

## ORIGINAL ARTICLE

# Stability and succession of the rhizosphere microbiota depends upon plant type and soil composition

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**We examined succession of the rhizosphere microbiota of three model plants (*Arabidopsis*, *Medicago* and *Brachypodium*) in compost and sand and three crops (*Brassica*, *Pisum* and *Triticum*) in compost alone. We used serial inoculation of 24 independent replicate microcosms over three plant generations for each plant/soil combination. Stochastic variation between replicates was surprisingly weak and by the third generation, replicate microcosms for each plant had communities that were very similar to each other but different to those of other plants or unplanted soil. Microbiota diversity remained high in compost, but declined drastically in sand, with bacterial opportunists and putative autotrophs becoming dominant. These dramatic differences indicate that many microbes cannot thrive on plant exudates alone and presumably also require carbon sources and/or nutrients from soil. *Arabidopsis* had the weakest influence on its microbiota and in compost replicate microcosms converged on three alternative community compositions rather than a single distinctive community. Organisms selected in rhizospheres can have positive or negative effects. Two abundant bacteria are shown to promote plant growth, but in *Brassica* the pathogen *Olpidium brassicae* came to dominate the fungal community. So plants exert strong selection on the rhizosphere microbiota but soil composition is critical to its stability. microbial succession/ plant–microbe interactions/rhizosphere microbiota/selection.**

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## Introduction

The rhizosphere is a critical zone of soil surrounding roots that is directly influenced by the plant and into which plants secrete as much as 11% of net fixed carbon (Jones *et al.*, 2009; Dennis *et al.*, 2010). It is a ‘hot spot’ of microbial activity, with increased microbial numbers, microbial interactions and genetic exchange (Bulgarelli *et al.*, 2013; Turner *et al.*, 2013a). There is a close two-way interaction between the microbial community (microbiota) and plant host that is an essential determinant of plant health and productivity. This is very apparent in symbiotic associations between rhizobia and legumes, resulting in fixation of 40–50% of the biosphere’s nitrogen (Terpolilli *et al.*, 2012; Udvardi and Poole, 2013) as well as widespread acquisition of nutrients such as phosphate by mycorrhizae (Lanfranco and Young, 2012; Nuccio *et al.*, 2013).

However, interaction between plants and microbes is more general with plants shown to shape the rhizosphere microbiota in legumes, potatoes and suppressive soils (Sharma *et al.*, 2005; Lebreton *et al.*, 2007; Manter *et al.*, 2010; Kinkel *et al.*, 2011; Mendes *et al.*, 2011; Turner *et al.*, 2013b). Some microbial root colonists are saprophytes, which also colonise splinters of wood inserted into soil (Bulgarelli *et al.*, 2012), but others are selected by exudation of nutrient sources and phytoalexins, such as glucosinolates and avenacins (Sonderby *et al.*, 2007; Bressan *et al.*, 2009; Turner *et al.*, 2013b). Furthermore, by altering the rhizosphere microbiota, plants induce formation of suppressive soil where growth of plant pathogens is inhibited (Lebreton *et al.*, 2007; Kinkel *et al.*, 2011; Mendes *et al.*, 2011). Microbes can increase plant growth by releasing phytohormones, biofertilization (Lugtenberg and Kamilova, 2009) and stimulation of both the induced systemic resistance and systemic-acquired resistance components of the plant immune system (Van der Ent *et al.*, 2009). It has been difficult to study these complex interactions at the community level but recently high-throughput sequencing methods have been used to show that *Arabidopsis* preferentially

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selects specific microbial endophytes, which colonise plant tissues, with some, albeit weaker, selection in the rhizosphere (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012).

Previous studies on the soil microbiota of plants grown in natural soil have shown that plant and soil are both important factors in community structure (Berg and Smalla, 2009). However, as shown by Lauber *et al.*, 2009, soil pH also has a dramatic influence on the soil microbiota. Studying field grown plants for their influence on the microbiota is hindered by high soil complexity and local variation in pH. Moreover, most previous studies used DNA fingerprinting methods with limited replication (<4 samples). As we show here it is vital to sample at least 10–15 rhizosphere samples (biological replicates) in order to establish statistical significance.

To study microbial succession we used a nutritionally defined medium that in the first generation consisted of a 10% soil inoculum mixed with either autoclaved sand or compost. In two succeeding generations, 25% of the growth medium was transferred from the preceding generation and mixed with 75% sand or compost. This allowed control of soil nutrients, as they were refreshed in each generation and separation of the effects of soil characteristics from those of the initial microbial community composition. This approach was used to examine rhizosphere succession over three generations in three model and three crop plants, belonging to distinct families. Sand and compost were used to allow assessment of the relative importance of root exudation and soil organic matter in supporting rhizosphere communities.

