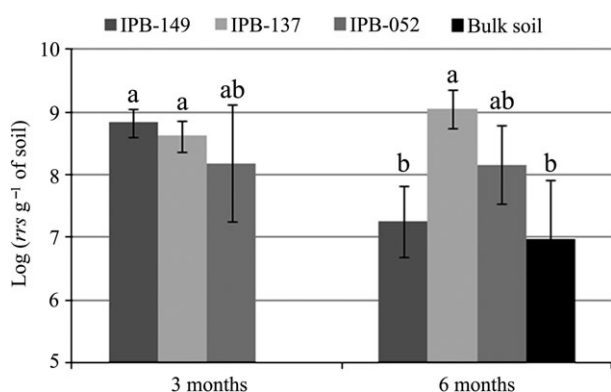


Fig. 1. Determination of 16S rRNA gene copy numbers by quantitative real-time PCR (qPCR) in TC-DNA from the tuber rhizosphere of IPB-149, IPB-137, and IPB-052 genotypes taken 3 and 6 months after sowing and bulk soil (6 months after sowing). Different letters (a and b) indicate significant differences of means in pairwise comparisons (Tukey test; $P < 0.05$).

IPB-137, and IPB-052 from t_1 did not show significant differences in 16S rRNA gene copy numbers and only genotype IPB-149 showed significantly lower 16S rRNA gene copy numbers at t_2 compared to IPB-137 and IPB-052. For t_2 samples, significantly higher 16S rRNA gene copy numbers were determined in TC-DNA from the tuber rhizosphere of IPB-137 genotype compared to bulk soil indicating a typical rhizosphere effect. In contrast, there were no significant differences between the 16S rRNA gene copy numbers determined in the TC-DNA from the tuber rhizosphere of both other genotypes and bulk soil ($P < 0.05$) (Fig. 1). A significantly higher 16S rRNA gene copy number was also observed in TC-DNA from the tuber rhizosphere of IPB-137 compared to that of IPB-149 genotype (Fig. 1).

Effects of plant age and genotypes on the structure of bacterial community analyzed by DGGE

Effects of plant age and genotypes on the structure of bacterial communities present in the tuber rhizosphere of sweet potato plants were analyzed using DGGE fingerprints of *Bacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*, *Pseudomonas*, and *Enterobacteriaceae* (Supporting Information, Fig. S1a–f). UPGMA analysis of bacterial community, *Alphaproteobacteria*, *Betaproteobacteria*, and *Actinobacteria* fingerprints clustered according to sampling time (Fig. 2 and Fig. S2a–c, respectively). Just analyzing *Bacteria* fingerprints, the replicates of all genotypes formed separate clusters according to the sampling times suggesting that the effect of the sampling time was stronger than that of the genotype (Fig. 2). The results of the UPGMA analysis of the *Alphaproteobacteria*, *Betaproteobacteria* (only for samples from t_2), and *Actinobacteria* fingerprints showed that the replicates of the fingerprints of each genotype formed groups of high similarity ($> 80\%$ similarity among profiles; Fig. S2a–c). UPGMA analysis of *Pseudomonas* and *Enterobacteriaceae* fingerprints revealed no influence of the sampling time (t_1 and t_2) or the genotype (Fig. S2d and e) as no separate clusters were observed. For bacterial community fingerprints, bulk soil clustered separately from the tuber rhizosphere samples suggesting a strong effect of sweet potato on total bacterial communities (Fig. 2).

When bacterial community fingerprints were statistically analyzed using the permutation test ($P < 0.05$; Table 2), the results showed that the plant age significantly influenced the bacterial community structure of all groups studied for all three genotypes. *Pseudomonas* community was less influenced by time while *Alphaproteobacteria* and *Bacteria* community fingerprints of IPB-137 genotype were strongly influenced by time (20.2% and

