SPECIES-SPECIFIC ROOT MICROBIOTA DYNAMICS IN RESPONSE TO PLANT-AVAILABLE PHOSPHORUS

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38   -   Figure S1 | Analysis steps
39   -   Figure S2 | Comparison of ITS PCR approaches for plant root samples
40   -   Figure S3 | Rarefaction curves for bacterial and fungal OTU richness
41   -   Figure S4 | Effects of plant species and P-levels on microbial richness,
42   diversity and evenness
43   -   Figure S5 | Beta-diversity analysis including the soil samples
44   -   Figure S6 | Identification of endobacteria by phylogenetic placement
45
46   -   Table S1 | Effects of plant species and P treatment on alpha diversity
47   (ANOVA)
48   -   Table S2 | Effects of plant species and P treatment on community composition
49   (PERMANOVA)
50   -   Table S3 | Effects P treatment on species-specific community compositions
51   (PERMANOVA)
52   -   Table S4 | Statistics from identifying phosphate sensitive microbes
53   -   Table S5 | Network characteristics
54
55   -   Methods S1 | Microbiota profiling and analysis
56
57   -   Notes S1 | Comparison of PCR approaches
58   -   Notes S2 | Bioinformatic scripts
59   -   Notes S3 | Data analysis in R
60   -   Notes S4 | Mapping endobacteria
61   -   Notes S5 | Comparison of ITS profiling approaches

SUMMARY

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63 • Phosphorus (P) is a limiting element for plant growth. Several root microbes,
64 including arbuscular mycorrhizal fungi (AMF), have the capacity to improve
65 plant nutrition and their abundance is known to depend on P fertility. However,
66 how complex root-associated bacterial and fungal communities respond to
67 changes in P availability remains ill-defined.

68 • We manipulated the availability of soil P in pots and compared the root
69 microbiota of non-mycorrhizal Arabidopsis with mycorrhizal Petunia plants.
70 Root bacteria and fungi were profiled using ribosomal operon gene fragment
71 sequencing, we searched for P sensitive microbes and tested whether a P
72 sensitive core microbiome could be identified.

73 • Root microbiota composition varied substantially by P availability. A P
74 sensitive core microbiome was not identified as different bacterial and fungal
75 groups responded to low-P conditions in Arabidopsis and Petunia. P sensitive
76 microbes included Mortierellomycotina in Arabidopsis, while these were AMF
77 and their symbiotic endobacteria in Petunia. Of note, their P-dependent root
78 colonization was reliably quantified by sequencing.

79 • The species-specific root microbiota dynamics suggest that Arabidopsis and
80 Petunia evolved different microbial associations under the selection pressure of
81 low P availability. This implies that the development of microbial products that
82 improve P availability requires the consideration of host-species specificity.

83 **KEY WORDS**

84 *Arabidopsis thaliana*, arbuscular mycorrhizal fungi (AMF), endobacteria, *Petunia*

85 *hybrida*, phosphate, root microbiota

INTRODUCTION

Phosphorus (P) presents one of the key nutrients for plant growth. While a small fraction of soil P is directly available for plant uptake, the larger fraction is complexed to organic and mineral soil components and therefore inaccessible for plants. The conventional agronomic solution to increase P availability for plants relies on supplementing mineral phosphate (PO_4^{3-}). However, yield optimization requires excess application of phosphate since less than thirty percent of applied P fertilizers effectively support plant growth, the rest of the applied phosphate readily transforms to plant non-available P forms (Cordell *et al.*, 2009). Such an overuse causes the rapid depletion of finite phosphorus reservoirs, is expensive and causes environmental harm, primarily with negative impacts on the aquatic environment by eutrophication of the surface water (Cordell *et al.*, 2009; Scholz & Wellmer, 2013; Reijnders, 2014). Therefore, next-generation agriculture requires novel sustainable solutions that reduce fertilizer inputs and increase the nutrient-use efficiency while maintaining high plant yields.

Numerous root-associated microbes have the capacity to mobilize soil P or metabolize recalcitrant forms (Gyaneshwar *et al.*, 2002; Jacoby *et al.*, 2017), thereby enhancing plant performance and agricultural yield especially under nutrient limiting conditions. For fungi, there is a continuum of functionally similar associations between different groups of root fungi and their host plant species (van der Heijden *et al.*, 2017). For instance, arbuscular mycorrhizal or ectomycorrhizal fungi are intimately connected to plant roots and support plant growth by mobilizing and transporting P from a larger soil volume and more distant pools of P thanks to their large hyphal network, (Jakobsen *et al.*, 1992). Aside from these classical mycorrhizal plants, also nonmycorrhizal plants such as *Arabidopsis thaliana* [hereafter: Arabidopsis] and *Arabis alpina* rely on fungal associations for nutrient acquisition (Cosme *et al.*, 2018). They rely on beneficial fungal endophytes including *Colletotrichum tofieldiae* (Hiruma *et al.*, 2016), *Serendipita indica* [formerly *Piriformospora indica*] (Yadav *et al.*, 2010) or a fungus of the order Helotiales (Almario *et al.*, 2017). Typically, the P availability in soil determines to which extent a plant is colonized by the fungal symbiotic partner with high levels of colonization under low-P conditions and little colonization in soils with high-P levels. In addition to fungi, many root bacteria are known to support plant nutrition with their abilities to solubilize inorganic P or to mineralize organic P (Rodríguez & Fraga, 1999; Alori *et al.*, 2017). Powerful P solubilizing bacteria include strains from the genera

119   *Bacillus*, *Pseudomonas* and *Rhizobium*; for a more comprehensive list as well as their

120   growth effects on crops, we refer to Alori *et al*. (2017). While a wide range of individual

121   rhizosphere microbes is known to support plant P nutrition, the effects of P availability

122   on the overall root microbiota remains less understood. A deeper understanding of

123   interactions between plants and their microbial allies in response to the bioavailability

124   of P is needed for developing microbe-dependent P fertilization solutions (Schlaeppi &

125   Bulgarelli, 2015; Busby *et al.*, 2017).

126   Interactions among microbes emerge as a critical component for the

127   maintenance of host-microbial homeostasis and for plant performance (Hassani *et al.*,

128   2018). Inter-kingdom microbial associations occur in the plant root microbiota as for

129   instance root fungi hosting endobacteria in their cells (Desirò *et al.*, 2014). Such ancient

130   fungi-endobacteria interactions (Bonfante & Desirò, 2017) include root fungi of the

131   Mucoromycota (Spatafora *et al.*, 2017) that host diverse bacterial endosymbionts

132   related to *Burkholderia* or *Mycoplasma*. An example of *Burkholderia*-related

133   endobacteria includes *Candidatus* Glomeribacter gigasporarum that is hosted by a

134   Glomeromycotina fungus (Bianciotto *et al.*, 2003). *Mycoplasma*-related endobacteria

135   have a broader host range with presence in Glomeromycotina (Naumann *et al*., 2010),

136   Mortierellomycotina (Desirò *et al*., 2018) and Mucoromycotina (Desirò *et al*., 2015).

137   The occurrence and functional contribution of fungal endobacteria adds a further level

138   of complexity to the interactions of plant with and among their associated microbes.

139   Plants are more and more recognized in context with their microbial

140   communities, where a multitude of microbes collectively function as a microbiome. P

141   fertilization as well as P depletion are known to induce shifts in soil microbial

142   communities (Wakelin *et al.*, 2012; Leff *et al.*, 2015; Huang *et al.*, 2016; Bergkemper

143   *et al.*, 2016; Ikoyi *et al.*, 2018). For example, grassland soil microbes consistently

144   responded to phosphate inputs with compositional community changes, as for instance

145   mycorrhizal fungi, oligotrophic bacteria and methanogenic Archaea decreased in

146   relative abundance with nutrient additions (Leff *et al.*, 2015). While the responses of

147   soil microbial communities to varying levels of different sources of P have been well

148   studied, the plant root-associated microbial communities have received less attention

149   (Silva *et al.*, 2017; Almario *et al.*, 2017; Robbins *et al.*, 2018). Robbins *et al.*, (2018)

150   investigated the effects of different levels of P fertilization on the Arabidopsis

151   rhizosphere and root microbiota. While phosphate applications had little effects on

152   microbial diversity, they affected more strongly the plant-associated microbiota

6

153 compared to bulk soil communities, suggesting plant-mediated cues for structuring the

154 plant microbiota in response to the nutritional status. The authors noted a weak P-

155 fertilization effect on root communities that was manifested by low-abundant root-

156 associated microbes. This suggests P to be a minimal driver in shaping microbial

157 communities compared to larger drivers such as compartment (soil vs. rhizosphere,

158 rhizoplane, and roots) or soil type (soils differing chemically, physically and with

159 regard to their microbiota; Hacquard *et al.*, 2015). The work on model non-mycorrhizal

160 plants revealed subtle responses of the root microbiota to the availability of soil P;

161 further work is needed to test whether mycorrhizal plants exhibit stronger root

162 microbiota responses thanks to their colonizing symbionts.