## Materials and methods

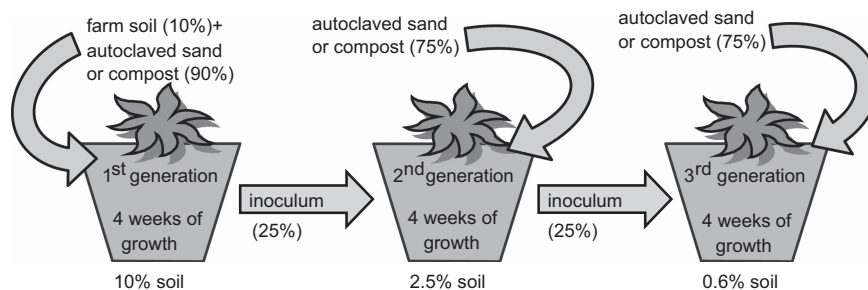
### *Soil collection and plant growth*

Soil used as a microbial inoculum was collected from a naturally grassed and unfertilised part of John Innes Centre Church Farm, Bawburgh, Norfolk, UK (52°37'39.35''N, 1°10'43''E). Covering vegetation was stripped off and soil collected from a depth of 10–30 cm. Chemical analyses (by MacaulaySoils, James Hutton Institute, Aberdeen, UK) showed that the soil is poor in nutrients ( $\text{NO}_3^-$  3.49 mg kg<sup>-1</sup>,  $\text{P}^{-3}$  120.5 mg kg<sup>-1</sup>,  $\text{K}^+$  168.2 mg kg<sup>-1</sup>,  $\text{Mg}^{2+}$  33.55 mg kg<sup>-1</sup>), is pH 7.5 and contains 2.92% organic matter. According to the UK Soil Observatory, the soil can be classified as Luvisol (WRB classification) and the soil texture was loamy with bits of chalk in the layer below the top layer. This soil was chosen as the history of its plant coverage is well documented and the microbial communities have been characterised in a previous study (Turner *et al.*, 2013b).

Seeds were surface sterilised by using ethanol (70% or 95%) and bleach (2–10%) and germinated on MS medium (Murashige and Skoog, 1962) containing essential macro- and microelements for plant growth

and sucrose (3%). For further details of the seed sterilisation method see Supplementary Table S1. Only seedlings coming from non-contaminated MS plates were planted. We assumed that the seed endophytic community is negligible compared to the soil community as already shown (Lundberg *et al.*, 2012). Plants were grown in a growth room in closed containers in 12 h/12 h light/dark conditions at 23 °C. Model plants (*Arabidopsis thaliana* Col-0, *Medicago truncatula* Jemalong A17 and *Brachypodium distachyon* Bd21) were grown in 50-ml sealed pots and crop plants (*Brassica rapa* R-O-18, *Pisum sativum* cv. Avola and *Triticum aestivum* cv. Paragon) in 100 ml sealed pots. Two artificial soils were used; poor soil consisting of silver sand (washed, lime free, horticultural grade, manufactured by J Arthur Bower's, Sinclair, Lincoln, UK) and referred to as 'sand', and a nutrient rich, commercially available compost (Everris Levington F2 Seed & Modular, Scotts, Ipswich, UK) referred to as 'compost' and containing 144 mg l<sup>-1</sup> total nitrogen, 73 mg l<sup>-1</sup> total phosphorous and 239 mg l<sup>-1</sup> total potassium (manufacturer's analysis). Example photographs of the plant growth are presented in the Supplementary Word file 1. Sand and compost were autoclaved and tested for any residual microorganisms using TY medium. Bacteria were not recovered although compost still possessed viable fungal spores. To each artificial soil a well-mixed Bawburgh soil inoculum was added and mixed well (10% vol/vol) (Figure 1). Plants in sand were watered once a week with plant mineral supplement (Poole *et al.*, 1994b).  $\text{KNO}_3$  (10 mg per pot) was added to each pot of sand at the start of week one of each generation. No nutrients were added to compost, as it is already a rich medium.

Plants were grown for 4 weeks over three generations from a 10% inoculum of Bawburgh soil. For generations two and three, 25% (vol/vol) of the growth medium from the preceding generation was mixed with either 75% sand or compost (Figure 1). The starting soil inoculum was diluted over successive generations so the community would increasingly depend on root exudates as a carbon source, especially in sand. With the alternative experimental strategy of replanting in the initial soil, changes in microbiota could be caused by gradual reductions in the nutrient status of the growth medium. In total, 24 independent replicates for each plant species and unplanted control were maintained. At harvest, the shoot was removed and root shaken to remove loosely adhering soil. Soil left in the pot (and fine fragments of roots, which could not be removed) was used as a microbial inoculum for further generations. Because of the small pot size all the soil in the pot was influenced by root exudates (Supplementary Word file 1). Roots were placed in a Falcon tube (50 ml) with phosphate buffered saline (30 ml) and vortexed (30 s) until the root was visibly clear from adhering soil, before roots were discarded and the soil suspension centrifuged at an RCF of 2880 for 5 min (Centrifuge 5810, Eppendorf, Stevenage, UK).



**Figure 1** Experimental design of multi-generation plant growth. Autoclaved sand or compost (90% by volume) was inoculated with Bawburgh grass soil (10% by volume) for the first generation of plant growth. In generations two and three, soil (25% by volume) from the previous generation was used as an inoculum. Bawburgh grass soil constituted 10, 2.5 and 0.6% (volume) in successive generations. Identical treatment was given to unplanted controls for three generations. After 4 weeks growth plants were harvested and DNA isolated from the rhizosphere for ARISA and 454Flx analysis of the microbiota.

The supernatant was decanted and DNA was isolated from 1 g (wet weight) of rhizosphere soil (Griffiths *et al.*, 2000) with humic acids removed by using the ZYMO research One-step PCR inhibitor removal kit (Zymo Research, Irvine, CA, USA).

#### Isolation of soil bacteria

To isolate soil microorganisms three different growth media were used: LB (Bertani, 1951), TY (O'Reilly and Niven, 1986) and acid minimum salts agar (Poole *et al.*, 1994a) supplemented with glucose (10 mM) and ammonia (10 mM). Rhizosphere soil (1 g) was mixed with acid minimum salts agar (no carbon or nitrogen source added) and shaken for 15 min, centrifuged at low speed to pellet the soil particles, the supernatant was transferred to a fresh tube and centrifuged at an RCF of 2880 for 5 min. The supernatant was discarded and the microbial pellet diluted in acid minimum salts agar (5 ml) and serially diluted on Petri plates containing LB, TY or acid minimum salts agar (Supplementary Word file 2).