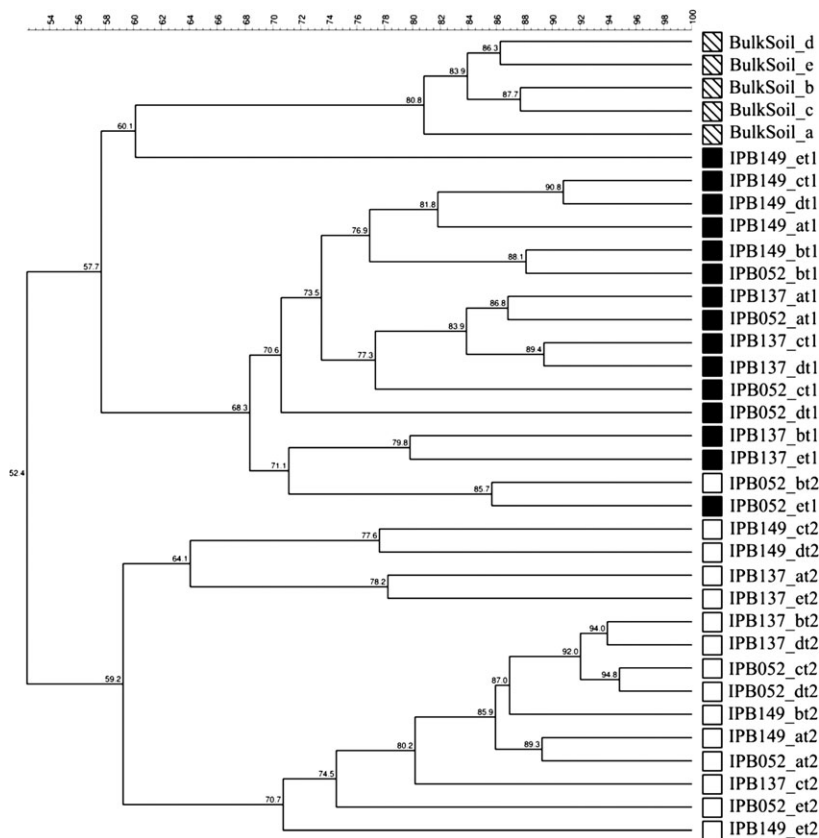


Fig. 2. UPGMA cluster analysis of the DGGE fingerprint of *Bacteria* community in the tuber rhizosphere (replicates a–e) of IPB-149, IPB-137, and IPB-052 genotypes and bulk soil (replicates a–e – striped squares) 3 (t_1 – black squares) and 6 (t_2 – white squares) months after sowing.

18.9% dissimilarity values average, respectively). Moreover, the majority of the communities analyzed showed significant differences ($P < 0.05$) between genotypes (Table 2). However, the dissimilarity values indicated that the effects of plant genotypes were lower at the second sampling time. For t_1 plant, genotype-dependent differences were clearer in the bacterial community fingerprints than in the other bacterial groups studied (Table 2). There were no significant statistical differences between *Pseudomonas* communities from any genotypes studied at t_2 . Bacterial communities present in bulk soil were significantly different ($P < 0.05$) from bacterial communities present in the tuber rhizosphere of all genotypes studied at t_2 indicating a typical rhizosphere effect (Table 2). Dissimilarity values for *Enterobacteriaceae* community could not be determined (Table 2) as there were no bands in bulk soil fingerprints, indicating that their abundance was below the detection limit (Fig. S1f). The genotype IPB-137 showed the highest dissimilarity values when compared to bulk soil samples for almost all the communities studied (Table 2). Finally, among these communities studied, *Betaproteobacteria* communities showed the highest dissimilarity values (around 60%) when compared to bulk soil samples (Table 2).

Bacterial community diversity and plant genotype-dependent rhizosphere responders

Three replicate tuber rhizosphere samples per plant genotype and bulk soil from t_2 were analyzed by barcoded pyrosequencing of 16S rRNA genes (V3–V4 region). A total of 54,479 sequences for all 12 samples were examined with a range of 2128–6439 reads per sample. More than 95% of the sequences could be classified to 19 phyla, while 56.7% sequences could be classified at the genus level (266 genera – Table S1). In general, the tuber rhizosphere from all plant genotypes and bulk soil showed a high richness (Fig. S3a: Chao1) and evenness (Fig. S3b: Pielou's evenness). However, a lower diversity was observed in tuber rhizosphere samples from IPB-137 compared to bulk soil.

A systematical analysis of taxonomic groups (from phylum to genus) with significantly different relative abundance between tuber rhizosphere from all genotypes and bulk soil was identified using multiple Tukey's tests under a generalized linear model via logistic function for binomial data. Table 3 gives an overview of bacterial groups with significantly different abundance in tuber rhizosphere compared to bulk soil samples. Several bacterial groups were significantly increased in the tuber rhizo-

Table 2. Percent dissimilarity of bacterial DGGE fingerprints of different taxonomic groups for tuber rhizosphere compared between sampling times (t_1 and t_2) or among IPB-149, IPB-137, and IPB-052 genotypes and bulk soil