163   *Petunia x hybrida* [hereafter: Petunia] is a model plant that is commonly used

164 for investigating the symbiosis with arbuscular mycorrhizal fungi (AMF, Wegmüller *et*

165 *al.*, 2008; Breuillin *et al.*, 2010). Due to its fast life cycle, modest size and the

166 availability of genetic tools made, Petunia is also a model to study plant development

167 (Vandenbussche *et al.*, 2016), plant nutrition (Liu *et al.*, 2018) and hormonal signaling

168 (Hamiaux *et al.*, 2012). Petunia belongs to the Solanaceae family, thus is related to

169 tomato, potato and eggplant so that root microbiota knowledge may be transferable to

170 these staple food crops. We therefore chose Petunia in comparison with non-

171 mycorrhizal Arabidopsis to study root microbiota dynamics in response to P availability

172 in soil and how plants cope with P limiting conditions.

173   In this study, we tested the hypothesis that the composition of the root

174 microbiota alters depending on the P availability and we asked whether a core

175 microbiome or plant species-specific microbiomes prevail in response to P deficiency.

176 We expected the Petunia root microbiota to enrich for AMF under low-P conditions,

177 whereas the Arabidopsis response to low-P remained unclear. Hence, while differential

178 fungal responses were anticipated for the two plant species, we were interested in their

179 bacterial responses and whether a different sets of bacteria will respond to the varying

180 levels of P availability. A particular goal of the study was to uncover the interplay

181 between root bacteria and fungi and we examined their co-occurrence patterns in

182 response to the varying P availability. We expected to find potential microbial

183 interactions and hypothesized that the root microbiota data contains paired sequence

184 information of fungal endobacteria and their corresponding host fungi. A technical goal

185 of this study was to quantify AMF colonization in the context of whole fungal diversity

7

186   based on DNA-based sequencing instead of the traditional morphological
187   quantification by microscopy.

188   This study reveals that root microbiota composition varies markedly by P
189   availability in soil and that different fungal and bacterial groups are responsive to low-
190   P conditions in Arabidopsis and Petunia. We find co-abundant groups of candidate
191   microbial cooperation partners, including AMF and their symbiotic endobacteria, both
192   known to support plant growth under low-P conditions. Our work suggests that
193   Arabidopsis and Petunia have evolved individual microbial solutions, involving
194   multitrophic microbial interactions, to cope with low-P conditions.

MATERIAL AND METHODS

PLANT GROWTH

The experiment was conducted in 400 ml pots lined with a mesh (Trenn-Vlies, Windhager, Thalgau, Germany). Soil was collected on April 4th 2014 from a field site (47°26'20" N 8°31'40" E), sieved to 2 mm and stored at 4°C until use. Soil was mixed 1:1 volume with sterilized quartz-sand. Chemical properties of the sand-soil mixture were analyzed at the Labor für Boden- und Umweltanalytik (Eric Schweizer AG, Thun, Switzerland): pH 6.8, 6/31/51% (clay/silt/sand) and 1.4/1.05/1.07 mg kg-1 (water-extractable N/P/K).

Petunia seeds were surface sterilized with 70% ethanol, washed with autoclaved water and plated on 1/2 strength Murashige and Skoog basal medium (Sigma, Buchs, Switzerland) supplemented with 1.5% sucrose and solidified with 1.5% agar. Plants were germinated under long-day conditions (16-h photoperiod) in climate chamber (Sanyo MLR-352H; Panasonic, Osaka, Japan) at 25°C and 60% relative humidity. After 7 days, seedlings were transferred to 400 ml pots filled with substrate. Plants were grown for two weeks in the same climate chamber then moved to an in-house climate chamber with same humidity, photoperiod and temperature. Plants were fertilized with a corrected Petunia nutrient solution (Reddy *et al.*, 2007), prepared with three concentrations of phosphate: 0.03 mM KH_2PO_4 (low-P), 1 mM KH_2PO_4 (medium-P) and 5 mM KH_2PO_4 (high-P). Each plant received 300 ml of the solution over the last six weeks before harvest. We conducted two separate experiments using the same treatments and growth conditions, the first to collect the plant root samples (DNA analyses and microscopy) and shoot biomass and a second experiment to quantify leaf nutrient levels.

SAMPLE COLLECTION

Plants were harvested at 10 weeks. The roots were separated from the shoot with a clean scalpel. The shoots were dried in a 60° C oven for dry weight analysis. The loosely attached soil was shaken from the roots, the roots were washed three times in PBS buffer (approximately 10 ml for 1 g of fresh weight) and then split into two equivalent subsamples. Samples for DNA extraction were stored at -80°C until processing. Samples for microscopy were stored in 50% ethanol. After staining with pen ink (Vierheilig *et al.*, 1998), root length colonization was determined using the

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227 magnified intersections method for 100 intersections per sample (McGonigle *et al.*,

228 1990). Soil from unplanted pots was collected by removing the top 1 cm layer and then

229 mixing the soil below, one sample (250 mg - 500 mg) was taken from each pot.

230 The dried shoots were weighed and milled. P and K concentrations were analyzed using

231 inductively coupled plasma-optical emission spectroscopy (ICP-OES) at the elemental

232 analytic department of Agroscope according to (VDLUFA-Verlag, 2006).

233 *MICROBIOTA PROFILING*

234 The protocol for microbiota profiling, including DNA extraction, PCR,

235 sequencing and bioinformatics, is described in detail in **Methods S1**. The comparison

236 of the PCR approaches is reported in **Notes S1**, which contains the bioinformatic script,

237 input data, analysis script and the markdown report. The bioinformatic analysis of the

238 main samples of the study is documented with the scripts, parameters, support and

239 report files in **Notes S2**. The raw sequencing data of the MiSeq runs and the SMRT

240 sequencing are available from the European Nucleotide Archive ENA under the study

241 accession PRJEB27162.

242 *STATISTICAL ANALYSES*

243 Statistical analyses were performed using R v3.3.2 (R Core Team, 2016) within

244 Rstudio (RStudio Team, 2015). The effects of P availability on dry weight, P content

245 and K content were assessed with a linear model. Dry weight data was log-transformed

246 to satisfy the assumptions of the linear model (normality of residuals and

247 homoscedasticity). To test for the effect of P availability on AMF colonization, a

248 generalized linear model was fitted with quasibinomial distribution to account for

249 overdispersion. Rarefaction curves were prepared with the function 'rarecurve' from

250 vegan (Oksanen *et al.*, 2018). For alpha diversity, the data was rarefied to 15'000

251 sequences 500 times. For each subsample, several diversity indices were estimated:

252 richness (S) is the number of OTUs, H is the Shannon index from which $D=exp(H)$ was

253 calculated (Jost, 2007), and Sheldon evenness is $E= exp(H)/S$ (Sheldon, 1969).

254 ANOVA was used to assess the effect of P a and plant species on the mean of the 500

255 subsamples for each sample. For the rest of the analysis, the data was filtered (at least

256 4 sequences per sample in 4 samples) to remove low abundant OTUs. The effects of P

257 availability and plant species on community composition were assessed with

258 permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis

259 dissimilarities and visualized with principal coordinate analysis (PCoA) using vegan

10

260   and phyloseq (McMurdie & Holmes, 2013). The effect of P availability on abundance

261   of each OTU was investigated with edgeR (Robinson *et al.*, 2010) on TMM-normalized

262   data (Robinson & Oshlack, 2010) and visualized with ternary plots. TMM-normalized

263   data was used to calculate Spearman rank correlations between OTUs for co-occurrence

264   networks. Positive (ρ>0.7) and significant relationships (P<0.001) were visualized with

265   igraph (Csardi & Nepusz, 2006). Scripts, functions and support files are available as

266   **Notes S3**. **Figure S1** visualizes the workflow of the analysis steps.

