Biology and Fertility of Soils (2021) 57:973–989
https://doi.org/10.1007/s00374-021-01590-0

ORIGINAL PAPER

Spatial analysis of the root system coupled to microbial community inoculation shed light on rhizosphere bacterial community assembly

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Received: 20 April 2021 / Revised: 23 July 2021 / Accepted: 31 July 2021 / Published online: 14 August 2021

Abstract

Although studied for more than a century, the spatial distribution of microorganisms in a root system still remains partly understood. In a repeated greenhouse experiment using the model plant *Brachypodium distachyon*, we investigated the composition and distribution of rhizosphere bacteria and their response to inoculation with artificially selected microbial communities, using two different sampling scales: root sections from distinct individual roots (apical, middle, and rear sections) and the remaining entire system recovered after homogenization. Using 16S rRNA gene sequencing, we identified that root section identity was the most influential factor on the microbiota composition ($R^2 = 44.4\%$), followed by batch ($R^2 = 34.4\%$), and plant identity ($R^2 = 15.2\%$). Apical sections were characterized by increased abundances for Firmicutes members, while the rear sections featured more Verrucomicrobia. Root section sampling showed better sensitivity at detecting significant effects of the inoculation on the microbiota composition (e.g., local influence of inoculation on rear sections), in contrast, the homogenized sampling showed improved reproducibility (e.g., smaller sample dispersion). The comparison of the two sampling strategies highlighted a clear tradeoff between reproducibility and sensitivity, encouraging to complement traditional approaches with fine-scale sampling to improve our capacity to understand biological effects that could be otherwise missed.

Keywords Microbial inoculation · Root axis · Rhizosphere microbiota · Root system · Sampling scale

Introduction

Improving plant productivity to meet the needs of the ever-growing human population is a crucial challenge. A sustainable way to achieve this goal is through manipulation of the microbial community surrounding plant roots (Day et al. 2011; Mueller and Sachs 2015), a zone termed "rhizosphere" (Hartmann et al. 2008). Indeed, the rhizosphere is hosting a wide diversity of microbes, including beneficial species supporting plant growth and health (Raaijmakers et al. 2009), and is regarded as an active hotspot of biochemical reactions providing essential nutrients for plants (Kuzyakov and Blagodatskaya 2015). Microbial inoculation to improve plant traits is a direct application of this knowledge, ranging from well-established methods using either single and multispecies (Magallon-Servin et al. 2020; de-Bashan et al. 2020) up to the direct manipulation and passing of entire rhizosphere microbial communities via artificial selection protocols (Swenson et al. 2000; Panke-Buisse et al. 2015). Nevertheless, in order to facilitate the successful utilization of rhizosphere microbes to improve crop yields, it is important to understand the hierarchy of factors influencing the spatial distribution of microbes inside the root system.

So far, the most popular way to investigate rhizosphere microbiota is by collecting and homogenizing a sample of the entire rhizosphere (Barillot et al. 2013). From this homogenized sampling, fundamental knowledge was gained on the rhizosphere microbiota. For instance, this approach allowed to identify the paramount importance

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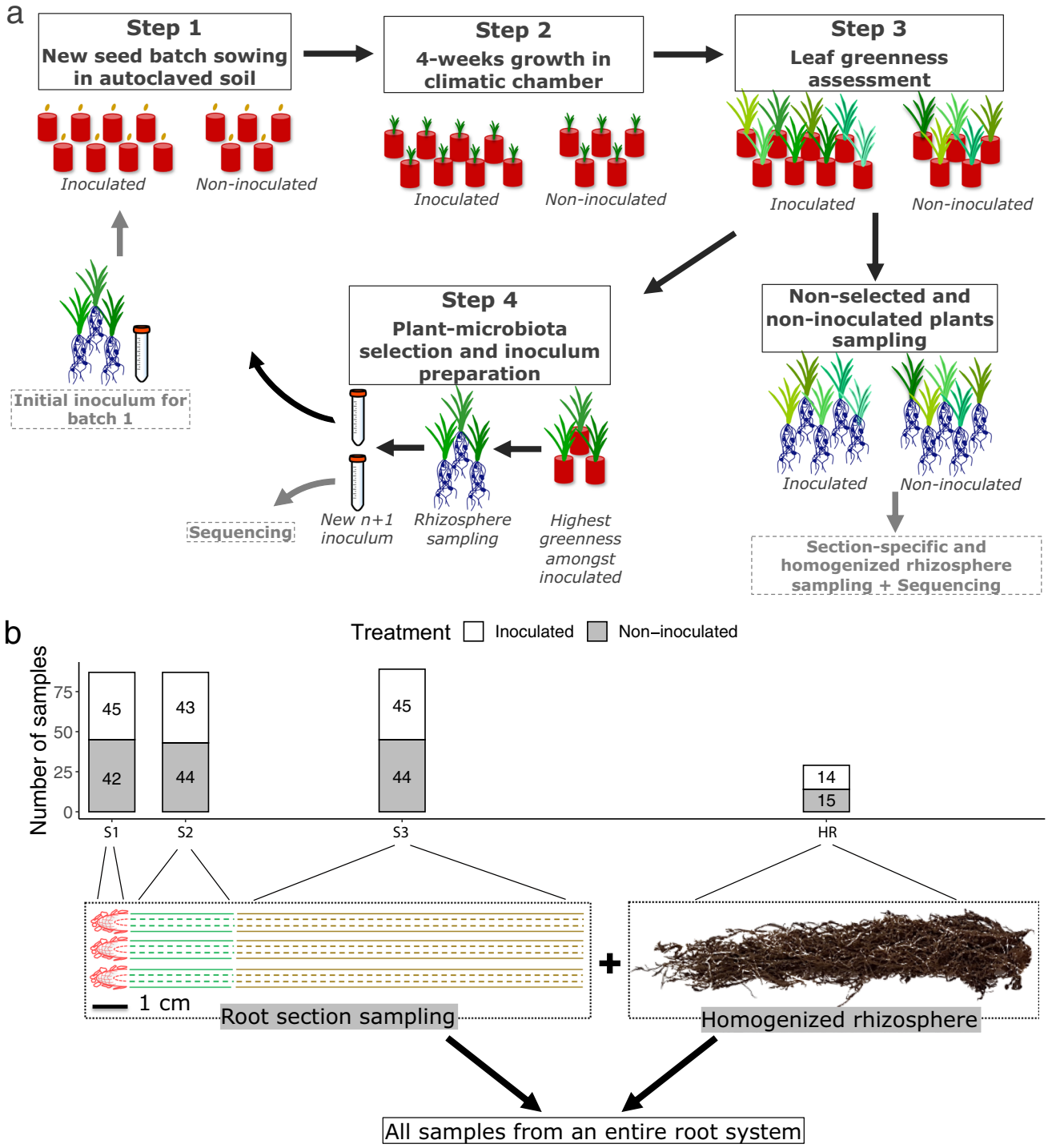

of soil properties in rhizosphere microbiota assembly (Fitzpatrick et al. 2018), as well as the characterization of "taxonomic core microbiota" of several plant species, being the commonly shared fraction of microorganisms between individual plants of the same species, although they may grow under different abiotic conditions (e.g., *Arabidopsis thaliana*, Lundberg et al. (2012); lettuce, Chowdhury et al. (2019); barley, Jacquiod et al. (2020)). While this homogenized sampling proves useful on many occasions, it neglects smaller scale aspects within the rhizosphere where microbial responses might occur. Such studies, performed at different spatial scales, are needed and valuable to investigate the rhizosphere functions (Vetterlein et al. 2020).