	DGGE gel					
	Bacteria	Alphaproteobacteria	Betaproteobacteria	Actinobacteria	Pseudomonas	Enterobacteriaceae
Sampling time						
IPB-149 ($t_1 \times t_2$)	21.4**	10.9**	8.9**	9.8**	9.3	7.4**
IPB-137 ($t_1 \times t_2$)	20.6**	29.2**	28.7**	17.3**	13.7*	4.4*
IPB-052 ($t_1 \times t_2$)	14.6**	20.5**	19.1**	4.2*	2.1	7*
Genotypes						
IPB-149 \times IPB-137 (t_1)	14.8**	6.3**	6.8*	6.8**	4.3*	4.1*
IPB-149 \times IPB-052 (t_1)	25.1**	9.6**	1.9	6.5**	11.6*	5.6*
IPB-137 \times IPB-052 (t_1)	15.4**	9.2**	3.6*	8.2**	7*	–1
IPB-149 \times IPB-137 (t_2)	0.5	9.7*	11**	14.9**	4.9	8.8**
IPB-149 \times IPB-052 (t_2)	8.3**	10.4**	15.6**	4.6**	1.7	10*
IPB-137 \times IPB-052 (t_2)	10.1**	12.4**	14.2**	7.4**	3.7	1.3
Bulk Soil						
Bulk Soil \times IPB-149	19.8**	8.1**	64.6**	11.7**	16.5*	Nd
Bulk Soil \times IPB-137	27.8**	18.4**	57.1**	17.5**	23.1**	Nd
Bulk soil \times IPB-052	23.2**	6.7**	61.1**	15**	20.9**	Nd

Nd, not determined.

*Significant difference ($P < 0.05$) and **highly significant difference ($P < 0.01$) as determined using the permutation test.

sphere of all genotypes when compared with bulk soil samples. These groups included different genera such as *Rhizobium*, *Novosphingobium*, *Sphingobium*, *Burkholderia*, *Streptomyces*, and *Bacillus* and the family *Oxalobacteraceae*. Interestingly, the genus *Bacillus* had strongly increased in the tuber rhizosphere of all three plant genotypes and had the highest relative abundance among all genera detected in the tuber rhizosphere. Some taxonomic groups were only enriched for one or two plant genotypes. In the tuber rhizosphere of IPB-052, the relative abundance of the genera *Caulobacter*, *Paenibacillus*, and *Acidobacterium* subdivision 4 was increased compared to bulk soil. The relative abundance of the genera *Brucella*, *Sphingobium*, *Comamonas*, *Methylophilus*, *Pantoea*, *Acinetobacter*, *Pseudomonas*, *Stenotrophomonas*, *Chryseobacterium*, and *Sphingobacterium* was significantly increased in the tuber rhizosphere of IPB-137 compared to IPB-052 and IPB-149 and bulk soil. The genus *Chitinophaga* was found to be enriched in the tuber rhizosphere soil of both IPB-052 and IPB-137.

In contrast, some taxonomic groups, for example *Patulibacter*, were significantly decreased in their relative abundance in the tuber rhizosphere of all genotypes when compared to bulk soil samples. The genus *Patulibacter* had the highest relative abundance among all genera in the bulk soil. For other taxa, the relative abundance was decreased in the tuber rhizosphere of one or two genotypes, for example the relative abundance of the genus *Terrabacter* and members of *Acidobacterium* subdivision 16 were decreased in the tuber rhizosphere of IPB-137 and IPB-149.

Bacterial community structure revealed by 16S rRNA gene amplicon sequencing

Acquired sequences were grouped into 6,429 OTUs (> 97% sequence identity). To compare DGGE and pyrosequencing data, a UPGMA cluster analysis based on the relative abundance of OTUs was performed to analyze the structure of *Bacteria*, *Actinobacteria*, *Firmicutes*, and *Proteobacteria* communities present in the tuber rhizosphere of different sweet potato genotypes and bulk soil at t_2 (Fig. 3a–d). Analyses of *Bacteria*, *Actinobacteria*, and *Firmicutes* sequences (Fig. 3a–c) revealed that bulk soil sample replicates clustered separately from tuber rhizosphere samples with high similarity between replicates (> 90% similarity). These results confirmed the strong rhizosphere effects of sweet potatoes indicated by DGGE analyses. The reliability of the clusters analyzed by bootstrap revealed that the community composition of bulk soil was clearly different from the tuber rhizosphere of the plant genotypes. In contrast to DGGE results, low similarity values were observed between tuber rhizosphere samples of all three plant genotypes in *Bacteria*, *Actinobacteria*, and *Proteobacteria* communities (Fig. 3a, b and d). Despite low similarity values, almost all replicates from IPB-052 and IPB-149 clustered in UPGMA analysis of *Bacteria* and *Firmicutes* sequences, with 58% and 65% of similarity, respectively (Fig. 3a and c). This result suggests that these communities from genotypes IPB-052 and IPB-149 are similar. Replicates clustering with high similarity (> 80%) of all plant genotypes were observed only in *Firmicutes* UPGMA analysis (Fig. 3c). Finally, the UPGMA