267   *IDENTIFICATION OF ENDOBACTERIA*

268   We describe the identification of endobacteria OTUs using a phylogenetic

269   placement approach in **Methods S1**. Briefly, we pre-selected candidates in the

270   microbiome dataset using two approaches and then validated their representative

271   sequences by fine mapping to a reference tree of known endobacteria sequences. The

272   first approach was based on sequence clustering and for the second, we employed co-

273   occurrence characteristics from network analysis. Command line and analysis code in

274   R (including markdown report) as well as the database with curated endobacteria

275   16S rDNA reference sequences are available as **Notes S4**.

RESULTS

EXPERIMENTAL SETUP FOR MANIPULATING PHOSPHATE LEVELS

We investigated the dynamics of the root-associated microbiota to the availability of soil P and compared the non-mycorrhizal model species Arabidopsis to Petunia, which forms symbiosis with AMF. Plants were sown in a field soil that was amended with sand and we manipulated soil P availability by applying low, medium or high levels of phosphate. Unplanted pots were included as controls to collect soil samples. We first confirmed the effectiveness of the applied phosphate levels and found that phosphate treatments positively affected plant growth (**Fig. 1a**), increased P levels in plant leaves (**Fig. 1b**), while reducing the AMF colonization levels in Petunia roots (**Fig. 1c**). As we manipulated soil P levels using simple K salts, we tested if the low-P condition would also be limited in potassium. Since the plants growing in low-P conditions were sufficiently supplied with K (**Fig. 1d**), we concluded that the simple approach of using $KH_2PO_4^{3-}$ solutions permitted to establish a P gradient without causing K limiting conditions.

PROFILING SOIL AND ROOT MICROBIAL COMMUNITIES

First, we evaluated the following PCR approaches to profile root fungal communities: ITS1F and ITS2 (McGuire *et al.*, 2013a), fITS7 and ITS4 (Ihrmark *et al.*, 2012a) and ITS1F with the reverse complement of fITS7. Community profiles were inspected for the proportions of plant and AMF sequences as well as for fungal diversity. We selected ITS1F and ITS2, because this PCR approach captured low levels of plant sequences at good coverage of AMF and highest levels of taxa richness (**Fig. S2**, See **Notes S1** for a detailed comparison of the PCR approaches).

We then characterized soil and plant root-associated bacterial and fungal communities by sequencing amplicons of the 16S rRNA gene and the internal transcribed spacer (ITS) region 1, respectively. We obtained 2'196'310 high-quality bacteria sequences with a median of 36'718 sequences per sample and 3'809'350 high-quality fungal sequences with a median of 54'337 sequences per sample. Bacterial and fungal sequences clustered into 3'701 bacterial operational taxonomic units (bOTUs) and 1'688 fungal OTUs (fOTUs), respectively.

Soil bacteria comprised abundant Acidobacteria, Actinobacteria, Firmicutes, Deltaproteobacteria and Verrucomicrobia, whereas plant roots were mainly colonized

12

308   by Betaproteobacteria, Gammaproteobacteria and Bacteroidetes (**Fig. 2**). The bacteria

309   community composition at Phylum rank was not markedly different between

310   Arabidopsis and Petunia.

311   For fungi, Ascomycota and Basidiomycota were abundant in soil and Petunia

312   root samples (**Fig. 2**). Mortierellomycotina were particularly abundant in soil fungal

313   communities, whereas a high number of Glomeromycotina was found in Petunia roots,

314   which varied as a function of the applied phosphate levels. Consistent with the levels

315   of AMF root colonization measured by microscopy (**Fig. 1c**), Glomeromycotina were

316   most abundant in Petunia roots under low-P conditions and decreased in proportion

317   with increasing P availability (**Fig. 3a**). The cumulative relative abundance of

318   Glomeromycotina sequences was significantly positively correlated (adj. $R^2 = 0.59$; *P*

319   < 0.001) with the rate of AMF root colonization as assessed by microscopy (**Fig. 3b**).

320   We noted that the root fungal community of Arabidopsis was dominated by sequences

321   belonging to Olpidiomycotina. Most of these sequences belonged to fOTU1 (assigned

322   to *Olpidium brassicae*), which accounted for 94.50% of the sequences in Arabidopsis

323   but only 0.79% of the sequences in Petunia samples. To exclude that this is a technical

324   peculiarity of MiSeq, we confirmed the dominance of *O. brassicae* by sequencing the

325   entire ITS region (PCR primers ITS1F and ITS4) using SMRT sequencing (**Fig. 2c**).

326   We refer to the **Notes S5** for the detailed comparison of the sequencing approaches. In

327   brief, the SMRT-sequencing based community profiles also avoided amplifying plant

328   sequences while abundantly capturing the AMF. Both methods have their inherent

329   technical advantages with the MiSeq approach offering enhanced throughput and

330   sampling depth, whilst the SMRT-sequencing method provides enhanced taxonomic

331   resolution. Albeit a few quantitative differences, the two approaches reproduce overall

332   similar taxonomic compositions and revealed remarkably similar biological patterns

333   (**Notes S5**).

334   *PHOSPHATE INDUCED VARIATION IN MICROBIAL DIVERSITY*

335   In the following, we used the MiSeq-based fungi profiles as they were obtained

336   using the same sequencing platform as the bacteria and because of the enhanced

337   sequencing depth. Bacteria and fungi richness was highest in unplanted soil, followed

338   by Petunia and then Arabidopsis roots (**Fig. S3**). ANOVA confirmed the effect of plant

339   species on alpha diversity for both bacteria and fungi and further uncovered an effect

340   by the different P availability on the bacteria community (**Fig. S4**, **Table S1**). Bacterial

341 richness, diversity and evenness were generally higher in Petunia compared to
342 Arabidopsis and generally increased with increasing P concentrations. With the
343 dominance of *O. brassicae,* fungal richness, diversity and evenness were markedly
344 lower in Arabidopsis compared to Petunia.

345 Utilizing principal coordinate analysis (PCoA) of Bray-Curtis dissimilarities,
346 we found compositional differences in microbial communities due to the tested
347 experimental factors sample type, plant species and P availability (**Fig. 4**). Consistent
348 with previous work (Bulgarelli *et al.*, 2012; Hartman *et al.*, 2018), bacteria and fungi
349 differed markedly between the sample types of unplanted soil and roots (**Fig. S5**). With
350 regard to plant species, fungal communities were more divergent between Arabidopsis
351 and Petunia roots compared to bacteria communities (**Fig. 4**), possibly reflecting their
352 opposite behavior with AMF. Permutational multivariate analysis of variance
353 (PERMANOVA), finding significant plant species effects on both bacterial and fungal
354 communities (**Table S2**), confirmed that plant species explained more variation for
355 fungi (53% of variation) compared to bacteria (14%).

356 The effects of P availability were apparent in bacterial communities of both
357 Arabidopsis and Petunia by clustering following the gradient in phosphate levels (**Fig.
358 4A**), whereas for fungi, this was only manifested in Petunia (**Fig. 4B**). PERMANOVA
359 confirmed a significant P availability effect for the bacteria (**Table S2**). To approximate
360 the effect sizes of P availability on the Petunia and Arabidopsis root microbial
361 communities, we inspected the R2 values of PERMANOVA applied to the data of each
362 plant separately. While P availability explained 14.1% and 13.3% of variation in
363 Petunia root bacterial and fungal communities, respectively, it accounted for 15.0% and
364 21.7% of variation in the Arabidopsis root microbial communities (**Table S3**). This
365 indicates that varying P availability in soil can account for about 15% of variation in
366 plant root microbiota composition.