At the scale of the root system, a high chemical and biochemical heterogeneity exists vertically along the root axis (e.g., variations in pH, water content, redox potential, and

◂**Fig. 1** Flowchart showing the study design (**a**) and sampling process (**b**). **a** Step 1–2: Thirteen *Brachypodium distachyon* plants were grown in individual pots for four weeks (five non-inoculated, five inoculated with a pooled microbial community from three *Brachypodium distachyon* rhizospheres, and additionally three inoculated plants used for inoculation of next generation). Step 3: Shoot colors of the inoculated plants were assessed by the routine pipeline of the INRAE 4PMI phenotyping platform (Akmouche et al. 2019) (https://www6.dijon.inrae.fr/plateforme4pmi_eng/Technical-description/High-Throughput-phenotyping). Step 4: The three plants from the inoculated treatment having the highest leaf greenness were selected, their rhizosphere microbiota were collected, pooled, and inoculated to the next batch (step 1). The whole process was iteratively repeated three times. During the artificial selection and inoculation process, the five inoculated plants with the lowest greenness and five non-inoculated plants were used for sampling. **b** The rhizosphere microbiota was investigated with both root section sampling and homogenized rhizosphere sampling. For section sampling of each plant, three roots were selected and cut into three sections, namely, S1, S2, and S3 referring to apical, middle, and rear parts, respectively. The homogenized rhizosphere (named HR) sampling refers to the entire remaining root system (not including the three selected roots). The bar-plot in the upper panel shows the sample numbers in each sample type/treatment

enzyme activity), which could either be the reason and/or the consequence for the concomitant heterogeneous distribution of the rhizosphere microbiota (Tarafdar and Jungk 1987; Hinsinger et al. 2009; Carminati 2013; Razavi et al. 2016; Kreuzeder et al. 2018). For instance, carbon availability, an important driving factor for microorganisms (Demoling et al. 2007), is strongly dependent on the vertical location and timing in the rhizosphere. Indeed, carbonated molecules released by roots (collectively named "rhizodeposits"), are more abundant at the root tips, then progressively decrease in the upper elongation and maturation zones (Iijima et al. 2000; Dennis et al. 2010; Doan et al. 2017). Therefore, studying the vertical distribution of microbiota along physiologically well-defined root sections may be adequate to identify functionally distinct microbiota, representing a relevant proxy of the quantity/quality fluctuation of root rhizodeposits, a difficult parameter to acquire and investigate (Philippot et al. 2013; Pausch and Kuzyakov 2018). Studies have already addressed the spatial problem within the rhizosphere of several plant species focusing on parameters such as root system architecture (Saleem et al. 2018), root morphology (Saleem et al. 2016), and root branching (Pervaiz et al. 2020). However, the significance of these fine-scale sampling methods compared to the traditional homogenized sampling strategy was never investigated.

Among acknowledged methods available to manipulate the plant–microbe association (Magallon-Servin et al. 2020; de-Bashan et al. 2020), inoculation of complex microbial consortia is currently receiving a lot of attention with the advent of agroecology and redefinition of agricultural practices (Panke-Buisse et al. 2015; Mueller and Sachs 2015). Indeed, unlike the traditional application of a single or multiple strains (e.g., plant growth-promoting bacteria), this method relies on the selection and inoculation of entire microbial communities to manipulate plant phenotypes (Swenson et al. 2000; Panke-Buisse et al. 2015). In these previously reported experiments, microbial communities associated with plant phenotype changes are selected and inoculated to a new generation/batch of plants. This artificial selection of host-associated microbiota (Mueller and Sachs 2015) potentially promises more robust and stable results compared to the single-strain approach (e.g., inconsistent performance and survival during field applications (Arora et al. 2010)). However, important knowledge on the establishment, survival and functioning of inoculum within the rhizosphere microbiota context are still lacking, hampering the success of field trials, and subsequent applications (Mahmood et al. 2016).

The objective of the present work was to assess the spatial variability of the bacterial microbiota in the rhizosphere of *Brachypodium distachyon*, a model plant for cereals (see Material and methods). We aimed to assess the relative contribution of structuring factors such as the root section identity, plant identity, the root axis identity, and batch repeatability on rhizosphere bacterial community composition of plants developing either from ambient conditions, or with an initial inoculation with a complex microbial community. We refined our analysis on bacteria due to their strong reliance on plant rhizodeposits and water availability unlike fungi (Barnard et al. 2013; Merino-Martín et al. 2020). For the inoculation procedure, we followed the typical artificial selection protocol established for rhizosphere microbiota selection (Swenson et al. 2000; Lau and Lennon 2012; Panke-Buisse et al. 2015), by selecting and mixing the microbiota from three plants at the end of one growth batch to generate the community that will be used to inoculate the next batch (Fig. 1a). To follow the development of the rhizosphere microbiota with and without inoculation, we used a complementing sampling strategy relying on (i) root section sampling, targeting specific and well-defined vertical root areas featuring well known functions (the apical root S1, middle root S2, rear root S3, Fig. 1b) together with (ii) the traditional homogenized rhizosphere (HR) sampling as a control using the remaining rhizosphere material from that same plant. We deliberately choose to apply a *census* on the entire root axis according to physiologically distinct root sections with increasing length instead of random samples of the same size to avoid subsampling biases that could have led to the missing of potential important ecological niches along the root axis. Four hypotheses were experimentally tested in this study: H1, the "variability hypothesis": bacterial community variability has an inverse relationship with the sampling scale; H2, the "intra-/inter- plant root variability hypothesis": intra-plant variability is higher than inter-plant variability on the rhizosphere bacterial community;

H3, the "developmental variability hypothesis": root sections along the axis differ in their rhizosphere bacterial community composition; H4, the "local influence hypothesis": communities inoculated to seedlings have a stronger effect on apical root sections, due to the higher amount of rhizodeposits.

Materials and methods

Soil and plant growth conditions

A moorland sandy soil classified as cambisol was used (organic C: 14.7 g kg^{-1}; total N: 1.19 g kg^{-1}; pH: 5.22; clay: 6.9%; loam: 19.0%; sand: 74.1%; origin: CEREEP, Saint-Pierre-Lès-Nemours, France, sampling: February 2016). Soil was transferred to the greenhouse facility (INRAE Plant Phenotyping Platform for Plant and Microorganism Interaction (4PMI) platform, Agroécologie, INRAE Center Dijon, France), air-dried (room temperature, one week), sieved (2 mm), and stored in a sealed box. To reduce the density and heterogeneity of the endemic microbial community that might have fluctuated during storage and facilitate the settlement of the microbial inoculum, the soil was autoclaved before each plant growth batch (115 °C, 45 min, one dry cycle). In addition, the soil was allowed to rest for 72 h before the seedling transplantation and inoculation to avoid microbial community shift linked to necromass consumption. Our autoclaving procedure was not intended to sterilize the soil. 350 g of dried autoclaved soil were transferred in small pots and watered by sub-irrigation in individual cups with demineralized water (80% of the water holding capacity, WHC).

We choose the plant *Brachypodium distachyon* Bd21 (wild type), a well-known model for cereals (Watt et al. 2009; Girin et al. 2014), with a small size genome fully sequenced and a well-documented morphology (Chochois et al. 2015; Agapit et al. 2020) and physiology (David et al. 2019). Its autogamy makes it an interesting choice to get homogeneous seeds for growth reproducibility, since we used the same seed lot for the three batches. *Brachypodium distachyon* seeds came from a stable genetic breed grown in the *Brachypodium* resource center (Institut Jean Pierre-Bourgin, INRAE-Versailles, France). Non sterilized seeds were placed into parafilm-sealed transparent boxes with humidified blotting paper and exposed to vernalization (4 °C in the dark, 24 h). Afterwards, boxes were placed in a germinator (18 °C in the dark 48 h, then 20 °C at full light 96 h, Fitoclima 600 PL/PLH, Aralab). Seedlings were transplanted into humidified pots and placed immediately into a climatic chamber (22 °C, 12 h light, intensity: 200 µmol.s^{-1}.m^{-2}, 70% air humidity, Walk-in EH, Aralab). Subirrigation in individual cups was applied twice a week with the same amount of water for all the pots, and readjusted once a week to 80% WHC by individual weighting. No fertilizer was added to avoid disturbing naturally occurring plant-microbes interactions. Pots were manually randomized once a week.

Study design

To investigate the distribution of rhizosphere microbiota along root axis, we used five *Brachypodium distachyon* grown under nonsterile climatic chamber conditions to account for environmental/ambient colonization processes of the rhizosphere. These five plants are named "non-inoculated" and served as our control group to test the effect of the artificial microbiota selection and iterative inoculation. In that regard, it was important to work under nonsterile conditions in order to accurately estimate this ambient colonization effect coming from our experimental conditions (e.g., seeds, autoclaving procedure, soil, air, and irrigation water).

The artificial selection of rhizosphere microbiota and inoculation process was adapted from previous studies (Swenson et al. 2000; Lau and Lennon 2012; Panke-Buisse et al. 2015). Briefly, these studies make the hypothesis that plant phenotype can be modified by selecting the plant-associated microbiota based on the value of a desired plant trait (e.g., biomass, flowering time), inoculating it to another batch of plants coming from the same seed collection (to avoid plant genotype evolution), and repeating this procedure iteratively for several generations. The three best plants are selected, and their rhizosphere microbiota are extracted and pooled to guarantee enough diversity for the next generation in order to have a sufficient variation level for selection to operate (Raynaud et al. 2019). Here, eight *Brachypodium distachyon* plants were grown in a generation, of which three plants were selected (based on leaf greenness) and their rhizosphere microbiota were pooled and used as the inoculant for the next generation. The remaining five inoculated plants were sampled and named "inoculated group" (Fig. 1a). These five "inoculated" plants enable us to measure the effect of community inoculation against the five "non-inoculated" control group that was colonized by our ambient conditions. The eight inoculated plants from the first generation received an inoculum of three pooled rhizospheres from pre-grown *Brachypodium distachyon* under the same conditions. The effect of our artificial selection on the leaf greenness is shown in Fig. S1.