#### Plant growth promotion assay

Seeds of *Arabidopsis*, *Medicago* and *Brachypodium* (24 replicates) were surface sterilised as above. Plants were grown in sterilised silver sand and rooting solution and nitrogen added to the same levels given above. Seedlings were inoculated after the first week of growth with either *Achromobacter xylosoxidans* or *Arthrobacter sp.* or left uninoculated. Bacteria were grown overnight in the liquid culture (TY) and the plants inoculated with  $10^8$  of viable cells (calculated by optical density measurement and confirmed by cell counting). Bacteria were spun down, washed free of media, re-suspended in water and applied to plant roots. Plants were grown for 4 weeks before the shoot was cut-off, dried for 48 h at 70 °C and weighed.

#### Data analysis

Community structure was analysed by Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Fisher and Triplett, 1999) by using fluorescently labelled ITSF (5'-GTCGTAACAAGGTAGCCGTA-3')

and ITSReub (5'-GCCAAGGCATCCACC-3') primers and PCR conditions described already (Cardinale *et al.*, 2004). 454Flx titanium pyrosequencing was performed at The Genome Analysis Centre, Norwich, UK with DNA from 24 individual plants binned into three pools of 8. For 454Flx sequencing the bacterial 16S rDNA (V1–V2 fragment) was amplified by using 27F and 338R primers and PCR conditions described already (Hamady and Knight, 2009). The fungal ITS region was amplified using ITS1 and ITS2 primers and PCR conditions described already (Buee *et al.*, 2009). Bacterial and fungal reads were separated by the primer sequence. Additional information about sequencing is provided in Supplementary Table S2.

Reads were de-noised, quality filtered and the 454 adapters removed using a Roche (Branford, CT, USA) data pipeline at The Genome Analysis Centre. The reads were split based on the PCR primers used (bacteria or fungi) and the barcode from the Roche MID adapters library. The total pool of reads was searched by custom-made script looking for a 100% match of the PCR primer and the barcode. All the reads with any mismatch or a gap in either PCR primer sequence or the barcode were removed. This approach selected for reads with both PCR primer sequences intact, so all the reads had the right length (there can be some small difference in the V1–V2 region of 16S rRNA (in case of bacteria) or ITS (in case of fungi) fragment itself, which either arose as a sequencing errors or are truly biological differences in this region length between different bacterial/fungal species). Our approach in combination with the initial analysis at the genomic centre provided us with high-quality reads ready for the downstream analyses. Screening for chimeras was initially carried out by using DECIPHER (Wright *et al.*, 2012). However, this gave a number of apparent false positives. These were relatively common (up to 0.5% of reads), occurred in several replicate samples, and matched a number of 16S sequences in the NR Genbank database at >95% similarity, including matches to close to full length 16S sequences, which in some cases, were from cultured strains of *Rhizobium* and *Bradyrhizobium*. So we adopted an alternative approach of removing from each data set



Operational Taxonomic Units (OTUs) that represented <0.1% of reads. This removed all sequences identified as chimeric by DECIPHER (as well as other rare sequences) but retained the 'false positives' referred to above.

Sequences were binned into OTUs by using USearch 6.0 (drive5) (Edgar 2010), set at 95% sequence identity between reads (Supplementary Table 2) (Wooley *et al.*, 2010). Bacterial 16S rRNA gene reads were aligned against SILVA (v.115; Max Planck Institute for Marine Microbiology, Bremen, Germany) and fungal ITS reads were aligned against the full Genbank database. OTUs were annotated with MEGAN software (University of Tübingen, Tübingen, Germany), using the default settings, apart from the settings in the Last Common Ancestor (min support = 1 and top percent = 1) (Huson *et al.*, 2009).

The fluorescence intensity of ARISA peaks and abundance of OTUs were exported to Microsoft Excel, aligned into size bins of 0.8 bp. using T-Align (Smith *et al.*, 2005) and exported into Primer 6.0 (PRIMER-E, Ivybridge, UK) and R (R Development Core Team, 2008) for analysis. Each value was standardized (as a % of the total), square root transformed and a similarity matrix constructed using the Bray–Curtis coefficient. Results were visualised by using non-metric multi-dimensional scaling. Differences between groups were tested for significance using a permutation based analysis of variance (multivariate analysis of variance) by using the *adonis* function of the *vegan* package in R (Osborne *et al.*, 2011), including plant species and generation as factors plus the interaction between them.

3D ternary plots were generated by using a bespoke Python (2.6; Python Software Foundation, Beaverton, OR, USA) script and the diagrams drawn by using Jmol (13.0.15, an open-source Java viewer for chemical structures in 3D; <http://www.jmol.org/>) under Mac OS X (10.6.4, Apple, Cupertino, CA, USA) and was further annotated using Adobe Illustrator (CS5, Adobe Systems, San Jose, CA, USA). The script used is freely available from the authors on request.