367 *IDENTIFYING PHOSPHATE SENSITIVE MICROBES*

368 Next, we identified P sensitive OTUs – OTUs being differentially abundant
369 between low and high-P conditions – using edgeR (Robinson *et al.*, 2010). In total we
370 found 2.2% bOTUs and 6.3% fOTUs responsive to the varying P availability in Petunia,
371 while 3.1% bOTUs and 13% fOTUs were sensitive in Arabidopsis (**Fig. 5a**, **Table S4**).
372 With the exception of four bOTUs, different sets of P sensitive bacteria and fungi OTUs
373 were found for Arabidopsis and Petunia, suggesting that the two plant species have

14

374   differential microbial responses to low-P conditions. Among the four shared bOTUs

375   was a prominent *Dechloromonas* sp. (bOTU2), which is more abundant under low-P

376   conditions in both plant species (**Fig. 5b**). Bacteria from different taxonomic lineages

377   were abundant under low or high-P conditions (**Table S4**). The most abundant

378   Arabidopsis root bacteria included also Burkholderiales, Bdellovibrionales and

379   Rhodocyclales under low-P conditions, whereas taxa from the Chthoniobacterales,

380   Planctomycetales and Verrucomicrobiales were enriched under high-P conditions.

381   Under low-P conditions, the abundant Petunia root bacteria included members of the

382   Burkholderiales and Rhodocyclales, whereas under high-P conditions, a slightly

383   different set of bacteria, including a Flavobacterium sp. (Flavobacteriales), a Tahibacter

384   sp. (Xanthomonadales) and members of the Verrucomicrobiales were abundant.

385   Examples of highly abundant and low-P specific Burkholderiales and Rhodocyclales

386   members include a *Dechloromonas* sp. (bOTU2) and a *Candidatus* Accumulibacter

387   (bOTU13, **Fig. 5b**). Among the Petunia root bacteria, which are enriched under low-P

388   conditions, we noticed an bOTU assigned to *Candidatus* Glomeribacter gigasporarum

389   (bOTU134, **Fig. 5b**), which presents an endobacterium associated with lineages in the

390   AMF family Gigasporaceae (Bianciotto *et al.*, 2003).

391   Similar to bacteria, different fungal lineages responded to low or high-P

392   conditions in Arabidopsis and Petunia (**Table S4**). In Arabidopsis, besides many low

393   abundant and often taxonomically poorly resolved fungi, the distinct group of

394   Mortierellomycotina (e.g., fOTU7, **Fig. 5c**) was enriched under low-P conditions.

395   Under high-P conditions, the abundant fungi *O. brassiceae* (Olpidiales, fOTU2, **Fig.

396   5c**), *Hygrophoraceae* sp. (Agaricales, fOTU10) and *Cadophora* sp. (Helotiales,

397   fOTU14) were found besides numerous low abundant fOTUs. While in Petunia only a

398   handful of diverse and low abundant fungi were enriched under high-P conditions, we

399   found a large group of 28 mycorrhizal fOTUs enriched in the low-P treatment (**Table

400   S4**). These mycorrhizal fOTUs belonged mostly to the order Glomerales and included

401   numerous abundant members such as *Funneliformis* and *Glomus* spp. (e.g., fOTU6 in

402   **Fig. 5c**).

403   *PHOSPHATE-INDUCED DYNAMICS IN MICROBIAL ABUNDANCE*

404   Finally, we utilized co-occurrence network analysis to find pairs or groups of

405   microbes with a similar abundance behavior along the gradient of plant-available P.

406   Co-abundance presents a pre-requisite for cooperation among microbes and we

407 speculated to identify possible candidate cooperation partners that may contribute to

408 support plant growth under low-P conditions. **Figure 6a** visualizes the significant

409 positive pairwise correlations between root microbiota members (bOTU-bOTU, fOTU-

410 fOTU and bOTU-fOTU) of Petunia and Arabidopsis growing in conditions with low,

411 medium or high-P availability. We then partitioned the network into discrete

412 community modules and mapped the P-sensitive bOTUs and fOTUs into the network

413 and modules. While we did not find groups of co-occurring OTUs (=modules) that were

414 responsive to high-P conditions, we found two major modules, 'M1' and 'M26', that

415 comprised high proportions of P-responsive OTUs (**Fig. 6b**) being specifically

416 abundant under low-P conditions (**Fig. 6c**). The module 'M1' comprised only bacteria,

417 mainly belonging to the Betaproteobacteria orders Burkholderiales and Rhodocyclales

418 (**Table S5**). In contrast, the module 'M26' grouped a set of five taxonomically diverse

419 bacteria lineages with a large set of fOTUs primarily belonging to the order Glomerales

420 (**Table S5**). These fOTUs represented almost all AMF fOTUs in the dataset (**Table S5**)

421 and interestingly, they co-occurred with the *Candidatus* Glomeribacter bOTU134.

422 The same analysis was conducted for Arabidopsis and revealed a module 'M27'

423 with co-occurring bacteria and fungi OTUs that were specifically abundant under high-

424 P conditions (**Fig. 6**, **Table S5**). This module grouped diverse bacteria members

425 including Planctomycetes and Verrucomicrobia and a diverse set of fungi. The module

426 'M19' held low abundant and taxonomically diverse bacteria and fungi that favored

427 intermediate P-levels. The module 'M4' comprised abundantly co-occurring bacteria

428 and fungi under low-P conditions, belonging mainly to diverse Proteobacteria and

429 Ascomycota or unknown fungi, respectively. With the exception of a few bacteria, the

430 low-P responsive modules of Arabidopsis and Petunia had specific compositions (**Fig.**

431 **6d**), which is consistent with the species-specific root microbiota dynamics to low-P

432 condition.

433 Microbes that simultaneously co-occur with many others are often referred to

434 keystone taxa as they may play an important ecological role by determining community

435 dynamics and microbiome functioning (Banerjee *et al.*, 2018). We identified keystone

436 OTUs, defined based on their high degree of co-occurrence, for the Arabidopsis and

437 Petunia root microbiota networks (**Table S5**). While all Arabidopsis keystone OTUs

438 belonged to the high-P module 'M27', we found keystone OTUs in the low-P

439 responsive module 'M26' of Petunia. These low-P responsive keystone OTUs were the

440 Glomeromycotina fOTU6 and fOTU111 as well as fOTU109 of unknown taxonomy.

441   *ENDOBACTERIA*

442   To understand whether bacterial communities associated with the Arabidopsis

443   or Petunia root microbiota could include fungal endobacteria, we aligned candidate

444   bOTU sequences to a database of curated endobacteria sequences (see methods).

445   Phylogenetic placement confirmed bOTU134 as *Candidatus* Glomeribacter closely

446   related to one hosted in *Scutellospora pellucida* (**Figs. 7a, S6, S7**). In addition, we

447   identified two bOTUs (330 and 778) mapping to *Mycoplasma*-related endobacteria

448   identified in the AMF species *Claroideoglomus claroideum* and *C. etunicatum*,

449   respectively. Although these two bOTUs do not belong to the 'AMF module M26' of

450   Petunia, they were, similar to bOTU134, significantly higher in abundance under low-

451   P conditions (**Table S3**). Phylogenetic placement analysis revealed six additional

452   *Burkholderia*- and two *Mycoplasma*-related endobacteria OTUs (**Figs. 7, S6**), however

453   as they were detected with only a handful of reads in a few samples, we did not include

454   them for network analysis (see **Notes S4** for details). The use of microbiota network

455   characteristics was generally not indicative for identifying endobacteria OTUs, by

456   contrast the clustering-based approach proved to function well (**Figs. S6, S7**, **Notes S4**).

457   In summary, the combined sequencing of bacteria and fungi permitted to identify three

458   endobacteria OTUs that had a consistent abundance behavior with their mycorrhizal

459   hosts along the P-gradient.

DISCUSSION

460

461   In this study, we revealed the dynamics of the root microbiota of non-
462   mycorrhizal Arabidopsis and mycorrhizal Petunia plants along a gradient of plant-
463   available P in soil. We demonstrate that the composition of the root microbiota alters
464   depending on P availability and we revealed species-specific microbial patterns in
465   response to low-P conditions. Under low-P conditions, we confirmed a substantial
466   colonization by AMF in Petunia together with numerous bacteria of the Burkholderiales
467   and Rhodocyclales, whereas Arabidopsis roots hosted mainly Mortierellomycotina
468   fungi and abundant bacteria from the Burkholderiales, Bdellovibrionales and
469   Rhodocyclales. However, the root microbiota of the two plants contained different
470   members (bOTUs) of these taxonomic lineages. These groups of P-sensitive microbes
471   responded simultaneously to the different levels of P availability. Among these co-
472   occurring microbes, we found fungal endobacteria and their corresponding hosts,
473   presenting a well-known example of highly specific multitrophic microbial
474   interactions.