Growth time was four weeks (12–15 leaves on average at harvest time) for both non-inoculated and inoculated plants and the process was repeated iteratively three times using new seeds from the same collection to limit plant genotypic variability (Fig. 1a). Leaf greenness was used as a measurement of plant host performance and estimated with the routine pipeline of the INRAE 4PMI phenotyping platform (Akmouche et al. (2019), https://www6.dijon.inrae.fr/plate

forme4pmi_eng/Technical-description/High-Throughput-phenotyping).

Root section sampling strategy

To collect rhizosphere samples (Fig. 1b), the root system was meticulously recovered from pots and loosely attached soil was gently shaken off. The remaining soil, tightly attached to the roots, was considered as the rhizosphere. For each system, three approximately 10 cm-length roots with a similar morphology were selected and lateral roots were removed to minimize variations coming from the plant organ and developmental stage. Besides, the frequency and intensity of mycorrhization was verified at the beginning and the end of the experiment, based on a root staining method described in (Vierheilig and Wyss 1998; Jacquiod et al. 2021). We did not detect any traces of arbuscular mycorrhization in our *Brachypodium* roots. Following existing methodology (Buendia et al. 2019), each root was cut into three fragments and named "S1" (Section 1: The apical root, first 1 cm, including root tip and young root tissues), "S2" (Section 2: The middle root, the next 3 cm above S1, a transition zone that was still young and started to mature) and "S3" (Section 3: The rear root, the remaining root axis of approximately 10 cm above S2, containing old and matured tissues). The remaining root system (not including the three selected roots) of each plant, named as "HR" (homogenized rhizosphere), was used normally to get the traditional homogeneous rhizosphere sample by thorough washing of the tight root-adhering soil (Barillot et al. 2013). The knife and tweezers were systematically sterilized before collection of root sections, roots, and plants. In total, we collected 10 different samples from each plant (3 × S1, 3 × S2, 3 × S3, and 1 × HR). The rhizosphere soil was collected by dipping samples (1 ml sterile 0.9% NaCl for S1, S2, and S3; 5 ml for HR) and vortexing horizontally (6000 rpm, 20 min for S1, S2, and S3 sections; 2 min for HR). We deliberately reduced the vortex time for HR in order to limit the release of contaminating plant plastid DNA that is freed due to the abrasiveness of our sandy soil.

Microbial community inoculation procedure

The rhizosphere microbiota from three *Brachypodium distachyon* plants of the previous batch were pooled, suspended in 200 ml demineralized water, and magnet-stirred (500 rpm, 30 min). Fifty milliliters of the so-obtained slurry was collected for DNA extraction, 50 ml was used for glycerol stock preservation, and the remaining 100 ml was used as the complex microbial inoculant to inoculate the next seedling batch. The inoculation process was done by transplanting the seedlings and loading 2 ml of the inoculant slurry to the topsoil of transplanted seedlings before going to the climatic chamber. The non-inoculated control seedlings were directly transplanted into the soil with 2 ml of irrigation water to mimic the inoculation steps.

DNA extraction, sequencing, and bioinformatic analysis

The 1 ml of washed rhizosphere microbiota from S1, S2, S3, and HR were centrifuged (16,000 g, 10 min), supernatant removed, and pellets suspended in PCR grade water using FastPrep-24 (4.0 m/s, 60 s). The microbial DNA was extracted using the DNeasy PowerSoil HTP 96 Kit (QIAGEN), and stored at − 20 °C. Here we focus on bacteria due to their strong reliance on plant rhizodeposits and water availability, unlike fungi that can retrieve resources away in soil via their hyphae (Barnard et al. 2013; Merino-Martín et al. 2020). The 16S rRNA gene fragment targeting the hypervariable V3-V4 region of the small unit in the prokaryotic ribosomal operon was amplified in two steps: First, amplification with the modified universal primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′- GGA CTACHVGGGTWTCTAAT-3′) (Dams et al. 1988; Takai and Horikoshi 2000; Yu et al. 2005); Second, adaptors and sequencing primers were added to the first amplicon products. Amplified products were purified with Agencourt AMPure XP Beads (Beckman Coulter Genomics, MA, USA), normalized with SequalPrep™ Normalization Plate (96) Kit (Invitrogen). The pooled library concentration was determined using the Quant-iT™ High-Sensitivity DNA Assay Kit (Life Technologies). Paired-end sequencing was performed with the Illumina MiSeq System (Illumina Inc., CA, USA) with 5.0% PhiX as the internal control. All reagents were from the MiSeq Reagent Kits v2 (Illumina Inc., CA, USA). Adaptors and sequencing primers of raw FASTQ files were removed using "cutadapt" (version: 2.10) (Martin 2011). Trimmed reads were analyzed with an adapted DADA2 pipeline on QIIME2 (version: qiime2-2018.2) where the default overlap length (for merging paired reads) of forward and reverse reads was decreased to 6 nucleotides (Callahan et al. 2016; Bolyen et al. 2019). Eight nucleotides were removed at the 5′ end of both forward and reverse reads to keep a good sequencing quality at the denoising step. Other parameters were set as default. With the DADA2 algorithm, taxonomic assignments were resolved with exact sequence features, called amplicon sequence variants (ASVs). Taxonomy was assigned using the Silva database (release 132) at 99% identity (Quast et al. 2012). As archaea represented a minor proportion in our dataset (0.27%), we refer to "bacteria" in the text for simplicity reasons. Chloroplast and mitochondria ASVs were discarded before any

analyzes (1.3 million reads, accounting for 15.3% of total reads).

Statistical analysis

Samples with sequencing depth below 2000 reads were discarded ($n = 8$, Fig. S2) (Caporaso et al. 2011), leaving balanced numbers of replicates in the non-inoculated and inoculated treatments (Fig. 1b). To alleviate influence coming from varying root section sizes or sample weights, less representative and statistically irrelevant ASVs were not considered (presence in less than 10 samples out of 292). Samples were rarefied to the same sequencing depth before alpha diversity analysis (observed richness, Shannon, and inverse Simpson index (1/D)).

The alpha diversity of microbiota was calculated with R-package "phyloseq" (McMurdie and Holmes 2013). Indices in root sections were compared with linear regression ("lm", R-package "stats", covariate = "batch"). When needed, indices were standardized as "z-scores" relative to HR samples in each batch: z-score = $(\text{Value}_{[S1/S2/S3]} - \text{Mean}_{[HR]})/\text{SD}_{[HR]}$. The standardized alpha diversity indices between root sections were compared with two-sided two-sample t-test. Beta-diversity was calculated with function "diversity beta-phylogenetic" in QIIME2 (Bolyen et al. 2019) and compared with permutational multivariate analysis of variance (PERMANOVA, "adonis", R-package "vegan") (Anderson 2001; Lozupone and Knight 2005; Oksanen et al. 2007). The dispersion of samples was tested with function "betadisper" (R-package "vegan"). The z-score batch-standardized beta diversity was obtained as described for the alpha diversity. Comparisons of ASVs (relative abundances larger than 0.01%) abundances were performed with the R-package "limma" and "DAtest" (paired by "plant identity", covariate = "batch") (Ritchie et al. 2015; Russel et al. 2018). At higher taxonomic levels, comparison of taxa abundance was performed with one-way ANCOVA ("aov", R-package "stats", covariate = "batch"). When multiple testing occurred, we applied the Benjamini–Hochberg *P*-values correction to account for the false discovery rate (FDR) ("p.adjust" in R-package "stats") (Benjamini and Hochberg 1995). The comparison of median and mean UniFrac distance between sample types was assessed with Wilcoxon rank-sum test and t-test, respectively. The homogeneity of variance of UniFrac distance across sample types was tested using Bartlett's test (R-package "stats"). The one-sided permutation test was used to compare the reduction of variance attributed to batch, by randomly shuffling the treatment labels of samples and repeating 1000 iterations to obtain an empirical *P* value. Two-sided permutation test was used to compare the alpha diversity between the inoculant and other sample types in the inoculated plants (1000 iterations). Most plots were generated with R-package "ggplot2" (Wickham 2011).

To have a general knowledge of *Brachypodium distachyon* rhizosphere microbiota, hypotheses 1, 2, and 3 were tested only with non-inoculated control plants. Hypothesis 4 was tested with both non-inoculated and inoculated plants.

