Soil Biology & Biochemistry 89 (2015) 206–209

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Short communication

Roots from distinct plant developmental stages are capable of rapidly selecting their own microbiome without the influence of environmental and soil edaphic factors

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ARTICLE INFO

Article history:
Received 19 March 2015
Received in revised form
10 July 2015
Accepted 14 July 2015
Available online 26 July 2015

Keywords:
Arabidopsis thaliana
Developmental stages
Microbiome
Phytohormone

ABSTRACT

Soil microbes live in close association with plants and are crucial for plant health and fitness. Recent literature revealed that specific microbes were cultured at distinct developmental stages of *Arabidopsis*. It is not clear how fast the roots, depending on their developmental stage, can alter the root-associated microbiome. In this study, *Arabidopsis*, grown under sterile conditions at precisely distinct developmental stages were supplied with a soil microbial slurry. Within four days, roots selected specific microorganisms depending on plant development, and Proteobacteria among other bacterial groups were found to colonize the roots irrespective of developmental stage. Moreover, exposure to a microbiome resulted in modulation of phytohormone levels at different stages of *Arabidopsis*.

Plants and soil beneficial microbes interact closely for disease suppression, health, and nutritional aspects (Chaparro et al., 2012; Lundberg et al., 2012). The rhizosphere microbial community is influenced by the physical and chemical properties of the soil (Gottel et al., 2011; Schreiter et al., 2014), as well as by the genetics of the host plant (Aira et al., 2010; Peiffer et al., 2013; Li et al., 2014). Recently, it has been shown that distinct developmental stages of *Arabidopsis* can culture specific microbial members of the rhizosphere microbiome. For instance, in the early stages (seedling and vegetative) *Arabidopsis* increases transcription of genes related to N cycling in *Nitrobacter*, *Rhodospirillum*, *Nitrosospira*, *Mesorhizobium*, and *Azorhizobium*, while in the later stages (bolting and flowering) the plant significantly induces transcription of genes belonging to plant growth promoting rhizobacteria (PGPRs) such as *Bacillus* and *Burkholderia* as well as *Cyanothece* spp. and *Bradyrhizobium* (Chaparro et al., 2013, 2014). Similarly, the rhizosphere microbiome identity of maize is altered by growth stage; *Massilia*, *Flavobacterium*, *Arenimonas*, and *Ohtaekwangia* were relatively abundant at early stages, while *Burkholderia*, *Ralstonia*, *Dyella*, *Chitinophaga*, *Sphingobium*, *Bradyrhizobium* and *Variovorax* populations were dominant at later stages (Li et al., 2014). Based on this information, we could only say that at the time points when those samples were taken the microbiome showed those characteristics; however, it is not clear how fast the microbial community is responding to root growth and root exudate changes associated with developmental stage, and if concomitant changes in the soil matrix are also important for the interaction. The aim of this study was to determine the root's direct contribution in configuring the microbiome irrespective of environmental and soil edaphic factors at different developmental stages.

The experimental system consisted of growing *Arabidopsis thaliana* (Col-0) plants to different developmental stages (seedling, vegetative, and bolting) in sterile liquid medium (without the addition of sucrose). Once the desired plant developmental stage was reached, the plants were transferred to new vessels and amended with a soil slurry (filtered and unfiltered) derived from a

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http://dx.doi.org/10.1016/j.soilbio.2015.07.009
0038-0717/

native *Arabidopsis* soil which has previously been characterized by Broeckling et al. (2008) and utilized in numerous experiments (Li et al., 2010; Badri et al., 2013; Chaparro et al., 2013, 2014). The soil slurries were also added to no plant controls (media only) to determine the potential role of the growing root on microbial growth. Plants (and no plant controls) were exposed to the soil slurry for a period of four days and roots/media were subsequently collected and colony-forming units (CFU) of the active culturable microbial community were measured using a gradient dilution (James, 1990). The treatments amended with non-filtered soil slurry showed bacterial growth as measured by CFU, while no CFUs were present in the no plant control (MS medium) or filtered soil slurry treatments. Samples from plants at bolting stage (roots and corresponding media) had significantly higher ($p < 0.05$, one-way ANOVA, Tukey HSD post-hoc) bacterial growth as measured by CFUs per three plants or per 1 mL media ($6.3 \pm 1.8 \times 10^5$ and $8.67 \pm 2.01 \times 10^6$) (data showed as means ± SD) compared to the seedling ($3.5 \pm 0.51 \times 10^5$ and $4.18 \pm 1.75 \times 10^6$) and the vegetative ($3.7 \pm 0.73 \times 10^5$ and $5.89 \pm 0.54 \times 10^6$) stages, as well as the slurry which was added to liquid MS without plant ($3.00 \pm 1.08 \times 10^3$). As expected, these results are indicative of the importance of plants to promote microbial growth as the treatment with media alone sustained significantly ($p < 0.05$, one-way ANOVA, Tukey HSD post-hoc) lower microbial growth when compared to the treatments where a plant was present.