475   *ENDOBACTERIA*

476   To our knowledge, this is the first report to identify endobacteria of
477   Mucoromycotina in a plant microbiota study. Above all, the experimental design with
478   low- to high-P conditions was instrumental to become aware of fungal endobacteria in
479   the dataset. A first hint for the presence of endobacteria came from the statistical
480   approach to find differentially abundant bOTUs between low and high-P conditions,
481   revealing the enrichment of a *Candidatus* Glomeribacter OTU under low-P conditions
482   (**Fig. 5b**). Of note, bOTU134 was almost overlooked, as a reliable taxonomy
483   assignment (confidence >0.7) was only available down to family level, while the deeper
484   data exploration indicated a link with endobacteria (genus-level assignment was
485   '*Candidatus* Glomeribacter', confidence 0.34). The second hint that pointed to
486   endobacteria and their hosts were the co-abundance patterns, which grouped bOTUs
487   including *Candidatus* Glomeribacter bOTU134 with numerous Glomeromycotina
488   fOTUs (**Table S5**). Here again, experimental design was instrumental because the co-
489   occurrence analysis groups microbes with similar abundances in samples from low- to
490   high-P conditions. Finally, we relied on phylogenetic placement to confirm that
491   bOTU134 represents a Candidatus *Glomeribacter*, which is phylogenetically related to
492   a previously isolated exemplar from the AMF species *Scutellospora pellucida* (**Fig. 7**).

18

493   The identification of bOTU134 as a fungal endobacterium prompted us to

494   search for additional potential endobacteria in the Arabidopsis or Petunia root

495   microbiota. We compared two strategies, co-occurrence characteristics and sequence

496   similarity to known endobacteria, for their usefulness to identify endobacteria from root

497   microbiota data. For the co-occurrence characteristics strategy, we selected all bOTUs

498   that significantly co-occurred with fOTUs from lineages known to host endobacteria,

499   and then placed the resulting 129 candidate bOTUs into the endobacteria reference tree.

500   For the second strategy, we clustered all sequences of the curated endobacteria database

501   with all representative bOTU sequences of the microbiota dataset, and then placed the

502   resulting 22 candidate bOTUs with the highest sequence similarity into the

503   endobacteria reference tree. Overall, in addition to bOTU134, two abundant (bOTUs

504   330 and 778) and 8 low-abundant endobacteria OTUs were identified (**Notes S4**).

505   Moreover, the relative abundance of the three OTUs along the P-gradient was

506   consistent with the one of their fungal hosts (**Table S4**). While both approaches

507   identified bOTUs 134 and 778, the clustering-based approach functioned more

508   efficiently as also low abundant candidates were identified.

509   There are probably multiple reasons why endobacteria and their hosts were

510   largely neglected in microbiota studies so far. Reasons include the low taxonomic

511   resolution of short-read community data or the underrepresentation of reference

512   endobacteria sequences in commonly used taxonomy databases. Here, only the

513   combined sequencing of bacteria and fungi together with the dedicated experimental

514   design to manipulate the abundance pattern of fungal hosts and the curated endobacteria

515   database permitted to identify endobacteria OTUs. Our study demonstrates that

516   microbiota and/or metagenomic datasets represent useful tools to investigate

517   endobacterial–fungal interactions in their true ecological context. Possibly, such

518   cultivation-independent methods can point to further endobacterial–fungal

519   partnerships. Since endobacteria are ecologically relevant for the fitness of their (plant-

520   associated) fungal hosts (Salvioli *et al.*, 2016; Uehling *et al.*, 2017; Desirò *et al.*, 2018),

521   they may also relay some benefits or detriments to the host plant of the fungus

522   (Bonfante & Desirò, 2017). Hence, mycorrhizal plants, their colonizing fungi along

523   with their endobacteria form an entity of a multi-kingdom symbiosis. As put forward

524   by the holobiont concept (Vandenkoornhuyse *et al.*, 2015), the importance of multi-

525   kingdom microbe-microbe interactions for plant performance is not only true for

526   endobacteria and their fungal hosts but also in general between root bacteria and root

19

527 fungi. For instance, root bacteria are essential to protect plants against pathogenic root

528 fungi (Durán *et al.*, 2018).

529 *QUANTIFYING AMF IN PLANT ROOTS*

530 Although specific sequencing methods to quantify AMF in plant roots (Öpik *et*

531 *al.*, 2009; Schlaeppi *et al.*, 2016) are available, we sought to establish a sequencing-

532 based approach to assess AMF in plant roots in the context of the whole fungal

533 diversity. In the search for a PCR approach that avoided amplification of plant ITS

534 sequences, we also wanted that AMF would be well captured in plant roots unlike other

535 plant root-fungi profiling methods (Ihrmark *et al.*, 2012b; Hartman *et al.*, 2018). The

536 Illumina approach by McGuire *et al.* (2013b), although reporting soil fungal profiles,

537 indicated that PCR primers ITS1F and ITS2 would permit to abundantly capture AMF

538 and this approach turned out to be successful on plant roots, too (**Fig. S2**).

539 Prior to community sequencing, we had evaluated the effectiveness of our

540 experimental P availability gradient by confirming that AMF abundantly colonize

541 Petunia roots under low-P but not under high-P conditions (**Fig. 1c**). We performed this

542 quality control using the traditional 'magnified intersection' (McGonigle *et al.*, 1990)

543 microscopy method on equivalent subsamples as the ones that were used for the

544 sequencing. The microscopy method relies on staining cleared roots, interpreting and

545 enumerating the different fungal structures following a defined counting scheme under

546 the microscope. Compared to the sequencing-based quantification of AMF in plant

547 roots, the microscopy method appears disadvantageous as it is prone to operator-to-

548 operator variation, is time consuming and lacks throughput, discrimination between

549 AMF species as well as the context of the whole fungal diversity. Nevertheless, we

550 found a reliable agreement between the two methods with a significant positive

551 correlation (**Fig. 3b**). In summary, the MiSeq-based community profiling approach

552 with the PCR primers ITS1F and ITS2 avoided amplification of plant ITS sequences,

553 abundantly displayed the AMF and independently reproduces AMF colonization

554 patterns of plant roots.

555 *AMF AS KEYSTONE SPECIES*

556 Keystone taxa are thought to own central positions in microbial networks, with

557 many links to other species; therefore, they may play important ecological roles by

558 determining community dynamics and microbiome functioning (Banerjee *et al.*, 2018).

559 AMF were postulated to be keystone species (van der Heijden & Hartmann, 2016) and

20

560   indeed, we find two Glomeromycotina OTUs (fOTU6 and fOTU111) as being

561   keystones in the low-P responsive Petunia module 'M26' (**Fig. 6**). Technically, the two

562   Glomeromycotina OTUs fulfilled the network-based criteria defining keystone OTUs

563   – keystone OTUs have high degree of co-occurrence, high closeness centrality and low

564   betweenness centrality (Banerjee *et al.*, 2018). The two identified keystone OTUs have

565   high degrees of co-occurrence implying they are co-abundant with many other (mostly

566   Glomeromycotina) OTUs in the network. However, with regard to these network-

567   topology based criteria, there is a major caveat linked to Glomeromycotina fungi:

568   several Glomeromycotina species have particularly high intraspecies genetic diversity

569   at the rRNA operon (Stockinger *et al.*, 2010; Lekberg *et al.*, 2014) and, as a

570   consequence, these fungi require multiple OTUs (at >97% sequence identity) to

571   represent a single species. For example, three OTUs described the AMF *Rhizoglomus*

572   *irregulare* (Schlaeppi *et al.*, 2016). Therefore, multiple OTUs per fungal species

573   artificially inflate the number of co-occurring OTUs for an AMF species (the 3 OTUs

574   of *R. irregulare* will consistently co-occur with each other). While we agree that AMF

575   fulfil a key role in root microbiome functioning and this is especially true under low-P

576   conditions, we anticipate that the mathematical criteria for identifying AMF keystones

577   from network data may need to be improved and validated using functional analyses.

578   Instead of assigning keystone status to some pseudo-replicated sequence groups (e.g.,

579   the 3 OTUs for *R. irregulare*) based on network topology, we rather favor the idea of

580   empirically nominate keystone microbes because of their known key function(s) in a

581   given condition (e.g., low-P availability).