Results

Analysis of variance on rhizosphere microbiota

Several PERMANOVA models were tested to investigate variance partition in rhizosphere microbiota based on the weighted UniFrac distance (Table 1). Models in "Comparison 1" allowed ranking the significance and importance of tested factors (batch, plant identity, root identity, and section identity) for non-inoculated control plants based on the mean sum of squares (MeanSqs), being first the root section identity ($R^2 = 44.4\%$, $P < 0.001$), followed by the batch ($R^2 = 34.4\%$, $P < 0.001$), plant identity ($R^2 = 15.2\%$, $P < 0.001$), and root identity ($R^2 = 3.1\%$, $P = 0.996$).

We then used the root section sampling to identify differences between the non-inoculated and inoculated treatments (Table 1, Comparison 1). The inoculation procedure significantly reduced 8.7% of the variance explained by "batch" (from 34.4% [non-inoculated] to 25.7% [inoculated], $P = 0.046$, one-sided permutation test, 1000 iterations). But the reduction of variance explained by "batch" due to inoculation procedure was not captured with the homogenized sampling (from 72.5% [non-inoculated] to 62.2% [inoculated], $P = 0.156$, one-sided permutation test, 1000 iterations).

Finally, we compared the root section and HR to evaluate their capacity to estimate the variance partition for the batch and the treatment factors when using both inoculated and non-inoculated plants (Table 1, Comparison 2). The batch and treatment interaction was significant when using section sampling (factor: Batch:Treatment, $R^2 = 19.3\%$, $P < 0.001$), but not with the homogenized sampling ($R^2 = 17.2\%$, $P = 0.173$).

Similarly, when including the dismissed rare ASVs into the analysis (presence in less than 10 samples), consistent results were observed (Table S1). (i) The root section explained the largest proportion of variance ($R^2 = 42.2\%$). (ii) Inoculation procedure significantly reduced 9.6% of the variance explained by "batch" (from 36.3% [non-inoculated] to 26.7% [inoculated], $P = 0.021$, one-sided permutation test, 1000 iterations). Besides, exclusion of plastid sequences (ASVs from the chloroplast and mitochondria) did not influence our analysis. (i) The percentage of plastid sequences in samples was not correlated (Spearman's rho) with the alpha diversity (rho = −0.1, $P = 0.249$, richness; rho = −0.14,

Table 1 Variance partition and multiple PERMANOVA testing (weighted UniFrac, 10,000 permutations)

Comparison 1: Structuring factors and inoculation effect						
Factors	Noninoculated			Inoculated		
	Root section			Root section		
	MeanSqs	R^2 (%)	Significance, *P* value	MeanSqs	R^2 (%)	Significance, *P* value
Batch	0.449	34.4	***, < 0.001	0.246	25.7	***, < 0.001
Plant identity	0.199	15.2	***, < 0.001	0.203	21.2	***, < 0.001
Root identity	0.04	3.1	ns, 0.996	0.038	4.0	ns, 0.907
Section identity	0.579	44.4	***, < 0.001	0.438	45.7	***, < 0.001
Residuals	0.038	2.9		0.034	3.5	
Comparison 2: Sampling level						
Factors	Root section			Homogenized rhizosphere		
	MeanSqs	R^2 (%)	Significance, *P* value	MeanSqs	R^2 (%)	Significance, *P* value
Batch	0.446	34.8	***, < 0.001	0.064	36.8	***, < 0.001
Treatment	0.529	41.3	***, < 0.001	0.057	32.8	*, < 0.011
Batch:Treatment	0.247	19.3	***, < 0.001	0.03	17.2	ns, 0.173
Residuals	0.059	4.6		0.023	13.2	

MeanSqs (mean sum of squares) is the sum of squares for a factor divided by its degree of freedom. R^2 is expressed as the proportion of MeanSqs obtained from PERMANOVA (ns: $P > 0.05$; *: $0.01 < P < 0.05$; **: $0.001 < P < 0.01$; ***: $P < 0.001$)

$P = 0.090$, Shannon index; rho = −0.13, $P = 0.128$, inverse Simpson index) of samples without plastid sequences (see Fig. 2a in the following). (ii) Variance partitions were highly consistent regardless of including plastid sequences (Table S2) or not (Table 1).

Microbial community variations along the root axis

Along the root axis of non-inoculated plants, we observed high alpha diversity in S3 (Fig. 2a). In detail, the bacterial richness in S1 was significantly lower than S2 and S3 (mean ± SD, 225.6 ± 82.0 [S1] *vs.* 284.0 ± 70.0 [S2], $P < 0.001$; 225.6 ± 82.0 [S1] *vs.* 281.2 ± 88.7 [S3], $P = 0.003$). S2 and S3 were similar in bacterial richness ($P = 0.865$). Shannon diversity showed the same trend, as S1 had the lowest diversity (4.2 ± 0.7 [S1] *vs.* 4.6 ± 0.5 [S2], $P = 0.006$; 4.2 ± 0.7 [S1] *vs.* 4.7 ± 0.5 [S3], $P < 0.001$), while S2 and S3 did not differ ($P = 0.402$). For inverse Simpson diversity, the trend between root sections was the same, except that S1 were no longer different from S2 (42.0 ± 30.3 [S1] *vs.* 51.0 ± 28.2 [S2], $P = 0.112$), but still significantly lower than S3 (42.0 ± 30.3 [S1] *vs.* 57.8 ± 32.0 [S3], $P = 0.011$). For all tested indices, S1 were significantly lower than HR samples (observed richness, 225.6 ± 82.0 [S1] *vs.* 292.7 ± 40.5 [HR], $P = 0.005$; Shannon diversity, 4.2 ± 0.7 [S1] *vs.* 4.9 ± 0.2 [HR], $P = 0.003$; inverse Simpson diversity, 42.0 ± 30.3 [S1] *vs.* 64.0 ± 20.7 [HR], $P = 0.011$). S2 and S3 were not different from HR in any of these alpha indices ($P > 0.05$ in all comparisons). The rhizosphere microbiota composition in root sections was assessed by weighted and unweighted UniFrac distance with principal coordinates analysis (PCoA) (Fig. 2b, Fig. S3). Although partially overlapped, sections differed significantly from each other in their bacterial community composition (Table S3, $P < 0.001$, in all comparisons). HR samples were significantly less dispersed than all root section samples (weighted UniFrac distance, Fig. 2b, Fig. S4). In contrast, S1 was the most dispersed (unweighted UniFrac distance, Fig. S3, Fig. S4).

Taxonomic differences among root sections and HR samples from non-inoculated plants are shown in Fig. 3a. Overall, Proteobacteria dominated (41.5%, visualized at class level), followed by Firmicutes (13.9%), Chloroflexi (13.1%), Patescibacteria (7.7%) and Bacteroidetes (6.5%), altogether accounting for 82.7% of sequences. Firmicutes were more abundant in S1 (23.6%) than S2 (10.2%) or S3 (5.4%). In contrast, Patescibacteria and Verrucomicrobia were more abundant in S3 (13.1% and 6.7%) than S1 (3.5% and 2.3%) and S2 (6.3% and 3.0%). Next, we identified taxa that differed quantitatively among the three sections. In total, 9 phyla, 14 classes, 24 orders, 32 families, and 39 genera were differentially enriched across root sections (Table S4). Discriminant ASVs identified are shown in Fig. 3b and Fig. S5. Consistent with the visual phylum bar-plot inspection (Fig. 3a), many Firmicutes ASVs were enriched in S1 but depleted in S3. A number of ASVs from Patescibacteria and Verrucomicrobia were only enriched in S3 (Fig. S5).

Intra- and inter-plant microbiota variability

To evaluate the extent of microbiota variation among root sections of the same plant (intra-plant variability) and among root sections from different plants (inter-plant variability) for the non-inoculated treatment, we assessed