Table 3. Taxonomic groups differing in relative abundance observed in the tuber rhizosphere of IPB-052, IPB-137, and IPB-149 sweet potato genotypes and in bulk soil, 6 months after planting (t_2)

Phylum	Class	Order	Family	Genus	Bulk Soil	IPB-052	IPB-137	IPB-149	
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	<i>Caulobacter</i>	*0 ± 0b	0.3 ± 0a	0.1 ± 0ab	0 ± 0b	
				<i>Phenylobacterium</i>	0.6 ± 0ab	0.6 ± 0ab	0.3 ± 0b	1 ± 0a	
		Rhizobiales	Bradyrhizobiales	Bradyrhizobiaceae		1.5 ± 0b	1 ± 0bc	0.9 ± 0c	1.7 ± 0a
				Brucellaceae	<i>Brucella</i>	Nd	0 ± 0b	0.7 ± 1a	0 ± 0b
			Hyphomicrobiales	Hyphomicrobiaceae	<i>Hyphomicrobium</i>	0.2 ± 0ab	0.5 ± 0a	0.1 ± 0b	0.2 ± 0ab
			Methylocystales	Methylocystaceae		0 ± 0b	0.1 ± 0b	0.2 ± 0ab	0.3 ± 0a
			Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	0 ± 0c	0.5 ± 0ab	0.7 ± 0a	0.3 ± 0b
			Xanthobacterales	Xanthobacteraceae		0 ± 0a	0.1 ± 0ab	0.3 ± 0a	0 ± 0b
		Rhodospirillales	Acetobacteraceae		0.7 ± 0a	0.5 ± 0a	0.1 ± 0b	1.1 ± 0a	
		Sphingomonadales	Sphingomonadaceae	<i>Novosphingobium</i>	0 ± 0c	0.4 ± 0b	1.7 ± 1a	1.4 ± 2a	
	<i>Sphingobium</i>			0 ± 0c	1.8 ± 1b	3.9 ± 3a	1.6 ± 3b		
	Betaproteobacteria	Burkholderiales	Alcaligenaceae		0 ± 0b	0 ± 0ab	0.3 ± 0a	0.1 ± 0ab	
				<i>Burkholderia</i>	0.4 ± 0b	0.7 ± 0a	0.9 ± 1a	1.2 ± 0a	
			Comamonadaceae	<i>Comamonas</i>	0.1 ± 0b	0 ± 0ab	0.3 ± 1a	0 ± 0b	
			Oxalobacteraceae		0 ± 0c	0.3 ± 0ab	0.5 ± 1a	0.2 ± 0bc	
	Methylophilales	Methylophilaceae	<i>Methylophilus</i>	0 ± 0b	0 ± 0b	0.3 ± 0a	0 ± 0b		
				0 ± 0c	0.6 ± 0ab	0.2 ± 0b	0.6 ± 0a		
	Deltaproteobacteria	Myxococcales	Cystobacteraceae		0.3 ± 0b	0.6 ± 0ab	0.2 ± 0b	0.6 ± 0a	
				0 ± 0b	0.1 ± 0ab	0.5 ± 0a	0 ± 0b		
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	<i>Pantoea</i>	0 ± 0b	0.1 ± 0ab	0.5 ± 0a	0 ± 0b	
			0.9 ± 0a	Nd	Nd	Nd			
Oceanospirillales		Halomonadaceae	<i>Halomonas</i>	0.1 ± 0b	0.1 ± 0b	1.2 ± 1a	0 ± 0b		
		Moraxellaceae	<i>Acinetobacter</i>	0.4 ± 0c	0.5 ± 0c	7.2 ± 6a	2.1 ± 3b		
Pseudomonadales		Pseudomonadaceae	<i>Pseudomonas</i>	1 ± 0a	0.2 ± 0b	0.1 ± 0b	0.2 ± 0b		
			0 ± 0b	0 ± 0ab	0.3 ± 0a	0 ± 0b			
Xanthomonadales	Sinobacteraceae		1 ± 0a	0.2 ± 0b	0.1 ± 0b	0.2 ± 0b			
	Xanthomonadaceae	<i>Stenotrophomonas</i>	0 ± 0b	0 ± 0ab	0.3 ± 0a	0 ± 0b			
Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	<i>Terrabacter</i>	0.5 ± 0a	0.2 ± 0ab	0 ± 0b	0 ± 0b	