Representative reads from all individual OTUs were aligned by using ClustalW (EMBL-EBI, Hinxton, UK) and imported into MEGA5 (Centre for Evolutionary Medicine and Informatics, Tempe, AZ, USA) to produce a dendrogram. Based on the dendrogram, the OTUs were placed according to their phylogenetic relation and their ratio of abundance in the rhizosphere against unplanted control (calculated for each generation) visualised using heat maps. These maps were constructed using the online tool *matrix2png* (<http://www.chibi.ubc.ca/matrix2png/>). The Shannon index of diversity and community richness were calculated in Microsoft Excel for 454Flx data.

## Results

### *Experimental selection system*

Our experimental selection system allows us to examine the influence of plants on the rhizosphere

microbiota over several generations. Two sterile artificial growth media were used (see Materials and methods); silver sand with complete mineral solution added (sand) and a commercial compost rich in nutrients and organic material (compost). The starting microbiota (inoculum) was introduced by adding 10% Bawburgh soil to both artificial, sterilised growth media (sand or compost). An inoculum of 10% was chosen to minimise the influence of the soil inoculum on the nutrient status of the growth medium. These soils were low in infective rhizobia and neither *Medicago* nor *Pisum* were nodulated in any of the experiments reported below. However, in a separate test, nodules formed when appropriate rhizobia were added (data not shown). No mycorrhization was observed (data not shown). Bawburgh soil inoculum was only used to prepare the initial plant growth medium (generation 1) and successive generations were started using the same growth medium (either sand or compost) and microbial inoculum from the preceding generation (25% of the volume). A volume of 25% was used to ensure that the microbial community was transferred efficiently, while minimising the stochastic loss of rarer species (Bent and Forney, 2008). The alternative approach of growing plants in the same soil could lead to depletion of nutrients.

### *Microbiota succession in sand*

For clarity we first consider the microbiota of plants grown in sand. To check the stability of the starting inoculum over time, *Arabidopsis* plants were grown by using Bawburgh soil inocula (10% in sand) freshly sampled more than 1 year apart (November 2009 and July 2011). There were no major differences, apart from the absence of *Achromobacter* in the second year, in the abundance of the 10 most common bacterial OTUs or the Shannon diversity index (Supplementary Figure S1), showing that the starting inocula were very stable.

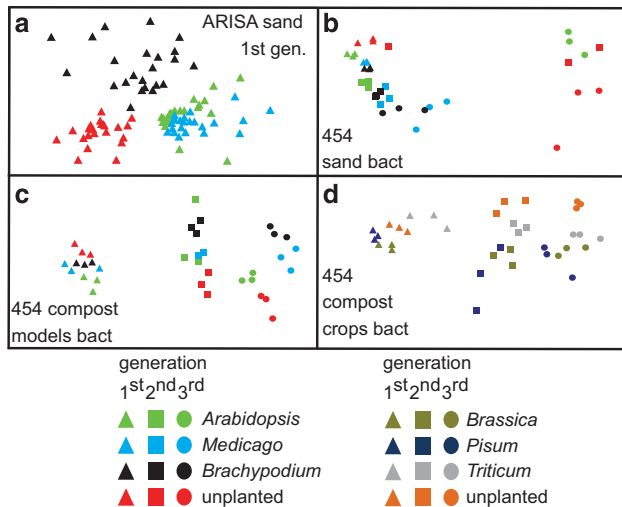
To begin the succession experiment, *Arabidopsis*, *Medicago* and *Brachypodium* (a model non-legume dicot, legume and monocot cereal, respectively) were grown in sand with a common Bawburgh soil inoculum. After only one generation of growth, there was a strong effect, with each plant's microbiota differing from all others and from the unplanted soil (most readily visible in the bacterial ARISA data where replication is greatest Figure 2a). The microbiota of all plants and unplanted soil samples show a similar shift in composition over generations, with points representing the first generation lying towards the left of Supplementary Figures S2a and d, and those representing the third generation towards the right. The microbiota of each plant species in each generation are clustered together, as are the microbiota of unplanted soil samples, with the unplanted microbiota usually at the periphery of the distribution of points in each generation, showing the effect of plant selection. The differences between times were

greater than those between plants (Table 1), although the effects of both, and the interaction between them were all significant. We hypothesise that a considerable component of this change over time is caused by a natural Bawburgh soil community adapting to soil dilution over three generations. Moreover the environmental conditions (for example, temperature, watering) differ greatly between the original soil (to which the community was adjusted) and the controlled laboratory conditions. Multivariate analysis of

variance performed on the sand experiment confirms the multi-dimensional scaling observations, that the differences between the rhizospheres and unplanted soil are real (Supplementary Figure S3).

ARISA enabled large numbers of replicate plants to be tested (~24 for each generation), and provides high taxonomic resolution due to variability of the bacterial 16S-23S rDNA intergenic spacer region (Ngugi and Stingl, 2012), but only a fingerprint of community structure. Therefore high-throughput 454Flx pyrosequencing of the bacterial V1–V2 16S rDNA or fungal intergenic spacer region was used to group the rhizosphere microbiota into OTUs. OTU data from 454Flx sequencing confirmed that plant rhizosphere microbiota diverged strongly from each other and over generations. They also confirmed that *Arabidopsis* in the third generation was closer to unplanted soil than either *Medicago* or *Brachypodium* (Figure 2b), suggesting *Arabidopsis* biased the rhizosphere microbiota less than the other plants. The fungal microbiota followed a similar pattern with rhizosphere communities close together in the first generation but more divergent in the second and third generations (Supplementary Figure 2g). The 454 data showed less variation between replicates than did ARISA, with the two factors and their interaction explaining between 56% and 82% of the total variance (Table 1), probably because DNA from eight individual plants was pooled into each replicate before pyrosequencing.