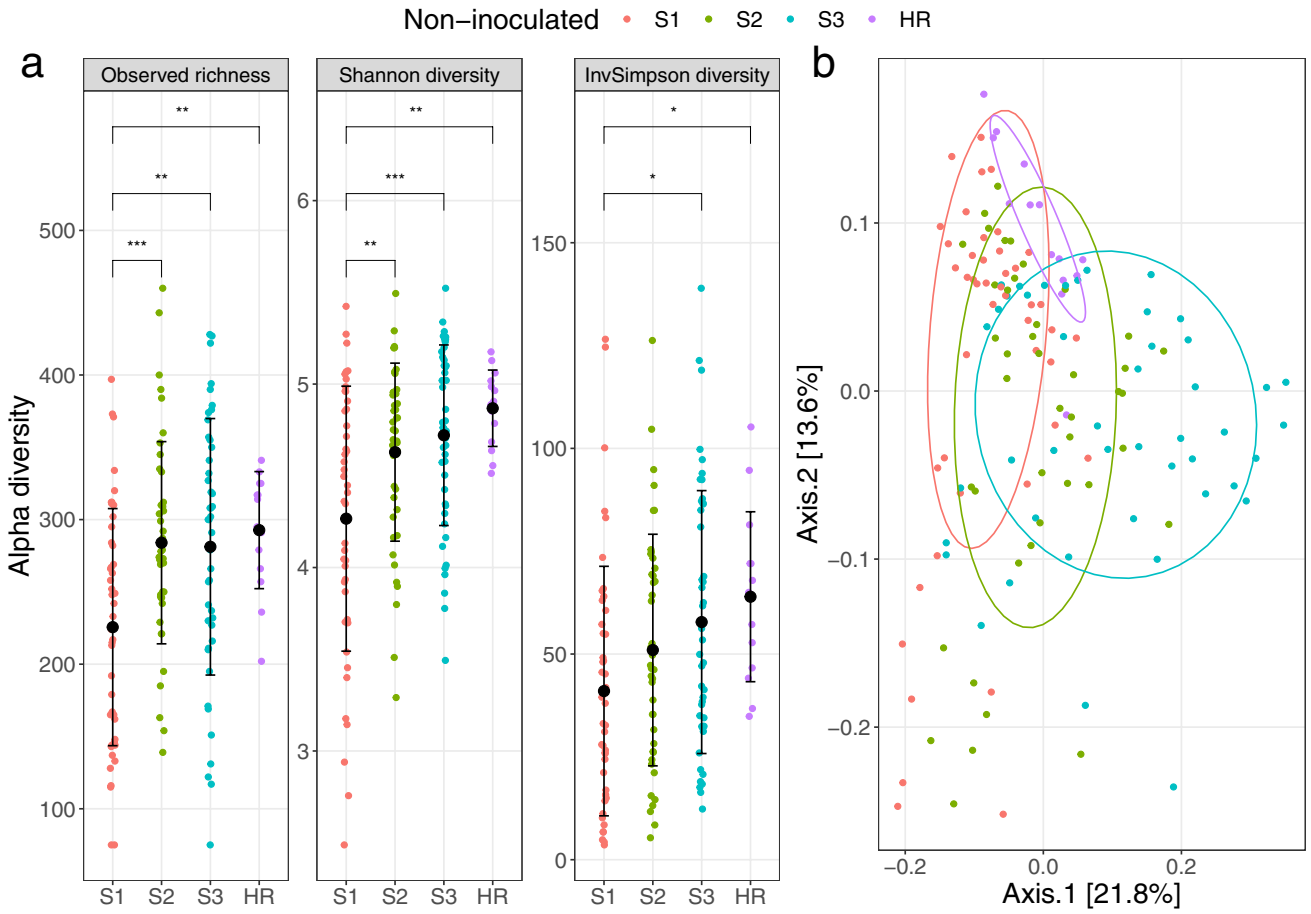

Fig. 2 Alpha diversity and beta diversity of the rhizosphere microbiota from non-inoculated plants. **a** Alpha diversity (observed richness, Shannon and inverse Simpson diversity) of apical (S1), middle (S2), rear (S3) root, and homogenized rhizosphere (HR). The black dots refer to the mean and error bars are the standard deviation. Significance in **a** was estimated from linear regression (covariate = "batch"; *: $0.01 < P < 0.05$; **: $0.001 < P < 0.01$; ***: $P < 0.001$). **b** Distribution of samples based on weighted UniFrac distance visualized with principal coordinates analysis (PCoA) with ellipse encircling 70% of samples per condition

pairwise UniFrac distances between these combinations (Fig. 4, Fig. S6). A diagram explaining the intra- and inter-plant comparisons is shown in Fig. S7. Generally, for each comparison, the inter-plant distance was always larger than intra-plant. For instance, the inter-plant weighted UniFrac distance comparisons of S1 ~ S1, S2 ~ S2, and S3 ~ S3 were always higher than the intra-plant ones. However, the inter-plant variability was not stronger compared to intra-plant. For example, the median UniFrac distance of inter-plant S1 ~ S1 was even smaller than the intra-plant S1 ~ S3 distance (median ± IQR [interquartile range], 0.340 ± 0.106 *vs.* 0.363 ± 0.131, $P = 0.011$). Similarly, for S3, that inter-plant S3 ~ S3 was significantly smaller than its difference to S1 (median ± IQR, 0.339 ± 0.122 *vs.* 0.363 ± 0.131, $P = 0.046$). In other words, similar sections from different plants have more similar communities than different sections inside the same plant root system. However, a stronger plant identity was observed when using unweighted UniFrac distance, as inter-plant comparisons were always higher than intra-plant (max. intra-plant = 0.52; min. inter-plant = 0.52; excluding HR; Fig. S6).

Impact of microbial community inoculation on the microbiota of root sections

We investigated if the rhizosphere microbiota composition in root sections was altered when spiked with a complex microbial community compared to the ambient development observed in non-inoculated control plants. First, we compared the standardized alpha diversity of each root section between inoculated and non-inoculated plants (Fig. 5a). We observed that inoculated plants presented higher bacterial richness (standardized mean ± SD, 1.330 ± 2.107 *vs.* -0.373 ± 2.101, $P < 0.001$) and Shannon diversity (0.449 ± 1.453 *vs.* -0.678 ± 2.373, $P = 0.008$) than the non-inoculated plants only in S3, not in S1 and S2. For inverse Simpson, inoculated plants showed a lower diversity

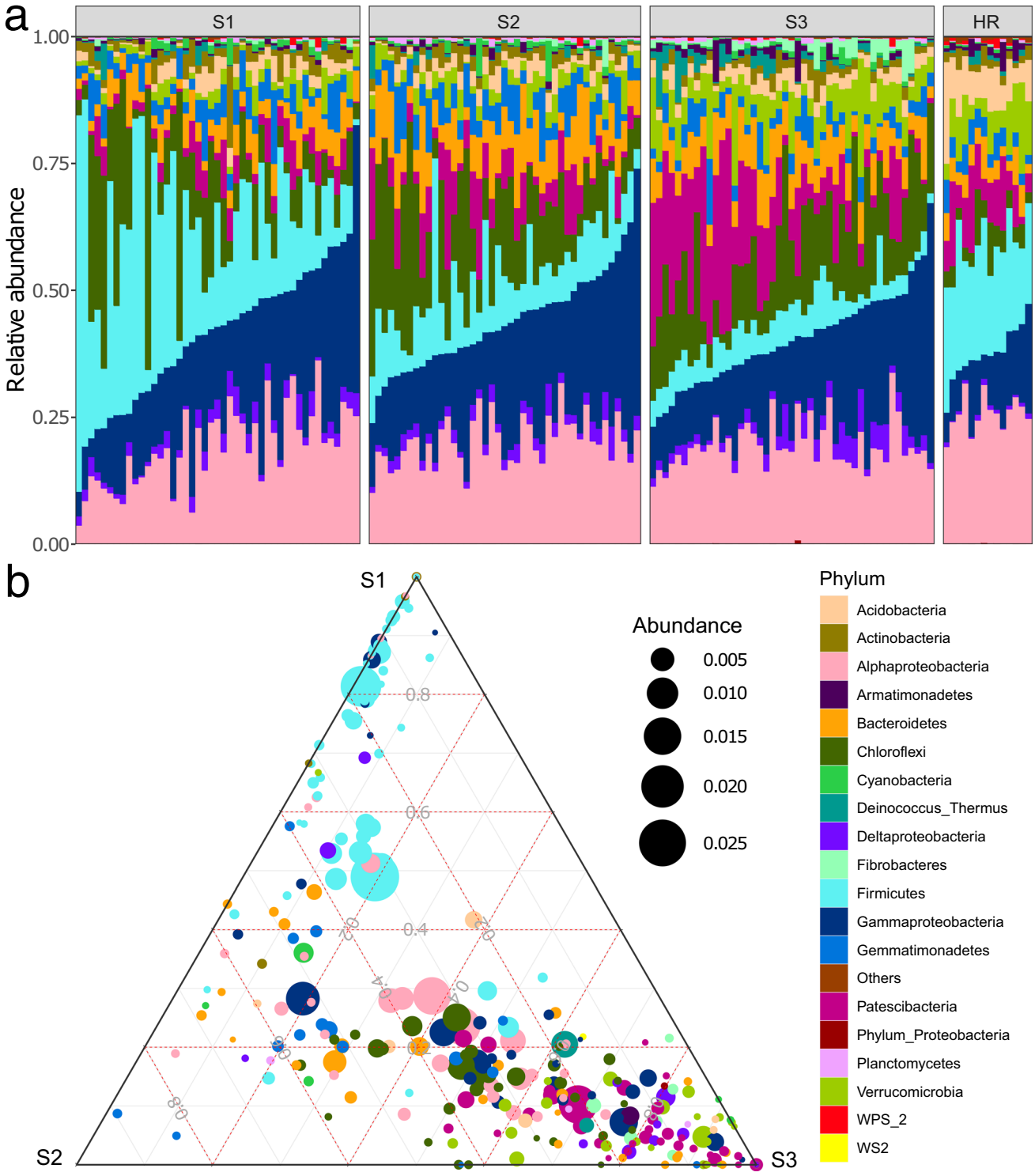

Fig. 3 Taxonomic composition of rhizosphere microbiota at phylum level (**a**) and the differentially enriched ASVs among root sections from non-inoculated plants (**b**). **a** Bar-plot of the 15 most abundant phyla. The remaining phyla were merged as "Others". Proteobacteria are shown at the class level. **b** Ternary plot showing the significantly enriched ASVs (relative abundance larger than 0.01%) in S1, S2, and S3. Each dot depicts one individual ASV, the dot size refers to its overall relative abundance, and the dot color refers to the phylum where an ASV is from. The positions of dots are determined as the contribution of the indicated root sections to the total relative abundance