			Microbacteriaceae		0.1 ± 0b	0.3 ± 0ab	0.5 ± 0a	0.3 ± 0a	
			Micrococcaceae		0.2 ± 0ab	0.2 ± 0ab	0.4 ± 1a	0.1 ± 0b	
			Micromonosporaceae		0.2 ± 0b	0.6 ± 0a	0.1 ± 0b	0.4 ± 0ab	
			Nocardioideales	Nocardioideae	<i>Nocardioides</i>	1.2 ± 0a	1.3 ± 0a	1.1 ± 0a	0.5 ± 0b
			Pseudonocardiales	Pseudonocardaceae	<i>Pseudonocardia</i>	1.4 ± 0a	0.1 ± 0b	0.1 ± 0b	0 ± 0b
			Streptomycetales	Streptomycetaceae	<i>Streptomyces</i>	0.5 ± 0d	3.7 ± 1a	1.9 ± 1b	1 ± 0c
			Solirubrobacterales	Conexibacteraceae	<i>Conexibacter</i>	0.4 ± 0ab	0.2 ± 0bc	0.1 ± 0c	0.5 ± 0a
				Patulibacteraceae	<i>Patulibacter</i>	16.1 ± 2a	0 ± 0b	0 ± 0b	Nd
			Bacteroidetes	Flavobacteria	Flavobacteriales	Cryomorphaceae		0 ± 0b	0 ± 0ab
Flavobacteriaceae	<i>Chryseobacterium</i>	0 ± 0b				0.1 ± 0b	2.8 ± 4a	0 ± 0b	
	<i>Flavobacterium</i>	0.4 ± 0a				0.1 ± 0ab	0.3 ± 0a	0.1 ± 0b	
	0.4 ± 0a	0.1 ± 0ab				0.3 ± 0a	0.1 ± 0b		
Sphingobacteria	Sphingobacteriales	Chitinophagaceae		<i>Chitinophaga</i>	0 ± 0c	0.2 ± 0b	2.7 ± 4a	0 ± 0c	
		Cytophagaceae			0.1 ± 0b	0.3 ± 0ab	0.4 ± 0a	0.1 ± 0b	
		Sphingobacteriaceae		<i>Mucilaginibacter</i>	1.5 ± 0a	0.2 ± 0b	0 ± 0b	0.2 ± 0b	
				<i>Pedobacter</i>	0.4 ± 0a	0 ± 0b	0.1 ± 0ab	Nd	
		<i>Sphingobacterium</i>	Nd	Nd	1.2 ± 1a	0 ± 0b			
Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	7 ± 0c	21.8 ± 5a	21.6 ± 14ab	19.2 ± 12b	
				<i>Tumebacillus</i>	0.6 ± 0a	0.1 ± 0b	0.1 ± 0b	0.3 ± 0b	
			Bacillales_incertae_sedis	<i>Pullulanibacillus</i>	0.7 ± 0b	0.8 ± 0b	0.2 ± 0c	2.5 ± 2a	
				Paenibacillaceae	<i>Cohnella</i>	0.7 ± 0a	0.4 ± 0b	0.1 ± 0b	0.4 ± 0b
				<i>Oxalophagus</i>	0.4 ± 0a	0.5 ± 0a	0.1 ± 0b	0.7 ± 0a	
				<i>Paenibacillus</i>	1.8 ± 0b	2.4 ± 0a	1.7 ± 0b	1.5 ± 1b	
	Staphylococcaceae		0 ± 0b	0.2 ± 0ab	0.5 ± 0a	0.2 ± 0ab			
Acidobacterium	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium</i>	1.3 ± 0a	0.4 ± 0b	0 ± 0c	0.5 ± 0b	
				Subdivision 1	4 ± 0b	2.7 ± 1c	0.4 ± 0d	4.8 ± 2a	
				Subdivision 16	0.5 ± 0a	0.3 ± 0ab	0.1 ± 0b	0.2 ± 0b	
				Subdivision 2	0.7 ± 0a	0.5 ± 0a	0 ± 0b	0.6 ± 0a	
				Subdivision 3	2.3 ± 0a	2.6 ± 1a	0.6 ± 1b	2.9 ± 1a	
				Subdivision 4	0.2 ± 0b	0.6 ± 0a	0.3 ± 0ab	0.3 ± 0ab	
				Subdivision 6	0.5 ± 0a	0.5 ± 0a	0.1 ± 0b	0.6 ± 0a	
Cyanobacteria	Cyanobacteria		Chloroplast	Streptophyta	0 ± 0b	0.6 ± 0a	0.8 ± 0a	0.8 ± 1a	
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales			0.2 ± 0ab	0.4 ± 0ab	0.1 ± 0b	0.3 ± 0a	