582   *OLPIDIUM BRASSICAE*

583   The root microbiota of Arabidopsis but not Petunia, whether profiled with

584   MiSeq or SMRT-sequencing, comprised abundant sequences assigned to *O. brassicae*,

585   which is a common root-infecting fungal pathogen of *Brassicaceae* plants (Lay *et al.*,

586   2018). As biotrophic fungus, *O. brassicae* does not cause tissue maceration. Although

587   the Arabidopsis roots were without signs of disease when harvested, we learned after

588   the community sequencing that the fungus had spread in the root tissue. Nevertheless,

589   such dominance of *O. brassicae* OTUs was observed in previous studies (Tkacz *et al.*,

590   2015; Durán *et al.*, 2018; Lay *et al.*, 2018). Similar to our study, Lay *et al.* (2018)

591   examining canola, wheat and pea roots, also found a Brassicaceae-specific enrichment

592   of an *O. brassicae* OTU. Because these findings originate from different Brassicaceae

21

593 species and different soil types, we consider the dominance of *O. brassicae* in

594 Arabidopsis roots in our study rather a true biological observation than a technical

595 artifact. Although, we confirmed this observation with SMRT sequencing, we cannot

596 fully exclude that the frequent amplification of *O. brassicae* OTUs may be linked to

597 the PCR primer ITS1f, as all these studies target the ITS1 region and have the use of

598 ITS1 or ITS1f PCR primers in common.

599 *P-SENSITIVE MICROBIOTA*

600 We found species-specific microbial patterns in response to low-P conditions

601 consisting of abundant Mortierellomycotina and Glomeromycotina fungi in

602 Arabidopsis and Petunia, respectively (**Fig. 5, Table S4**). Similar to the functioning of

603 AMF in plant P provision, there are some reports that *Mortierella* spp. support P

604 nutrition of plants (Alori *et al.*, 2017). Although represented by different sequence

605 groups, bacteria of the Burkholderiales and Rhodocyclales were abundant in both

606 species. Bacteria of both orders, including members that attach to AMF hyphae, are

607 well known for their ability to solubilize and mobilize P (Sharma *et al.*, 2013; Taktek

608 *et al.*, 2015; Alori *et al.*, 2017). *Candidatus* Accumulibacter being abundant in Petunia

609 under low-P conditions (bOTU13, **Fig. 5b**) as well as the *Dechloromonas* sp. (bOTU2,

610 enriched in low-P, both plant species) are both intriguing root bacteria, as they are

611 capable of polyphosphate metabolism, which has been implicated in stress response to

612 low nutrients in the environment (Rao & Kornberg, 1996; Flowers *et al.*, 2013). In

613 summary, it appears that non-mycorrhizal Arabidopsis and mycorrhizal Petunia rely on

614 different microbial associations to cope with low-P conditions.

615 We revealed species-specific and in contrast to Robbins *et al*. (2018), we found

616 marked root microbiota dynamics of the two plants along a gradient of plant-available.

617 A plausible reason for stronger P effects on the root microbiota is that our approach

618 created stronger P limiting conditions (1.05 mg P kg^{-1} soil) compared to the low-P

619 control soil (P1, did not receive phosphate amendments over the last 65 years) of the

620 long-term P fertilization experiment studied by Robbins et al. (2.3 mg P kg^{-1} soil, values

621 are directly comparable as they were analyzed in the same professional soil laboratory

622 with the same method). Moreover, we established a steeper gradient between low and

623 high-P conditions with a P availability of ~113 mg P kg^{-1} soil as high-P condition

624 compared to ~12 mg P kg^{-1} by Robbins *et al*. (2018). The stronger P limiting conditions

625 in our study were also reflected in the different rosette biomass data in both studies as

626   we find a twofold reduction in median rosette biomass whereas they measured at

627   maximum a 1.5x effect in low-P soils.

628   *CONCLUDING REMARKS*

629   The analysis of root microbiota dynamics of Arabidopsis and Petunia to low-P

630   conditions revealed a number of plant-species specific root microbes that are

631   preferentially selected at low soil P availability. With regard to agricultural

632   applications, this works suggests that for supporting different plant species in P

633   nutrition, different P-solubilizing and/or mineralizing bacteria strains are needed.

634   Possibly this explains the high context dependency of successful field applications with

635   P-solubilizing and/or mineralizing bacterial products.

AUTHOR CONTRIBUTIONS

648   NB, MvdH and KS designed the experiments. NB performed the experiment, did the

649   molecular work and analyzed the data. JCW, VS, AD and KS contributed to data

650   analysis. NB, LB, MvdH and KS wrote the manuscript. All authors approved the final

651   manuscript.

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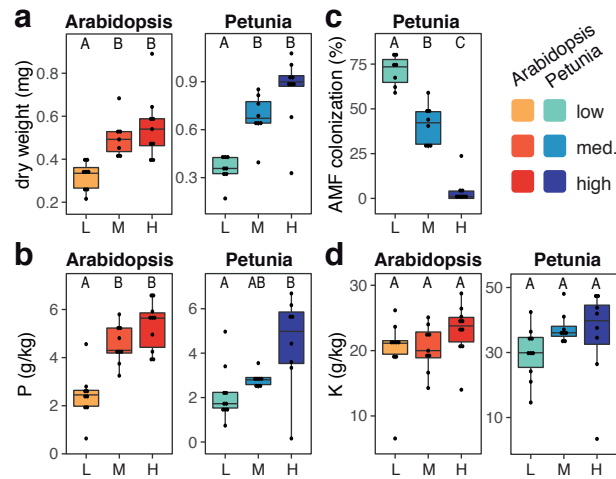
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3 **Figure 1 | Effectiveness of manipulated phosphate levels on plant growth, leaf nutrient**
4 **levels and levels of AMF root colonization**
5 Arabidopsis (reddish colors, see legend) and Petunia (blueish colors) were grown at low (L),
6 medium (M) and high (H, increasing hue) levels of P availability and basic plant parameters
7 were recorded to confirm that the experimental setup. Parameters included (**a**) above-ground
8 plant biomass, (**b**) leaf phosphorus levels, (**c**) Petunia root colonization by arbuscular
9 mycorrhizal fungi (AMF) and (**d**) leaf potassium levels. A linear model was used to test for
10 effects of P availability for panels (**a**) (log-transformed data), (**b**) and (**d**) and a quasibinomial
11 generalized linear model for panel (**c**). Different letters indicate significant pairwise
12 differences among sample groups (P < 0.05, Tukey HSD).
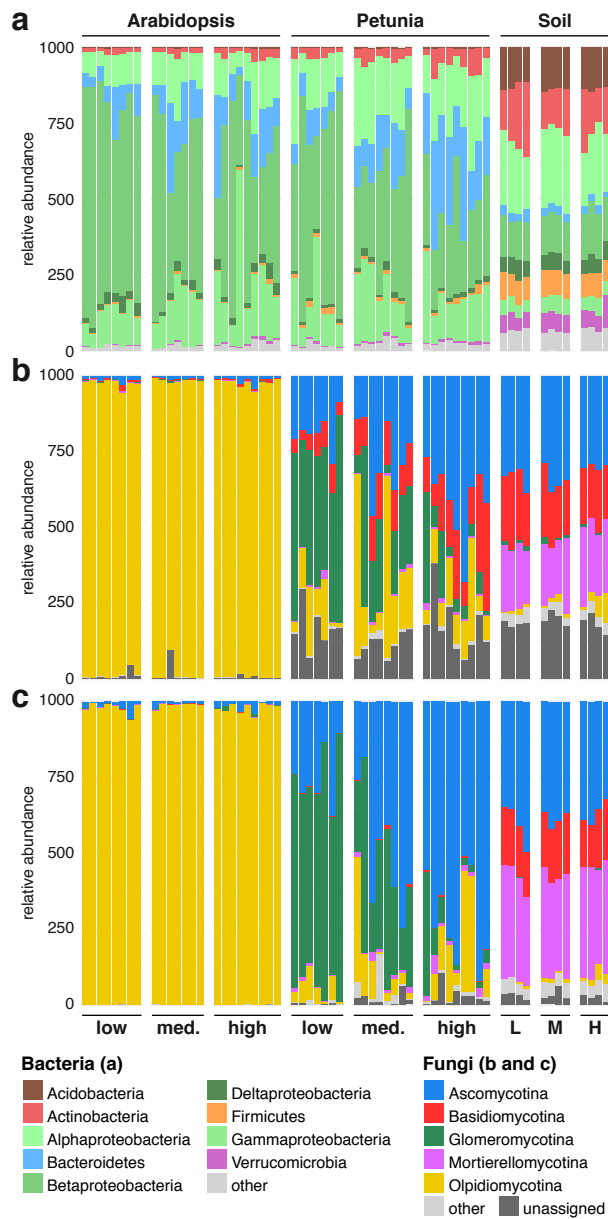
Figure 2 | Taxonomic profiles of microbial communities at phylum level
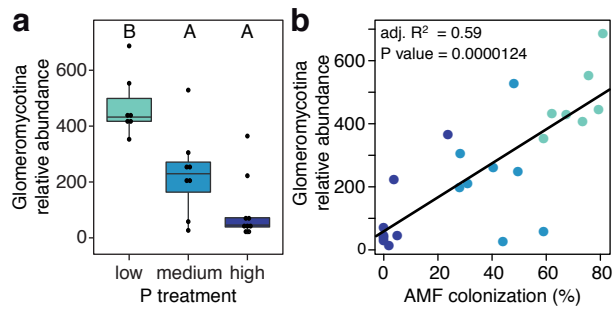(**a**) Bacteria profiles were obtained using MiSeq sequencing while fungal profiles were determined using (**b**) MiSeq and (**c**) SMRT sequencing. Phyla with relative abundances lower than 1% were summarized with 'other'. Levels of P-availability are indicated with low (L), medium (med.; M) or high (H).