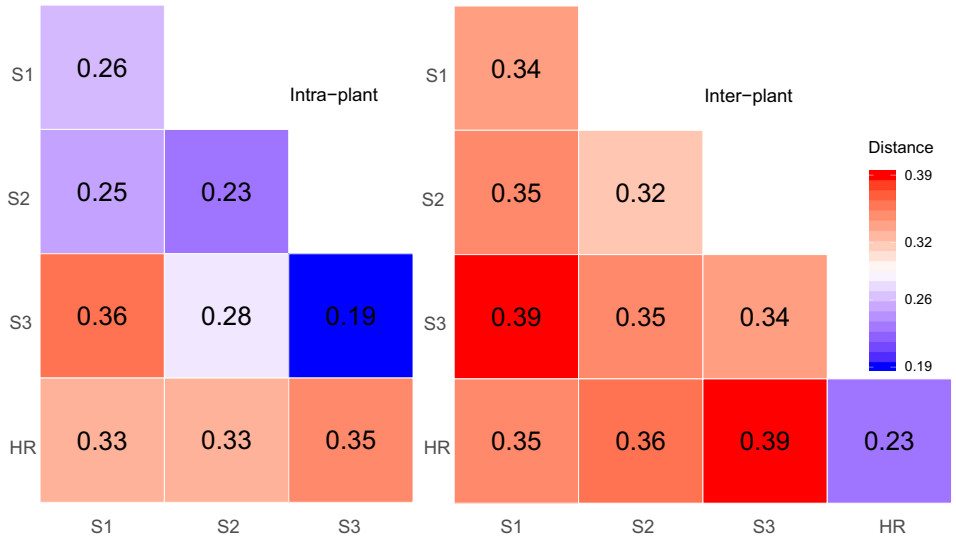

Fig. 4 Pairwise weighted UniFrac distance between samples from the same plant (intra-plant) and different plants (inter-plant) from the non-inoculated treatment. Numbers shown are the median value of weighted UniFrac distance under corresponding comparisons. UniFrac distance varies from 0 to 1 and quantifies how dissimilar microbial communities are (0 = identical, 1 = completely different)

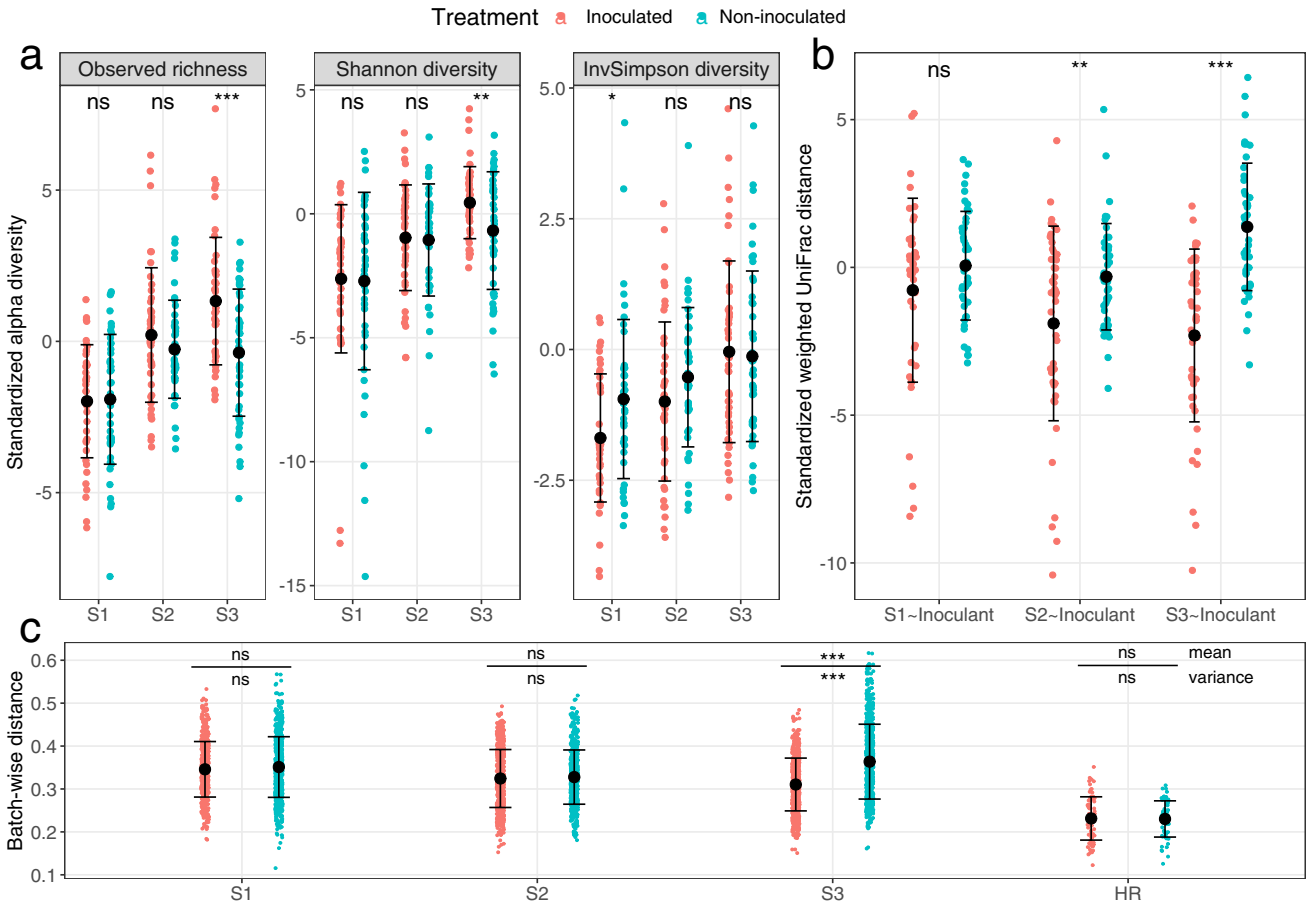

Fig. 5 Effect of inoculation on microbial communities in each root section between treatments (inoculated and non-inoculated). **a** The standardized alpha diversity of root sections between inoculated and non-inoculated treatments. **b** The standardized weighted UniFrac distance between each root section and the applied inoculants. Note that the microbiota of non-inoculated root sections were also compared to the applied inoculant as a reference. **c** The mean and variance of batch-wise weighted UniFrac distance for each sample type between treatments. In all panels, black dots and error bars are showing the means and standard deviations, respectively. The equality of mean and homogeneity of variance in **c** were tested with two-sample t-test and Bartlett's test, respectively. Significance is defined as ns: $P > 0.05$; *: $0.01 < P < 0.05$; **: $0.001 < P < 0.01$; ***: $P < 0.001$

in S1, compared to non-inoculated plants (− 1.691 ± 1.225 *vs.* − 0.948 ± 1.521, $P = 0.012$).

To confirm the differential influence of inoculation along the root axis, we compared their weighted UniFrac distance between each section and the corresponding inoculant. Although control plants were not inoculated, they were included for comparison purposes (Fig. 5b). In line with alpha diversity, the mean standardized distance of S1 ~ Inoculant did not differ between inoculated and non-inoculated plants. However, S2 ~ Inoculant (standardized mean ± SD, − 1.897 ± 3.292 [inoculated] *vs.* − 0.317 ± 1.801 [non-inoculated], $P = 0.007$) and S3 ~ Inoculant (-2.304 ± 2.923 [inoculated] *vs.* 1.371 ± 2.156 [non-inoculated], $P < 0.001$) were significantly smaller in inoculated than non-inoculated plants (Fig. 5b), indicating higher resemblance in S2 and S3 sections compared to inoculants. Besides, the alpha diversity and bacterial compositions of used inoculants were compared with the inoculated plants (Fig. S8). Our inoculants were observed to contain collections of highly diverse microbes.