Poor soil conditions over successive generations favour fast growing Proteobacteria (Lambert *et al.*, 1990; Demoling *et al.*, 2007), consistent with some groups becoming increasingly dominant in generations two and three (Supplementary Figure S4a). The most abundant bacterial OTUs selected were  $\alpha$ -proteobacteria (Rhizobiaceae family),  $\beta$ -proteobacteria (especially the genus *Massilia*), and Bacteroidetes, whereas the second most abundant group, the actinobacteria, decreased in the rhizosphere over generations (Supplementary Figures S5a and b). Selection is displayed most dramatically in ternary plots (see Supplementary Figure S6 for an explanation), where each sphere represents a single OTU, size represents abundance, position corresponds to the relative abundance in the three rhizospheres and colour reflects the rhizosphere abundance relative to unplanted soil (Figure 3 and Supplementary Figure S7). Sphere colour represents is based on the relative abundance (rhizosphere/unplanted control) of a particular OTU for each generation. Thus, blue-green OTUs are primarily soil organisms, whereas orange-red OTUs are rhizosphere specific. A few bacterial and fungal OTUs, that are abundant and/or of special interest are annotated (Figure 3 and Supplementary Figure S7). In the first generation, there were some generalist- (red spheres in the middle) and plant-specific rhizosphere OTUs (red spheres in the corners), but the majority were found in both rhizosphere and soil (green and yellow spheres). However, in the second generation, most OTUs are



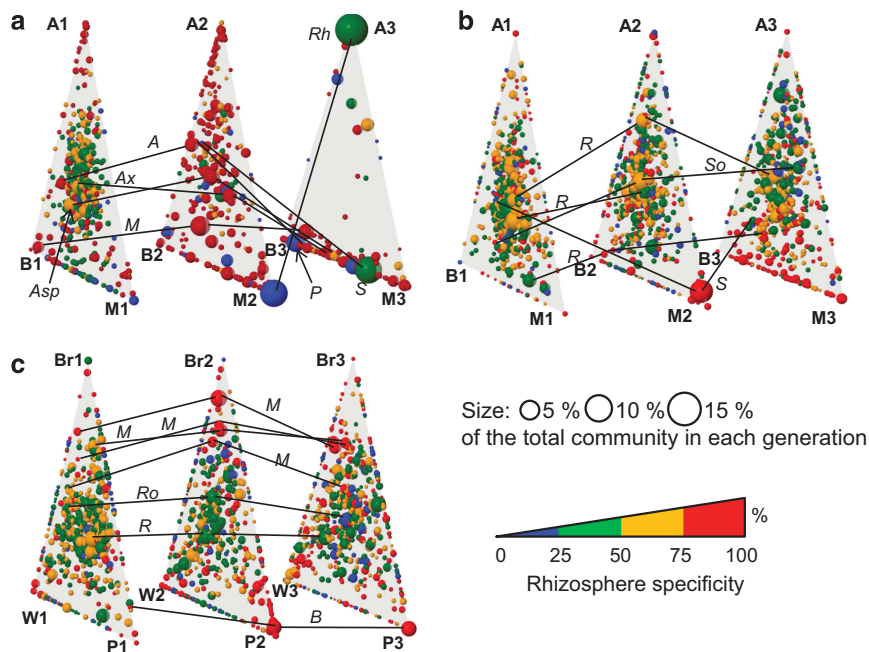
**Figure 2** Different model and crop plants grown in sand and compost establish distinct rhizosphere microbiota. (a) Sand: ARISA analysis of first generation of plants. (b) Sand: 454Flx pyrosequencing analysis of bacteria from all three generations. (c) Compost: 454Flx pyrosequencing analysis of bacteria from three generations of model plants. (d) Compost: 454Flx pyrosequencing analysis of bacteria from three generations of crop plants. Data shown as *n* multi-dimensional scaling plots (based on Bray–Curtis dissimilarity), which lack axes and where each data point represents one plant microbiota.

**Table 1** Percentage of variance in microbiota community composition explained by plant species, generation and the interaction between them for ARISA and 454 data

	Species	Generation	Interaction	Residual
ARISA	12	20	14	54
bacteria–compost	11	28	10	52
ARISA	18	39	16	26
bacteria–sand	27	34	20	18
454	29	23	21	27
bacteria–compost	13	26	17	44
454				
Bacteria–sand				
454				
Fungi–compost				
454				
Fungi–sand				

Abbreviation: ARISA, Automated Ribosomal Intergenic Spacer Analysis.

All effects are significant at  $P < 0.001$ , except for the interaction term for fungi in sand, where  $P = 0.003$ . For compost experiments, all six plant species and both unplanted controls are analysed simultaneously. Sand experiments used only model plants.



**Figure 3** The effect on the bacterial rhizosphere microbiota of plants grown in sand compared with compost. Each sphere represents a single OTU, size represents abundance, position corresponds to the relative abundance in the three rhizospheres and colour reflects the rhizosphere abundance relative to unplanted soil calculated for each generation separately. Please refer to Supplementary Figure S6 for an example explanation. (a) Bacterial microbiota of model plants grown in sand. (b) Bacterial microbiota of model plants grown in compost. (c) Bacterial microbiota of crop plants grown in compost. Only OTUs with a relative abundance of greater than 0.1% were used. *Achromobacter xylosoxidans* (Ax), *Acidovorax* (A), *Arthrobacter* sp. (Asp), *Massilia* (M), *Pseudomonas* (P), *Rhizobium* (three separate OTUs are annotated) (R), *Rhodopseudomonas* (Rh), *Rodanobacter* (Ro), *Solirubrobacter* (So) *Stenotrophomonas* (S) and *Arabidopsis* (A), *Medicago* (M), *Brachypodium* (B), *Brassica* (Br), *Pisum* (P), *Triticum* (W) and generation (1, 2, 3).