2

Figure 3 | Abundance of AMF in Petunia roots

(**a**) Quantification of AMF based on the relative abundance of Glomeromycotina sequences in the microbiota profiles. Relative abundances were calculated from total sum normalized data. A quasibinomial generalized linear model was used to test for effects due to P availability. Different letters indicate significant pairwise differences between sample groups (P < 0.05, Tukey HSD). This data was correlated (**b**) with the levels of AMF root colonization as measured in the same samples by microscopy (data presented in **Fig. 1c**).

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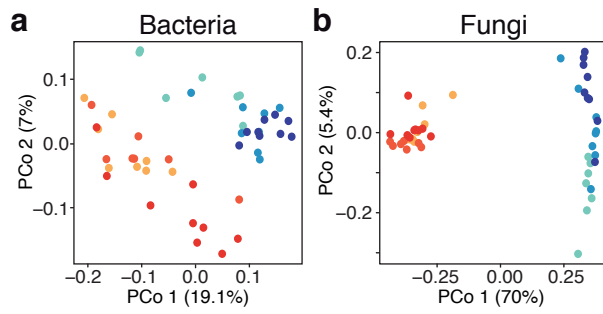
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**Figure 4 | Effects of plant species and P-levels on community composition**
Unconstrained ordinations with PCoA using Bray-Curtis dissimilarities were performed on the (**a**) bacterial and (**b**) fungal communities associated with roots. Samples were colored following the color scheme defined in **Fig. 1** (Arabidopsis and Petunia with reddish and blueish colors, respectively, and the increasing P availability (low, medium to high) are marked with increasing hue.
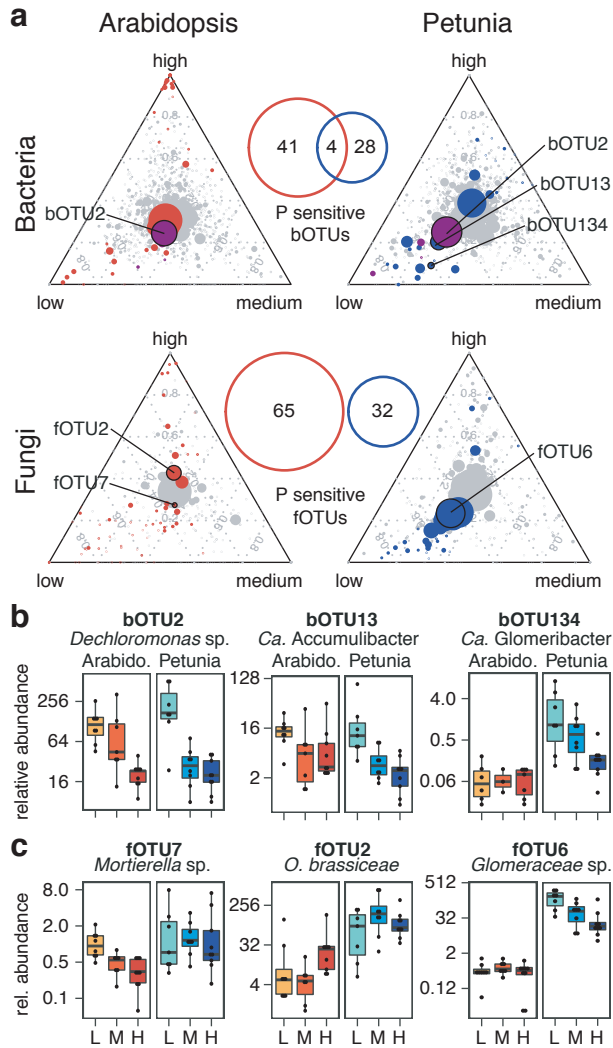
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**39 Figure 5 | Identifying phosphate sensitive microbes**
40 P sensitive OTUs (differentially abundant between low and high P conditions) were separately
41 identified for the bacteria and fungi, both in Arabidopsis and Petunia (edgeR analysis, high vs
42 low P, FDR < 0.05). (**a**) The ternary plots depict individual OTUs (in circles), sized by their
43 relative abundance, and the position of the OTUs in the triangle reflects their proportional
44 abundance in low, medium and high P samples. P sensitive OTUs are colored in red
45 (Arabidopsis), blue (Petunia) or purple (found in both species) and non-affected OTUs are
46 colored in gray. The number of P sensitive OTUs and their overlap between species is given
47 with the venn diagrams. Panels **b** and **c** illustrate the relative abundances (per milles) of a few
48 representative P sensitive bacterial and fungal OTUs in low (L), medium (M) and high (H) P
49 conditions (They are also indicated in the ternary plots).