To investigate how inoculation affected the change of microbiota composition in root sections across batches, we compared the within-section weighted UniFrac distance between inoculated and non-inoculated plants (Fig. 5c). In S3, inoculation not only significantly reduced the mean distance across batches in inoculated plants (mean ± SD, 0.310 ± 0.062 [inoculated] *vs.* 0.364 ± 0.087 [non-inoculated], $P < 0.001$), but also significantly decreased its variance (0.004 [inoculated] *vs.* 0.008 [non-inoculated], $P < 0.001$), compared to non-inoculated plants. No effect was observed in other root sections. Noteworthy, HR samples showed the smallest mean distance.

To understand the specific taxonomic changes occurring in root sections due to the microbial inoculation, we applied linear models to excavate ASVs that differed in relative abundance between the inoculated and non-inoculated plants (Fig. 6a). We found 144 ASVs with significantly altered abundance in S3 due to the inoculation (13 were not present in non-inoculated plants), while only 24 and two ASVs were observed in S2 and S1, respectively (Fig. 6b). For HR samples, only nine ASVs were significantly altered by inoculation.

Discussion

In the present study, we investigated the *Brachypodium distachyon* rhizosphere microbiota composition and distribution by combining two analysis scales, namely root sections from individual plants and the traditional homogenized rhizosphere sampling. Under a nonsterile greenhouse environment, plants were grown either with an initial microbial community inoculum ("inoculated" group), or without inoculation ("non-inoculated" control group) to detect effects linked to the ambient microbial colonization sources (e.g., soil, autoclaving procedure, air, seed surface, and irrigation water).

Rhizosphere microbiota variability depends on the sampling scale

According to our "variability hypothesis" (H1), the bacterial community variability was expected to be negatively correlated with the sampling scale. Indeed, many of our results supported this hypothesis. For instance, the dispersion analysis showed that HR samples were less dispersed, and therefore more reproducible, compared to root section samples (Fig. 2b, Fig. S4). It indicates that homogenizing the entire root system results in less variable and more reproducible microbiota profiles, but at the expense of missing variations at a smaller scale that might be relevant. This was clearly illustrated by the root section sampling, which enabled the detection of significant effects associated with the inoculation, especially its interaction with our three iterated plant growth batches, which was missed by the traditional approach (Table 1). Besides, at the root section scale, microbiota variability was conversely related with the section size (Fig. 4), showing in other words that the smaller the scale, the more variable the rhizosphere bacterial community. Therefore, our experimental design allowed unraveling a compromise between the sampling scale and the variability of microbiota composition in the rhizosphere. The strength of fine-scale approaches in the rhizosphere was also evidenced previously using in situ hybridization and fluorescence (FISH) (Bloemberg et al. 2000; Bulgarelli et al. 2012), but never via community analysis. This relationship between scale and pattern is well known in ecology (Levin 1992), and we demonstrated here that going at the root section scale is necessary to assess important effects that could be otherwise missed using conventional means.

Intra-plant root microbiota variability matters

According to our "intra-/inter- plant root variability hypothesis" (H2), we expected higher variability within an individual plant than between plants. Indeed, a large proportion of the variance was explained by intra-plant factors, e.g., root section identity (while inter-plant variability attributed to plant identity was three-times lower, Table 1). Besides, if plant identity had a strong influence on the rhizosphere bacterial community, a larger inter-plant distance should be observed for a given section than the largest intra-plant difference observed for that same section. This was not the case, as intra-plant differences were more important than any inter-plant comparisons for a given section (Fig. 4), thus reinforcing the relevance of our sampling of physiological distinct root sections, as they feature significant bacterial community composition differences that are likely linked

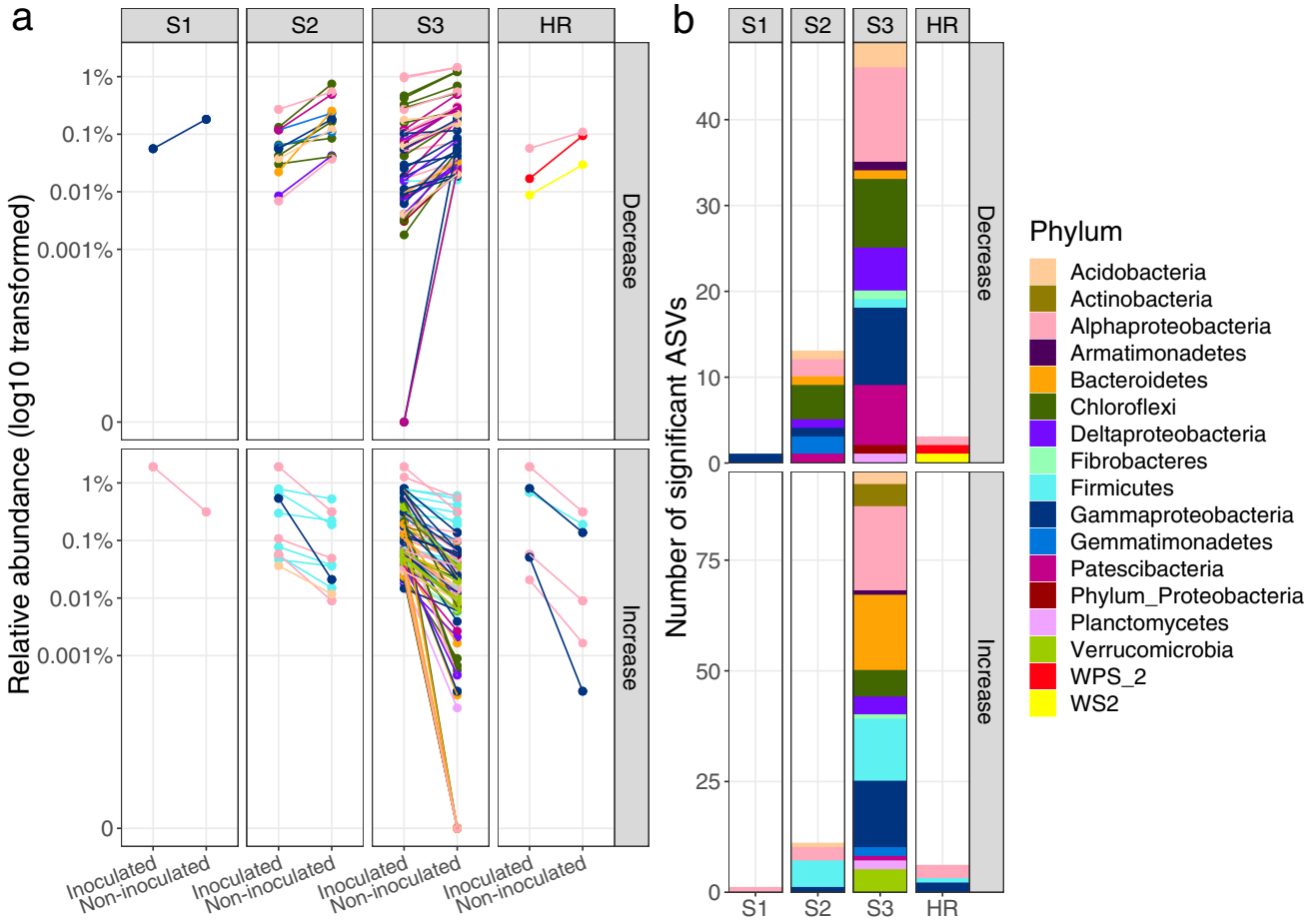

Fig. 6 ASVs significantly enriched or depleted by inoculation between treatments (inoculated and non-inoculated). **a** The relative abundance (log_{10} transformed for visualization) of significant ASVs between treatments. Dots are the ASVs colored by their phyla (Proteobacteria are shown at the class level), and linked dots are showing the abundance trend for each ASV between treatments. **b** Bar-plot showing the number of significant ASVs in each sampling type and colored by their respective phyla. An ASV is grouped into "Increase" if it is more abundant in inoculated compared to the non-inoculated plants, otherwise grouped into "Decrease"

to important vertical gradients (Dennis et al. 2010; Doan et al. 2017).

Since no effect the sampled plant root identity were observed on the bacterial community composition (Table 1), the variance attributed to plant identity likely comes from (i) plant genotypic/epigenetic/phenotypic/physiological variations resulting in altered microbe recruitment frequencies (Reinhold-Hurek et al. 2015); (ii) seed endophytes or seed surface-carried microbes, which are known to fluctuate between seeds (Barret et al. 2015; Johnston-Monje et al. 2016), although their relative importance to the rhizosphere microbiota remains uncertain (Normander and Prosser 2000; Ofek et al. 2011; Sarniguet et al. 2020). Notably, plant identity did show stronger variations when using the unweighted UniFrac distance, which indicates that these effects between plants were mainly resulting from low abundance and rare species, thus indicating that this variance might be partly attributed to random sampling effects.