red, indicating a greater abundance in the rhizosphere. In the third generation, the diversity of the *Arabidopsis* microbiota collapsed, with invasion of apparent opportunists that were also common in unplanted soil (green and blue spheres) (Figure 3a). The dominant bacterial species in the third generation were predominantly putative autotrophs such as *Rhodopseudomonas* in *Arabidopsis* and *Stenotrophomonas* in *Medicago* and *Brachypodium* (Figure 3a). Isolates of these species are either able to fix CO<sub>2</sub> and gain energy from photosynthesis and use nitrate as an electron acceptor (present in plant mineral supplement) or fix N<sub>2</sub> from the atmosphere (Taghavi *et al.*, 2009; Yuan *et al.*, 2012). However, the fact that the dominant opportunists present in the third generation of *Arabidopsis* were different from *Medicago* and *Brachypodium* suggests that plant selection still operated. The putative autotrophic lifestyles of these opportunists may give an advantage over species utilising plant-derived compounds and/or soil residues. This is particularly the case when the percentage of Bawburgh soil in sand decreased from 10% to 2.5% to 0.6% over the three generations, with a commensurate reduction in organic material (Figure 1). *Brachypodium* and *Medicago* grown in sand also showed a reduced diversity, although to a lesser extent than for *Arabidopsis* (Supplementary Figure S4b). A similar reduction in diversity in the third generation also occurred for fungi (Supplementary Figures S4b and S8a).

#### Plants select for plant growth promoting bacteria

At the end of the third generation we isolated soil bacteria from rhizosphere samples. One advantage of this experimental selection experiment is that 454Flx analyses revealed the abundance of different OTUs for each plant in each generation. Thus it was known that the genera *Achromobacter*, *Arthrobacter* and *Massilia* were among the most abundant and strongly selected bacteria. To see if these could be isolated, 150 bacterial strains from the third generation of *Arabidopsis*, *Medicago* and *Brachypodium* were cultured (50 isolates from each plant species) using different growth media and individual colonies were screened by using 16S rRNA sequencing (Supplementary Word file 2). *Achromobacter xylosoxidans* and *Arthrobacter* sp. but not *Massilia* were successfully isolated (annotated as Ax and Asp on Figure 3a, respectively). *A. xylosoxidans* and *A. sp.* were isolated from *Medicago* and *Brachypodium* rhizospheres, respectively. The corresponding OTUs appear to be soil borne bacteria as they were also found at low abundance in the unplanted control samples (Supplementary Figure S9). While both organisms were present in soil they were strongly selected in the rhizosphere apart from the third generation of *Arabidopsis*. When re-inoculated as pure cultures onto plants grown in sterile sand, *Achromobacter* increased growth of all plants, while *Arthrobacter* increased the growth of *Brachypodium* from which it was initially isolated (Supplementary



Figure S10). Thus these isolates are plant growth promoting rhizobacteria that form a mutualistic relation with a plant, promoting plant growth and presumably developing a favourable rhizosphere niche for their own growth. Thus while this experimental approach is not intended to replicate natural soil it is a very powerful way to investigate plant–microbe interactions and select plant-dependent bacteria.

#### *Microbiota selection in compost*

Selection of the rhizosphere microbiota in sand (Figures 2a and b and Supplementary Figures S2a, d and g) creates conditions in which plant exudate is the dominant organic nutrient source. This makes it particularly suited to identification of the role of plants in selection of microbiota, independent of soil factors. However, it is important to test a more complex soil to compare the effect of both growth media and plant species in selection of microbiota. Therefore the impact of plants on rhizosphere communities over three generations was measured in nutritionally rich compost. Again the microbiota was influenced by plants (Figures 2c, d, 3b and c, Supplementary Figures S2b, c, e, f, h, i, k, S5c, d), with  $\alpha$ -proteobacteria (particularly the order Rhizobiales) strongly selected. However, actinobacteria increased in abundance in compost. Out of 41 actinobacterial OTUs showing a significant difference between the rhizosphere and unplanted control, 29 were more common in rhizospheres, in contrast to their depletion in sand (out of 38 Actinobacterial OTUs, 24 were depleted in sand; Supplementary Figures S5b and c).

Model plant species are well characterised genetically and have been chosen for their small size and rapid growth. However, these growth habits might be unusual in causing strong effects on their rhizosphere microbiota. We therefore tested larger, widely planted crop plants *Brassica*, *Pisum* and *Triticum*, which belong to the families Brassicaceae, Fabaceae and Poaceae, respectively. Crop plants, grown in compost, had strong shifts in their rhizosphere microbiota over three generations (Figure 2d). Indeed, crop species had a greater influence on the rhizosphere than model plants, selecting more rhizosphere-specific bacterial OTUs (Supplementary Figures S2k, S5c and S11). This is probably explained by the more extensive root systems of crop plants, leading to increased root exudation and root turnover (Hawes *et al.*, 2000).