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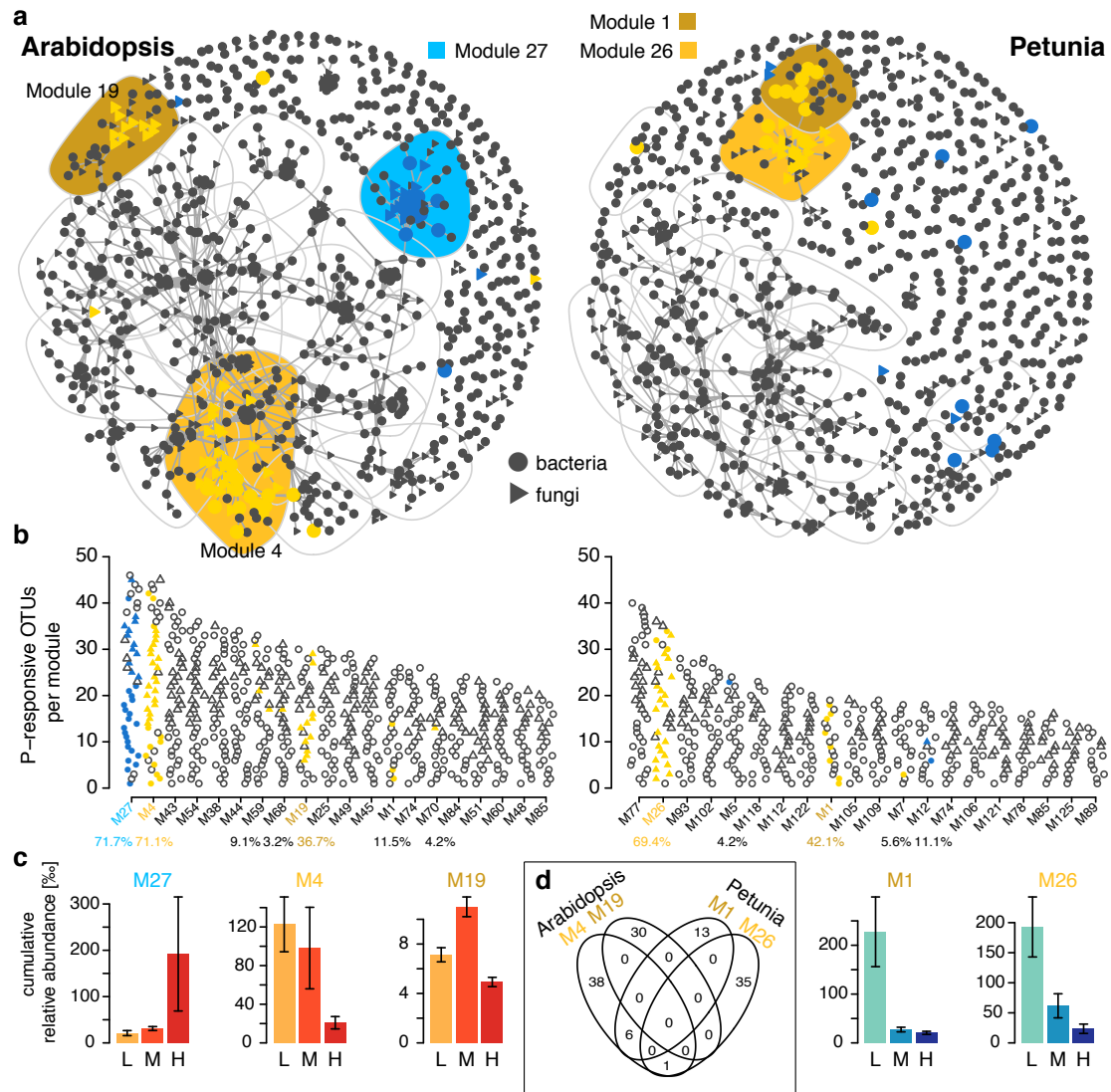
Figure 6 | Microbial co-occurrence patterns along the gradient of plant-available phosphate
(**a**) Co-occurrence networks visualize the significant positive pairwise correlations (ρ > 0.7, *P* < 0.001; indicated by links between OTUs) between bacteria (circles) and fungi (triangles) OTUs in Arabidopsis and Petunia root communities. P sensitive OTUs, which are abundant under low and high P conditions, are colored in yellow and blue, respectively. The twenty network modules comprising highest numbers of OTUs are rimmed with grey lines with the modules containing high proportions of P-responsive OTUs being shaded in yellow and blue. (**b**) Top twenty most populated modules, ranked by decreasing numbers of OTUs (bOTUs in circles; fOTUs as triangles) with low and high P sensitive OTUs being colored in yellow and blue, respectively. Percentages below the x-axis report the proportion of P sensitive OTUs present in each module. (**c**) Cumulative relative abundance (as permilles) of all bacteria and fungi OTUs in the P sensitive modules in low (L), medium (M) and high (H) P conditions. The cumulative relative abundance indicates the overall response of the microbes in the P sensitive modules. (**d**) Number and overlap of OTUs in the low P sensitive modules of Arabidopsis and Petunia are shown with the venn diagram.

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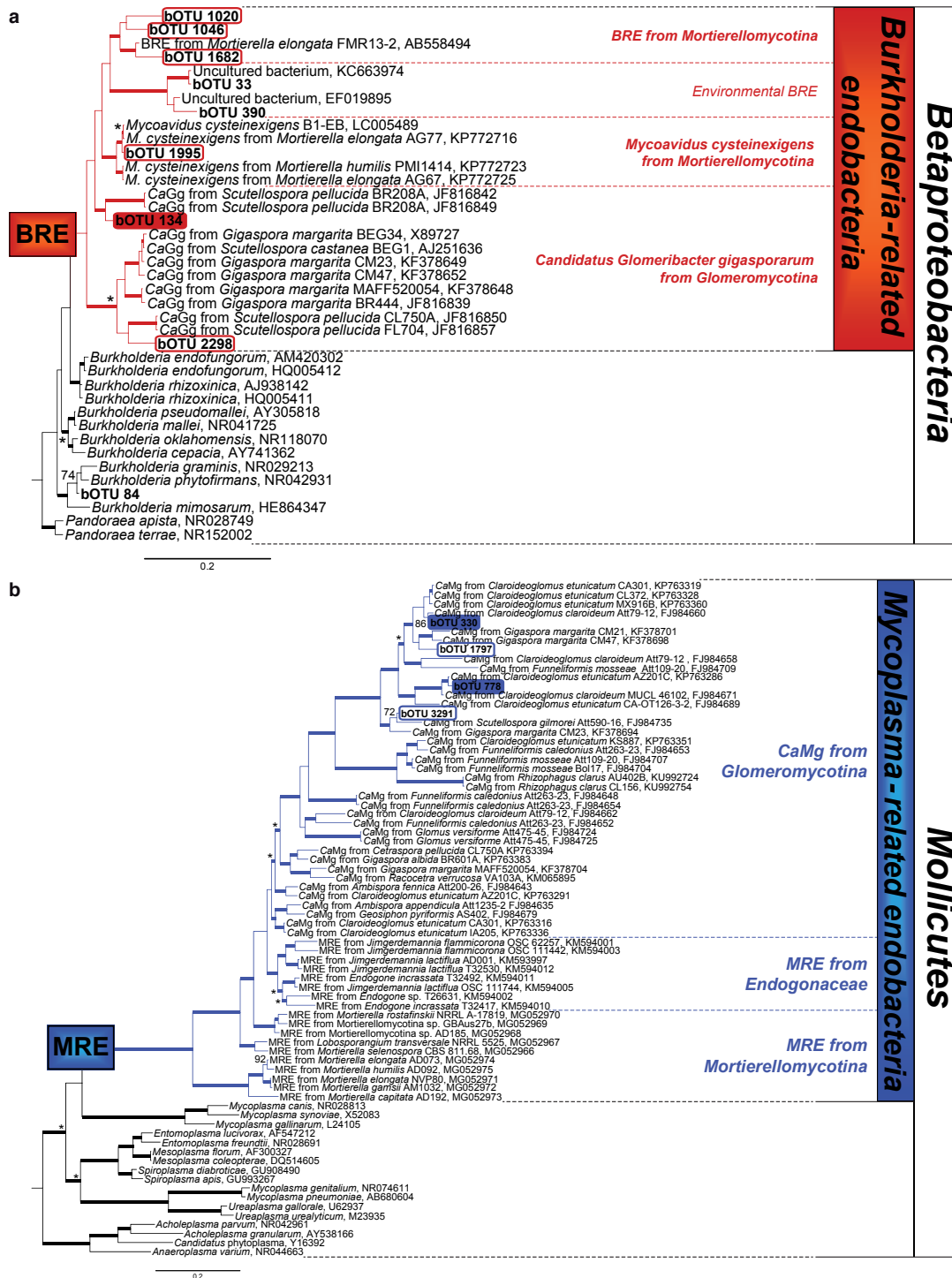
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Figure 7 | Phylogenetic placement of endobacteria OTUs from candidates identified by the clustering approach

Simplified trees summarizing the confirmed Burkholderia-related endobacteria (BRE, **a**) and Mollicutes-related endobacteria (MRE, **b**); the detailed tree is available as **Fig. S6**. **(a)** Four candidate BRE OTUs cluster within two clades encompassing BRE sequences from Mortierellomycotina fungi. In detail, bOTU 1995 is sister to the type strain of *Mycoavidus cysteinexigens*, whereas bOTUs 1020, 1046 and 1682 cluster with an undescribed BRE hosted in *Mortierella elongata*. Two bOTUs (134 and 2298) cluster within two clades encompassing *Candidatus* Glomeribacter gigasporarum (*Ca*Gg) sequences retrieved from *Scutellospora pellucida* (Glomeromycotina). Two bOTUs (33 and 390) cluster within a new BRE clade,

7

79   together with putative environmental BRE sequences. **(b)** Four candidate MRE OTUs cluster
80   within different clades encompassing *Candidatus* Moeniiplasma glomeromycotorum (*Ca*Mg)
81   sequences from Glomeromycotina fungi. In detail, bOTUs 330 and 778 cluster with *Ca*Mg
82   hosted in several strains of Claroideoglomus spp., whereas bOTUs 1797 and 3291 cluster with
83   *Ca*Mg hosted in several strains of *Gigaspora margarita* and *Scutellospora pellucida*. The trees
84   show the topology obtained with the Bayesian method. Branches with Bayesian posterior
85   probabilities (BPP) ≥0.95 and ML bootstrap support values ≥70 are thickened; asterisks (*)
86   indicate branches with BPP ≥0.95 but ML bootstrap support values <70; ML bootstrap support
87   values ≥70 are shown for branches having BPP <0.95. Sequences generated in this study are
88   in bold.