Nd, not detected.

Relative abundance (%) ± standard deviation – Different online letters indicate significant differences ($P < 0.05$).

*Relative abundance (%) < 0.05%.

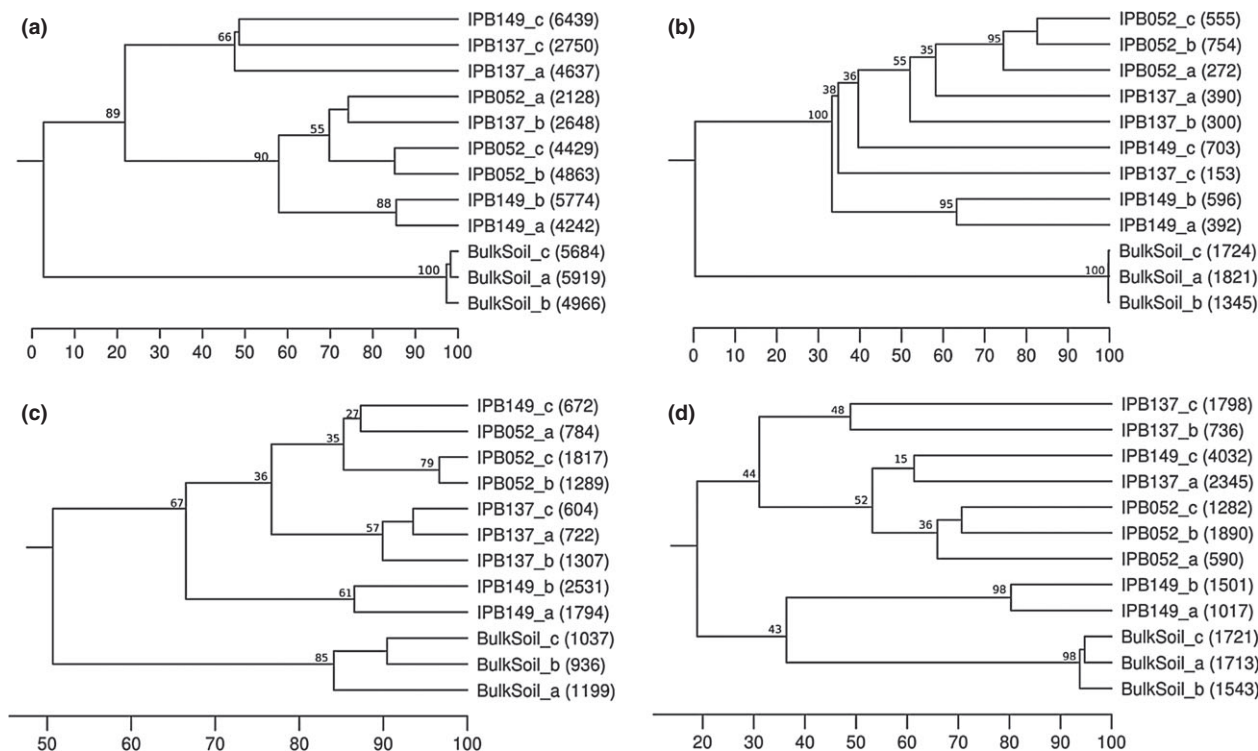


Fig. 3. UPGMA cluster analysis of bacterial community structure in the tuber rhizosphere of IPB-149, IPB-137, and IPB-052 and bulk soil (replicates a–c) 6 months after sowing using pyrosequencing analysis data based on Pearson correlation using OTUs (> 97% sequence similarity) as the species data for *Bacteria* – a, *Actinobacteria* – b, *Firmicutes* – c, and *Proteobacteria* – d. Number in brackets: number of sequences obtained for the sample. Numbers on node: bootstrapping value for each node means the percent frequency of all samples under the node grouping exclusively together.

cluster analysis (Fig. S4a) and the principal coordination analysis (Fig. S4b – PCoA) based on weighted UniFrac distance were also performed to analyze *Bacteria* structure of tuber rhizosphere samples from different sweet potato genotypes and bulk soil in t_2 , and similar trends as for UPGMA cluster analysis (based on Pearson correlation) were revealed.

Discussion

Biotic and abiotic factors such as soil type, seasons, plant developmental stage, proximity to root, root architecture, plant species, and cultivars can affect the structure of microbial communities in the rhizosphere (reviewed by Berg & Smalla, 2009; Buée *et al.*, 2009). Plant species can select specific microbial populations from the bulk soil by root exudates excretion (reviewed by Bais *et al.*, 2006; Hai-Char *et al.*, 2008). Differences in the composition of root exudates but also of the root architecture are assumed to affect the relative abundance of soil microorganisms in the vicinity of the root (Somers *et al.*, 2004). In several studies, the influence not only of the plant species but also of the plant genotypes on the rhizosphere microbial communities

was demonstrated (reviewed by Berg & Smalla, 2009; Smalla *et al.*, 2001; van Overbeek & van Elsas, 2008; Andreote *et al.*, 2009; Weinert *et al.*, 2009, 2010). In the present study, for the first time, the structure of bacterial communities in soil adhering to the tuberous roots – a rather particular rhizosphere – of three sweet potato genotypes was investigated using molecular approaches.