In addition, for all six species of plants the microbial community was much more stable during growth in compost compared with sand, with no loss of diversity over generations as indicated by the Shannon index of diversity (Supplementary Figures S4c and d). The statistical analysis performed on the richness values (Supplementary Figure S4e) indicates that indeed there is a disturbance in the community of sand and also to some extent in the

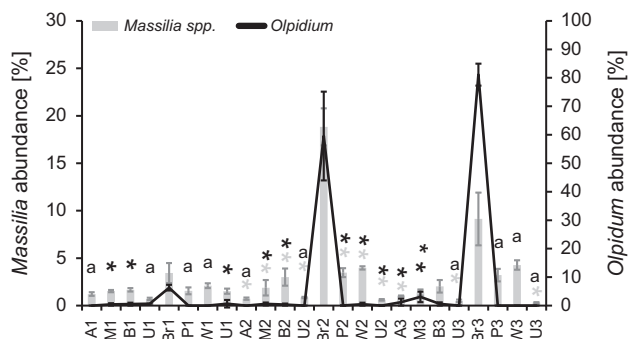
compost after the initial generation. Moreover, there are clear differences between compost and sand community richness in the following generations. In generation two and three compost maintained a relatively stable microbiota, although the microbiota of *Arabidopsis* in the third generation split into three very different communities of bacteria, suggesting stochastic variation (Supplementary Figure S2j). Potentially, these sub-communities could diverge further in subsequent generations, leading to formation of microbiota with very different properties. This effect may be the cause of the alterations in growth of *Arabidopsis* grown over multiple generations observed by Swenson (Swenson *et al.*, 2000).

#### *The fungal microbiota and *olpidium brassicae* as a host-specific rhizosphere pathogen*

Fungal communities contained many fewer OTUs than the bacterial one (Supplementary Figure S7). In contrast to bacterial 16S sequences, many fewer fungal ITS sequences were available to use in taxonomic assignments. As a consequence, most fungal sequences could only be annotated to the division/subdivision level. With the exception of *Brassica*, all communities were dominated by Ascomycota, particularly Saccharomyceta (Supplementary Figure S7). Each plant species had a distinctive fungal community, with replicates from each generation of each species usually clustering together in the multi-dimensional scaling plots, although the effect of generation was not as strong as in the 454 bacterial data, and there was more variation between replicates in sand (Supplementary Figures S2g and i, and Table 1).

Basidiomycota were more abundant in compost than in sand, but there were few differences in the abundance of divisions and subdivisions between plant species (Supplementary Figure S8). The only large changes were observed for *Brassica*, where one OTU became steadily more abundant over time, increasing from 6% to 61% then to 81% of the fungal community over generations 1–3, resulting in a dramatic shift of the overall fungal population (Figure 4 and Supplementary Figures S2i and S7c —annotated as ‘O’). The consensus intergenic spacer of this OTU had 100% identity to *Olpidium brassicae* (Figure 4 and Supplementary Figures S12 and S7c —annotated as ‘O’). *O. brassicae* is a fungal pathogen abundant in *Brassica* rhizospheres (Hartwright *et al.*, 2010) that reduces production of pods and seeds (Hilton *et al.*, 2013). The organism in our samples is likely to be *O. brassicae*, or a close relative, although ultimately this would require confirmation by culturing or more extensive sequencing. The taxonomic position of *Olpidium* is still a matter of debate, but here it was classified into Fungi incertae sedis (other phyla) (Supplementary Figure S8).

This remarkable selection of an OTU with a taxonomic assignment to a pathogen in a susceptible host in the experimental selection system could



**Figure 4** An invasion of *Olpidium brassicae* on *Brassica rapa* corresponds with increased abundance of *Massilia*. The black line represents *O. brassicae* abundance and bars represent abundance of the genus *Massilia*. *B. rapa* was grown for three generations on compost, *A. thaliana* (A), *M. truncatula* (M), *Brassica distachyon* (B), *B. rapa* (Br), *Pisum sativum* (P), *Triticum aestivum* (W), unplanted control (U), generation (1, 2, 3). The abundance of *Massilia* and *Olpidium* in *Brassica* rhizospheres was compared against other rhizospheres using *t*-tests. Grey and black stars represent statistical significance result for *Massilia* and *Olpidium* abundance, respectively. \* $P < 0.05$ , a-absent. Error bars represent the standard error calculated from three biological replicates.

mirror the problems encountered with repeated plant monoculture in agriculture. There was also a similar increase in abundance of the bacterial genus *Massilia*, suggesting it may be related to this invasion. Since this genus grows on chitin as a carbon and nitrogen source (Faramarzi *et al.*, 2009; Adrangi *et al.*, 2010) there may be a causal link, with *Massilia* growing on material derived from the putative *O. brassicae* (Figure 4) (James *et al.*, 2006).

## Discussion

We established a system of experimental selection in microcosms by using a defined starting microbial inoculum and controlled soil structure to determine how different plant species and growth media interact to alter succession of the rhizosphere microbiota. Previous studies have compared the influence of plant and soil on the rhizosphere microbiota (Berg and Smalla, 2009), but for the first time we have used high replication to allow statistical analysis to be applied. In addition we compared six different plants and two soil systems to increase the robustness of our observations. Finally, 454Flx sequencing was used to determine the nature of the organisms selected by different plants. Our two key questions were: how strongly do different plants select the rhizosphere microbiota and how is this influenced by growth media?

With regard to the first question, it is clear that plants select very distinct bacterial and fungal rhizosphere microbiota and this is confirmed by multivariate analysis of variance analysis, the proportion of variance in community composition explained by plant species and its interaction with time (Table 1). While fingerprinting techniques such as ARISA and T-RFLP (terminal restriction fragment

length polymorphism) have been used before and suggest plant dependent differences, our data show that to detect differences within a single generation more replicates are required (typically greater than 10) than are usually used (Knight *et al.*, 2012). Crop plants, with more extensive root system, shifted the community more than smaller model species, but all plants tested had very different microbiota from each other and from unplanted soil (Figures 2a and d, and Supplementary Figure S2).