We then examined the composition of the microbiome at each developmental stage of *Arabidopsis*. Total RNA from the plant roots that were exposed to native *Arabidopsis* soil slurry for a period of 4 days was isolated, subsequently converted to cDNA, amplified using the dual indexed bacterial 16S rRNA V4 universal primers, and subjected to Illumina MiSeq sequencing (Kozich et al., 2013) (see Supplementary Material). Raw sequences were analyzed using Mothur v1.32.0 (Schloss et al., 2009; Kozich et al., 2013). Sequences were normalized to 22,100 sequences per sample in order to ensure that differences in sequencing depth did not bias our analyses (Lozupone et al., 2011). We also quantified the copy number of 16S rRNA in each cDNA sample using qPCR of V1–V3 region of 16S rRNA (see Supplementary Material) and the results showed the same trend as with the CFU data; seedling stage showed the lowest ($p < 0.0005$, one-way ANOVA, Tukey HSD post-hoc) copy number of 16S rRNA (Fig. 1). Principal coordinates analysis (PCoA) of the microbial community revealed that the microbiome of the slurry alone was significantly different from those found in the roots at different developmental stages (AMOVA, Bonferonni adjustment, $p < 0.008$) (Fig. 2A) indicating that the root selected members of the

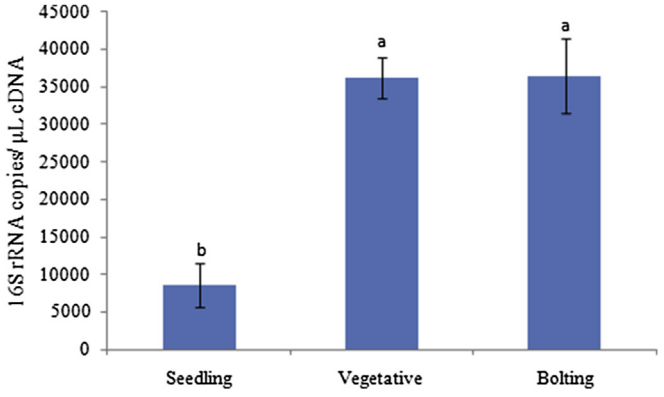

Fig. 1. Real-time PCR quantification of 16S rRNA gene. The template used in qPCR experiment was cDNA made from 200 ng RNA. The lower case letters indicate contrasts that are significantly different ($P < 0.05$) among treatments. Each sample was measured in triplicate. Bars in this figure represent mean ± standard deviations.

community in a short period of time. PCoA separated the communities associated with each developmental time point along PCoA 2 (Fig. 2A). Furthermore, we observed that the communities changed in a successive manner as the plants developed. Seedling and vegetative microbial communities were similar to each other, whereas the microbial community associated with bolting was distinct (Fig. 2A). This phenomenon was also observed when *Arabidopsis* was grown in soil (Chaparro et al., 2014). The microbial communities associated with the root were predominantly comprised of Proteobacteria (Fig. 2B), which are consistently reported to be associated with the rhizosphere microbial communities of *Arabidopsis* (Bulgarelli et al., 2012; Chaparro et al., 2014) and other plant species (Gottel et al., 2011; Peiffer et al., 2013; Turner et al., 2013; Chaparro et al., 2014), and appear to use plant cell-wall features to colonize the roots (Bulgarelli et al., 2012). Furthermore, the abundance of bacterial families within Proteobacteria belonging to *Burkholderiaceae*, *Oxalobacteraceae*, *Rhizobiaceae*, *Bacillaceae_1*, *Micrococcaceae*, and *Pseudomonadaceae* were significantly higher in the presence of a plant than in the initial soil slurry used as an inoculum (Supplemental Fig. S1). This indicates that the plant selectively attracts these bacterial families to colonize its roots. The increased abundance of these bacterial families in the soil have also been associated with soil disease suppression (Mendes et al., 2011). In contrast, *Sphingomonadaceae*, *Gemmatimonadaceae*, *Acidobacteria_Gp4_family_incertae_sedis*, and unclassified groups within Proteobacteria significantly decreased in abundance in the presence of the plant (Supplemental Fig. S1). It should be noted that some of the root-associated microbes identified in this study could be endophytes such as *Actinocorallia* sp. (Bulgarelli et al., 2012). However, due to our experimental set up the root associated microbes and the endophytes were extracted together. Interestingly, *Actinocorallia* sp. was not found in our study.

Root-associated microbial communities at each plant developmental stage were also distinct from one another. Specifically, at the phylum level, the microbial community associated with the seedling stage was significantly ($p < 0.05$, one-way ANOVA Tukey HSD post-hoc) more abundant in Firmicutes when compared to the vegetative and bolting stages (Fig. 2D), while the seedling stage was significantly less abundant ($p < 0.05$, one-way ANOVA Tukey HSD post-hoc) in Proteobacteria when compared to the vegetative and bolting stage (Fig. 2C). A closer look at the family level showed that *Bacillacea_1* was responsible for the increase in Firmicutes at the seedling stage (Supplemental Fig. S2F). Whereas, *Bradyrhizobiaceae*, *Comamonadaceae*, and *Rhizobiaceae* were responsible for the increase in abundance of Proteobacteria at late developmental stages (Supplemental Fig. S2A,C and E). Interestingly, *Burkholderiaceae* and *Erythrobacteraceae* did not follow the trend observed by Proteobacteria (Supplemental Fig. S2B and D).

Plant growth promoting rhizobacteria (PGPRs) are known to influence plant growth and defense by affecting hormonal and signaling processes. For instance, *Luteibacter rhizovicinus* MIMR1 (Guglielmetti et al., 2013) among other PGPRs (Hoffman et al., 2013) release indole-3-acetic acid (IAA) and its homologues into the rhizosphere promoting root and plant growth. Other PGPRs such as *Bacillus pumilus* SE34 and *Pseudomonas fluorescens* 89B61, increase plant defense by the induction of endogenous levels of salicylic acid (SA) or jasmonic acid (JA) (Yan et al., 2002). We tested plant phytohormone levels after plant exposure to soil slurries to determine whether bacteria colonizing the roots could provide any effects on the plant and some trends were observed (Fig. S3). For example, plants showed significantly increased ($p < 0.05$, two-way ANOVA, Tukey post-hoc) levels of SA and JA when exposed to the non-filtered soil slurry (Fig. S3D). Furthermore, when the plants aged, from vegetative to bolting, the levels of JA in the plant tissue significantly decreased ($p < 0.05$, two-way ANOVA, Tukey post-hoc)