The effect of the plant age on the bacterial community composition was indicated by DGGE fingerprints with plant genotype-dependent differences of the bacterial community fingerprints being higher at t_1 . Previously, plant age effects on the composition of bacterial communities in the rhizosphere were demonstrated in several studies (Smalla *et al.*, 2001; Heuer *et al.*, 2002; Herschkovitz *et al.*, 2005; Lerner *et al.*, 2006; Buée *et al.*, 2009), and the plant growth developmental stage was considered a main factor affecting bacterial communities in the potato rhizosphere (van Overbeek & van Elsas, 2008).

The sweet potato genotype-dependent composition of the bacterial communities in the tuber rhizosphere (soil adhering to tuberous roots) was observed by DGGE only after statistical analysis of the fingerprint data. In addition, plant genotype-dependent responders to the

tuber rhizosphere were identified by pyrosequencing analysis. This analysis which was performed only for the samples from the second sampling time as bulk soil samples were available only for this time point. Although different regions of the 16S rRNA gene were analyzed by DGGE or pyrosequencing, both methods have shown strong effects of sweet potato tuberous roots on the bacterial community structure in the tuber rhizosphere. DGGE fingerprints were generated not only for bacterial communities but also for different taxonomic groups typically associated with plants, and thus, their statistical evaluation provided also insights into the plant genotype-dependent rhizosphere effect of less abundant bacterial populations. Considering that different bacterial populations might be represented behind the same band and due to 16S rRNA gene heterogeneities resulting in more than one band for some bacteria, we did not determine diversity indices based on the DGGE fingerprints as carried out for pyrosequencing data. In contrast, pyrosequencing of 16S rRNA gene amplicons provided information on the taxonomic composition and allowed an OTU-based statistical analysis as well as the determination of diversity indices. Both techniques were previously used as powerful tools to study bacterial community structure and composition and provided similar results (Ding *et al.*, 2012, 2013).

Sweet potato genotypes IPB-149 and IPB-052 showed similar bacterial community composition in the tuber rhizosphere. IPB-149 and IPB-052 showed similar starch and ethanol production yields but also similar insect susceptibility (Table 1). In contrast, IPB-137 had a significantly lower number of tuberous roots and significantly lower starch content but was also more affected by insects (significantly higher insect endurance). All molecular methods applied indicated a stronger rhizosphere effect of the genotype IPB-137, which was correlated with a lower content of starch storage in the tuberous root compared to the genotypes IPB-149 and IPB-052. Only for IPB-137, the 16S rRNA gene copy numbers were significantly higher in TC-DNA compared to bulk soil, the differences between the DGGE fingerprints (*d*-values) for bulk soil and IPB-137 were highest, and the diversity based on OTU analysis was lower – all data coherently indicated a stronger rhizosphere effect. In addition, in comparison with bulk soil, significantly higher numbers of bacterial genera were enriched in the tuber rhizosphere of IPB-137, such as *Pseudomonas*, *Sphingobium*, *Acinetobacter*, *Chryseobacterium*, *Chitinophaga*, and *Sphingobacterium*. Isolates belonging to some of the genera enriched in the tuber rhizosphere of IPB-137, such as *Pseudomonas* and *Sphingobium*, were previously reported to be involved in the degradation of aromatic ring structures (Önneby *et al.*, 2014; Ruiz *et al.*, 2013; Tien *et al.*, 2013). It can only be specu-

lated that aromatic compounds might have been released from the tuberous roots eventually as a result of increased insect attacks. Typically, genera affiliated to the phylum *Proteobacteria* are enriched in the rhizosphere of potato plants (Inceoglu *et al.*, 2011; Dias *et al.*, 2013). Using 16S rRNA gene-based PCR-DGGE and clone library analyses, Dias *et al.* (2013) observed that two different potato cultivars [Modena – a genetically modified (GM) line with altered starch composition; and Karnico – a near-isogenic non-GM cultivar] selected different *Alphaproteobacteria* and *Betaproteobacteria* communities in the rhizosphere, while the genus *Pseudomonas* (*Gammaproteobacteria*) was selected only in the rhizosphere of the non-GM potato cultivar. However, in the present study, the genus *Bacillus* (phylum *Firmicutes*) was predominantly enriched in the tuber rhizosphere of all genotypes.