In sand, fast growing microbes (for example, *Achromobacter*, *Arthrobacter* and *Massilia*) that utilise plant-derived compounds had an advantage over slower growing microbes (Figure 3a). By the third generation, model plants grown in sand had rhizosphere microbiota dominated by bacterial opportunists as indicated by the abundance of the same OTUs in unplanted soil (Figure 3a and Supplementary Figure S4b). There was also a relative increase in Proteobacteria during the second and third generations that may reflect the disturbance caused by the selection system in sand (Figure 1). Crucially, the increase in Proteobacteria was much greater in unplanted sand in the second and third generations compared with plant rhizospheres (Supplementary Figure S4a). Thus even though diversity reduced substantially in the rhizosphere microbiota in generations two and three, plants still exerted a significant selection relative to soil. The increase in abundance of Proteobacteria, which are often fast growing, may be a signature of disturbance. Proteobacteria also increase in abundance in the human gut microbiota in patients infected by *Clostridium difficile*, which often follows antibiotic treatment, an effect reversed by faecal transplant (Hamilton *et al.*, 2013). We hypothesise that the loss of diversity and increased proteobacterial abundance in our study was greater in unplanted sand than in plant rhizospheres because of stronger disruption. This suggests the initial soil inoculum, from which the microbial community comes, is more adapted to the rhizosphere environment than to poor carbon conditions that occurred in the unplanted sand control.

With regard to our second question, it is apparent that each plant selected a largely different rhizosphere microbiota in sand compared to compost from the initial microbial inoculum (Bawburgh soil), (Supplementary Figure S5). Thus plant type interacts with soil to affect selection of the microbiota. Furthermore, the collapse in diversity that occurred in the third generation of plants grown in sand did not occur in compost, as shown by the stable Shannon index of diversity (Supplementary Figures S4c and d). Exudates produced by roots are clearly important in plant selection of microbiota (Eilers *et al.*, 2010), but a rich source of organic material in soil is critical for stability and maintenance of diversity.

Although compost helped maintain a stable community there was a remarkable invasion over plant generations of a fungal OTU that, based on ITS sequences, appears to be the pathogen *O. brassicae* or a close relative (Hartwright *et al.*, 2010). This only



occurred on its compatible host *B. rapa* (Figure 4). Intriguingly, there was also an increase in abundance of *Massilia* OTUs. *Massilia* is able to degrade chitin (Faramarzi *et al.*, 2009; Adrangi *et al.*, 2010) found in the fungus cell wall (James *et al.*, 2006). It may also explain the repeated observation that *Massilia* is abundant in the rhizosphere (Bodenhausen *et al.*, 2013) and found to be associated with soils colonised by pathogenic fungi like *Fusarium* and *Rhizoctonia solani* (Klein *et al.*, 2013; Yin *et al.*, 2013). Moreover, it forms at least commensal interactions with *Pythium aphanidermatum*, a phytopathogenic oomycete infecting cucumber seeds (Ofek *et al.*, 2012). However, our data do not allow us to determine whether the occurrence of *Olpidium* and *Massilia* in *Brassica* rhizospheres at much higher abundance than any other microorganism reflects a trophic relationship between them or simply a common preference for *Brassica* roots.

The *Arabidopsis* microbiota in sand collapsed in diversity in the third generation and although it remained highly diverse in compost, it divided into three divergent groups with significantly different microbiota, suggesting stochastic variation (Supplementary Figure S2j) and possibly the occurrence of multiple stable states in the rhizosphere community (Sutherland, 1974). Swenson *et al.*, 2000, conducted repeated replanting of *Arabidopsis thaliana* Landsberg *erecta* in compost for 16 generations, which led to significant divergence of plant growth particularly after 10 generations. In this case, plants were weighed at each generation and soil from the top 10% and bottom 10% of weights was used as a microbiota inoculum for each successive generation. Compost derived from the heaviest plants lead to enhanced growth in successive generations relative to compost from the lightest plants. In our study it was impractical to continue a detailed metagenomic analysis for 10–16 generations, but it suggests that repeated sub-culturing with plant selection may lead to very different microbiota becoming fixed with significant consequences for plant growth.

We believe that this is the first report using a multi-replicate system to study the influence of plants on the rhizosphere microbiota and its stability over plant generations. Using high-throughput sequencing methods it should now be possible to focus on the importance of rhizoplane and endosphere microbial communities using a similar experimental design. Future research should take into consideration that only by using a high number of biological replicates it is possible to use statistics to determine significance in studies of microbial ecology. Here we also show the power of selection experiment in rhizosphere enrichment with the isolation of plant growth promoting rhizobacteria species. We not only demonstrate their growth promoting potential but also show that these strains are very competitive in the rhizosphere and their influence on the plant is species specific. In order to successfully use microbial inocula in agricultural

systems it is essential to identify microorganisms that promote the growth of plants of interest and be competitive against other soil microorganisms (Lugtenberg and Kamilova, 2009).

Overall, the experimental selection system we used has proved particularly powerful at demonstrating that plant type and soil composition interact to control the rhizosphere microbiota, and allowing us to separate the influence of soil type from that of initial inoculum. Plants select distinct microbiota but their composition and stability also depend on soil.

## Conflict of Interest

The authors declare no conflict of interest.

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