The apical root section harbors a different community

According to our "developmental variability hypothesis" (H3), bacterial differences were expected across root sections along the axis due to different physiological states. Indeed, clear microbial differences were detected along root axis, but not between roots from the same plant (Table 1). Bacterial richness and evenness were lower at younger S1 but increased in older parts to similar levels between S2 and S3 (Fig. 2a). This concurs with Kawasaki et al. (2016), showing that root base had higher bacterial Shannon diversity than root tips. The microbial variability was also observed to be different between sections, being more important in S1 (Fig. S4). The preconceived assumption that larger samples could yield higher microbial diversity was not verified here, as no significant alpha-diversity differences were observed between S2 and S3 despite one being 3-times

larger than the other. Similarly, S2, S3, and HR differed in the amount of sampled material, but did not display significant alpha diversity differences. These observations further reinforce the relevance of the *census* sampling of the entire root via functionally distinct sections from a microbiological point of view.

The fact that more material was not confirmed to be associated with more diversity in our study might indicate that the low diversity and high community variability seen in S1 (the smallest samples taken in this study), could be rather due to functional aspects harbored by this specific root section. Indeed, among S1 enriched taxa, many are considered *r*-strategists e.g., *Bacillus* members from Firmicutes (Table S4, Fig. 3). They grow fast and respond quickly to nutrient resources (Cleveland et al. 2007), making them prone to be selected at the root apex where abundant rhizodeposits are found (Dennis et al. 2010), but also potentially via the remaining necromass nutrients released upon soil autoclaving (Nunes et al. 2018). Besides, root S1 is the part exploring soil and extending root length, thus more prone to random encounters, which makes it a section with variable microbial compositions. Altogether, results reflect the exploring function of the apical root section, being a highly dynamic niche dominated by *r*-strategists and with a less stable microbial community composition. S1 enriched Firmicutes may also be microbial survivors that resisted the heat treatment of our autoclaving procedure due to the thermotolerance of spore-forming Firmicutes members, e.g., *Bacillus* (Müller et al. 2014; Nunes et al. 2018).

In comparison, the matured S3 section is physiologically older, occupies more volume in the rhizosphere, and has fewer rhizodeposits. This offers stable conditions and more niches for microbial communities to adapt, diversify, and stabilize. This is in line with what we observed, as communities were more diverse and stable in this section. Therefore, this habitat would be more favorable for K-strategists. Indeed, members from Verrucomicrobia, a phylum containing representatives generally known to behave as K-strategists (low growth rates and efficient nutrient uptake systems) (Navarrete et al. 2015), and were enriched at the rear root S3 (Fig. 3). Meanwhile, the intermediate abundance of Firmicutes and Verrucomicrobia in the S2 section confirms its "transitional" status, as it features some characteristics from both extremities (relatively young tissues that are progressively differentiating and aging) (Fig. 3).

Overall, our data support our initial hypothesis that root sections harbor distinct microbial communities, and also suggests that root axis sampling may be a proxy for studying trophic aspects linked to rhizodeposition. Further research aiming at measuring the actual quantitative and qualitative signature of rhizodeposits along the root axis combined with microbial data would be required to confirm these preliminary observations.

Inoculated communities have a local influence on the rhizosphere microbiota along root axis

According to our "local influence hypothesis" (H4), inoculated microbes would preferentially have an effect on the younger root apex where more resources are available. However, we observed that it was the older root S3 that was the most influenced by the inoculation procedure via: (i) an increased alpha diversity in S3 (Fig. 5a); (ii) more beta-diversity similarity between inoculated communities and the communities in S3 (Fig. 5b); (iii) less divergent communities across our iterated batches in S3 (Fig. 5c); (iv) remarkably more ASVs significantly affected by inoculation compared to S1 and S2 (Fig. 6). Notably, we evidenced 13 ASVs out of the 144 that were not detected in the S3 of non-inoculated plants, thus representing potential "newcomers" introduced by the artificial selection procedure.

This suggests that the S3 section harbors a microbial community that is more susceptible to be affected by our inoculation procedure. This might be due to the fact that this section occupies more space in the root system and is the oldest part, providing more time and niches for microbial development. It is also possible that all these ASVs were present in all other sections at a much lower relative abundance, therefore, not seen by sequencing, while being specifically enhanced in relative abundance in S3. Our results are in line with previous observations showing denser colonization on older root parts (Chin-A-Woeng et al. 1997; Gamalero et al. 2004; Götz et al. 2006). Considering our experimental setup, several factors could also explain this trend such as the inoculation spot (inoculants were added from the top), irrigation (water was added from the bottom), and root movement through soil. Although most inoculation effects were mainly observed in S3, some marginal effects were noted in other sections (e.g., an abundant ASV from Gammaproteobacteria consistently enriched in all sections, shown in Fig. 6a, likely contributing to the decreased evenness in S1). Altogether, keeping in mind limitations introduced with our experimental choices (e.g., soil autoclaving and inoculation procedure), our design and results clearly indicated preferential niche occupation along the root axis, enabling the detection of inoculation effects at the mature rear sections of the root system.

The importance of root section sampling scale to complement traditional approach

A significant interaction between batch and treatment factors at the root section level was observed (Table 1). It indicates that rhizosphere microbiota under ambient and inoculated conditions did not develop the same way across batches. However, this interaction was not detected with

the homogenized sampling. It was further evidenced by a significant reduction of the batch effect variance portion between the two treatments. This significant reduction was successfully captured by our root section sampling, not with the homogenized sampling.

Furthermore, our root section sampling enabled a greater sensitivity to detect taxa that were significantly affected by our inoculation procedure, especially between S3 and HR. Overall, the root section sampling successfully detected the effect of microbial community inoculation by showing the decreased batch variability and altered taxa abundances. However, such observations were not captured or to a lesser extent when applying traditional homogenized sampling of the entire root system. Thus, our results indicate that root section sampling is a promising complement to traditional homogenization approach to probe biological effects associated with microbial inoculation, which are often quite difficult to observe.

Conclusions

Through the simultaneous testing of four hypotheses, we improved our understanding of the spatial variability of the rhizosphere microbiota. Microbial variability was clearly related to the observational scale. The sampled section along the root axis represented the main source of variability, then followed by the plant identity. We evidenced that root section sampling was more efficient in detecting biological effects associated with microbial inoculation within the rhizosphere and therefore could complement the traditional sampling approach. Our results have to be interpreted in light of the experimental choices applied in terms of inoculation and soil preparation. This study calls for further investigations to understand the functional implications behind the identified spatial patterns and potential repercussions on other microbial trophic levels (e.g., fungi and protists) as well as on the plant itself.

The online version contains supplementary material available at https://doi.org/10.1007/s00374-021-01590-0.

Acknowledgements We thank the Lundbeck Foundation (grant number R223-2016-179) for funding this study. S.W. was supported by the China Scholarship Council. S.J. was supported by the University of Bourgogne Franche-Comté via the FABER program (grant number 2017-9201AAO049S01302) and via the ISITE-BFC International Junior Fellowship award (AAP3: RA19028.AEC.IS). We express our gratitude to the members of the 4PMI platform lead by Christophe Salon and Céline Bernard for their expertise and help during plant phenotyping (For Plant and Microbe Interaction, INRA Center Dijon, France, https://www6.dijon.inrae.fr/plateforme4pmi_eng/Technical-description/High-Throughput-phenotyping). Respectively, we thank Damien Gironde, Frédéric Saignole, Noureddine El-Mjiyad and Karine Palavioux for helping during plant growth monitoring; Franck Zenk and Julien Martinet for image capture; and Mickael Lamboeuf for image processing; Sébastien Anselme, Richard Sibout, and Thomas Girin from the *Brachypodium* resources center at the Institut Jean Pierre-Bourgin, (INRAVersailles, France) for seeds provision; Beatriz Decencière, Amandine Hansart, and Florent Massol of the CEREEP—Ecotron IDF/UMS CNRS/ENS 3194 for soil provision.

Authors' contributions S.J.S., M.B., and L.P. designed the study. S.W. and S.J. carried out the study. S.W., S.J., M.B. contributed to the statistical analysis. M.B., S.J., S.J.S., L.P., and S.W. contributed to the concept and interpretation of the data. S.W. wrote the manuscript and S.J. made a substantial contribution to the revision of the manuscript.

Data availability The data that support the findings of this study are openly available in the Sequence Read Archive (SRA) repository at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA635875.

Code availability The code is available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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