Bacillus species are gram-positive spore forming cells, widely spread in soil environments and for many isolates, plant growth-promoting and biocontrol activity have been shown. They can promote plant growth via biological nitrogen fixation (Beneduzi *et al.*, 2008), indole acetic acid production (Araújo *et al.*, 2005), and they can perform phosphate solubilization (Beneduzi *et al.*, 2008). Moreover, different *Bacillus* species are alpha-amylase producers, the enzyme responsible for starch hydrolysis (Puspasari *et al.*, 2012). However, the strong enrichment of *Bacillus* seemed not to be correlated with the starch content of the different sweet potato genotypes. There are no data available so far correlating the high amounts of starch in sweet potato tuberous roots and the bacterial communities present in the tuber rhizosphere of sweet potato. In contrast, the effect of different starch concentrations in potato tubers on bacterial communities in the geocaulosphere (soil adhering to the potato tuber which in contrast to the sweet potato belongs to the stem) has been previously studied (Milling *et al.*, 2004; Inceoglu *et al.*, 2010, 2011, 2012; Dias *et al.*, 2013). The enrichment of the genus *Bacillus* in the tuber rhizosphere of sweet potato genotype could be due to the plant recruiting of specific beneficial microorganisms. *Bacillus* species can produce antimicrobial substances (AMS) which can avoid deleterious effects of phytopathogen agents (Araújo *et al.*, 2005; Tiwari *et al.*, 2010). Chen *et al.* (2007) demonstrated that more than 8.5% of the plant root-colonizing *B. amyloliquefaciens* FZB42 genome is devoted to synthesizing antibiotics and siderophores. Several studies have already shown that plant genotypes can affect the accumulation of microorganisms that help the plant to defend itself against pathogen attacks (reviewed in Berendsen *et al.*, 2012). Recruitment of *Bacillus subtilis* strains in the rhizosphere soil had already been demonstrated by Rudrappa *et al.* (2008) and Lee *et al.* (2012b) when pepper and *Arabidopsis* plants, respectively, were

infected with phytopathogens. Moreover, *Bacillus* and *Paenibacillus* seem to be of great importance for pathogen suppression under arid conditions (Köberl *et al.*, 2011, 2013). Finally, Rudrappa *et al.* (2008) also demonstrated that the excretion of malic acid via plant exudates was responsible for *Bacillus* recruitment.

In conclusion, despite of some limitation in the sampling design the present study provided the first detailed insights into the bacterial community associated with the tuber rhizosphere of three sweet potato genotypes based on a cultivation-independent approach. Pyrosequencing of 16S rRNA gene fragments revealed numerous taxonomic groups with increased abundance in the tuber rhizosphere, and most remarkable was the strongly increased abundance of the genus *Bacillus* in the tuber rhizosphere of all sweet potato genotypes. In future, these results can be used as valuable information for the selection and production of a PGPR bacterial consortium to enhance sweet potato productivity.

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Statement

Bacillus is dominant in the tuber rhizosphere of sweet potato.

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Supporting Information

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

Fig. S1. DGGE fingerprint analysis based on 16S rRNA gene fragments of *Bacteria* – a, *Alphaproteobacteria* – b, *Betaproteobacteria* – c, *Actinobacteria* – d, *Pseudomonas* – e, and *Enterobacteriaceae* – f communities present in the tuber rhizosphere (replicates A, B, C, D, and E) from IPB-149, IPB-137, and IPB-052 genotypes and in the bulk soil (replicates A, B, C, D, and E) 3 (t_1) and 6 (t_2) months after sowing.

Fig. S2. UPGMA cluster analysis of tuber rhizosphere (replicates a, b, c, d, and e) from IPB-149, IPB-137, and IPB-052 genotypes and bulk soil (replicates a, b, c, d, and e – striped squares) 3 (t_1 – black squares) and 6 (t_2 – white squares) months after sowing based on DGGE fingerprints for bacterial-specific groups (*Alphaproteobacteria* – a, *Betaproteobacteria* – b, *Actinobacteria* – c, *Enterobacteriaceae* – d, and *Pseudomonas* – e).

Fig. S3. Plot of curves of average diversity indices (a – Chao1 and b – Pielous's evenness) from IPB-149, IPB-137, and IPB-052 genotype tuber rhizosphere and bulk soil, 6 months after sowing.

Fig. S4. UPGMA cluster analysis of bacterial community structure from IPB-149, IPB-137, and IPB-052 genotype tuber rhizosphere and bulk soil (replicates a, b, and c) 6 months after sowing using pyrosequencing analysis data (a) and principle coordination analysis (PCoA) based on weighted UniFrac distance (b).

Table S1. *Bacteria* genera abundance present in the tuber rhizosphere from genotypes IPB-149, IPB-137, and IPB-052 and bulk soil.