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# Characterization of maize root microbiome in two different soils by minimizing plant DNA contamination in metabarcoding analysis

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## Abstract

A micropore-filtration method was used to reduce the proportion of plant DNA in microbial DNA samples isolated from roots prior to sequencing. We tested the impact of this pre-sequencing filtration methodology and used it to characterize the root microbiome of maize grown on two soils with different fertility levels. The micropore filtration reduced plant DNA contamination and unveiled potential in the N-poor soil for N fixation in roots and phosphate uptake by roots in the phosphate-poor soil. Our methodology and findings allude to the potential capability of plants to initiate plant-microbe interactions under sub-optimal soil fertility.

**Keywords** Rhizosphere microbiome · Plant genes, metabarcoding

## Introduction

Micro-organisms exist in communities with several levels of interactions within and between microbial taxa (Cao et al. 2018). This has necessitated the advancement of microbiology research from single-microbe studies to community-level microbiome investigations (O'Malley and Skillings 2018). The insights gained from these microbiome studies include the observation of how a suppressive interaction between two microbes can be beneficial to the plant host (Fávaro et al. 2012; Tshikantwa et al. 2018). In fact, there are reports showing the potential benefits of the root-associated microbiome in maize under different environmental conditions (Beirinckx et al. 2020; Gomes et al. 2018).

Microbiome studies have benefited from the advancement in DNA sequencing technology that enabled the analysis of community-level interactions in a culture-independent manner (Hugenholtz et al. 1998; Jany and Barbier 2008; Su et al. 2012). Among the two major approaches used in DNA sequencing,

whole-genome sequencing (metagenomics) and amplicon sequencing (metabarcoding), the latter that is cheaper and less-demanding on computing capacity involves the amplification of microbial marker genes like the *16S* rRNA and *ITS* (internal transcribed spacer) for bacteria and fungi, respectively, and sequencing of the amplicons (Banchi et al. 2018; Lasa et al. 2019).

Amplicon sequencing has been used in several studies to identify microbial composition and abundance of the microbiota from different sources including plant roots (Beckers et al. 2016; Ma et al. 2020). The major limitation of such metabarcoding studies on plant microbiome is that the microbial marker genes share an evolutionary origin, and hence nucleotide sequences, with plant organelles, including mitochondria, chloroplasts, and other plastids (Hanshew et al. 2013). Therefore, the primers used to generate the amplicons, amplify DNA from both microbes and plants for sequencing, eventually limiting microbial identification (Zaheer et al. 2018). To circumvent this limitation, we used a pre-DNA-isolation filtration protocol aimed at reducing the proportion of plant cells in ground root tissues using a size discrimination filtration technique and evaluated its efficacy in the characterization of maize root microbiome.

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## Materials and methods

### Planting and sample collection

Maize (cv. NK Falkone) was grown in 0.5 L pots containing two soil types, Soil\_A—from an experimental station, and

Soil\_B—an agricultural soil. The main properties of soil A were sand 7%; silt 63%; clay 23%; pH 7.2; organic C 1.9%; total N 2140 mg/kg; and available P 0.3%. The main properties of soil B were sand 43%; silt 40%; clay 13%; pH 5.9; organic C 1.3%; total N 1240 mg/kg; and available P 0.6%. Seeds sown directly into the pots germinated within 4 to 6 days post-planting and were subsequently grown for 3 weeks under greenhouse conditions of 16/8 h day/night and 27/21 °C day/night temperature from March to April 2019. Five pot-replicates per soil were used and each pot contained 3 plants. The plant roots were harvested at the V3–V4 stages (3 to 4 true leaves) and carefully but thoroughly washed in phosphate-buffered solution (PBS: 5.8 mM Na<sub>2</sub>HPO<sub>4</sub>•7H<sub>2</sub>O + 4.2 mM NaH<sub>2</sub>PO<sub>4</sub>•H<sub>2</sub>O + sterile water until a volume of 1 L) to remove any rhizosphere soil. Root samples were collected on ice and stored at –80 °C until DNA isolation.

### Sample processing and DNA isolation

Roots were ground in liquid nitrogen with mortar and pestle, and DNA was directly extracted from ~100 mg of the 5 replicates per soil using the QIAGEN DNeasy PowerSoil kit according to the manufacturer's specification. Also, 4.5 g of the ground root tissue from each of the 5 replicates per soil was thawed in 25 mL PBS and shaken vigorously to form a homogenous suspension (see Supplementary Fig. S1). To get rid of plant cells, which are generally larger than microbial cells, a 144 cm<sup>2</sup> piece of sterilized Miracloth (pore size 22 µm) folded into eight layers and trimmed to a dimension of 3 cm × 3 cm was inserted into a disassembled 50-mL syringe using a sterilized pair of forceps until the nozzle-base of the syringe was sealed by the Miracloth. The ground root in PBS suspension was subsequently carefully applied on the Miracloth and pressed through the Miracloth using the syringe piston. The filtrate was then centrifuged at 8500 g in a Hettich Rotina 420R refrigerated centrifuge for 20 min at 4 °C. The supernatant was discarded and the sediment containing mainly microbial cells was used for DNA isolation using the QIAGEN DNeasy UltraClean Microbial Kit according to the manufacturer's specification. A microbial kit was used instead of PowerSoil kit to avoid DNA shredding of the microbes which could occur if PowerSoil kit is used since there is no microbial cell protection by soil or plant root particles. The PowerSoil kit and Microbial kit are from the same manufacturer and employ the same technique in DNA extraction. DNA yield was measured using a NanoDrop ND1000 spectrophotometer.

### Metabarcoding and data analyses

For identification and quantification of bacterial and fungal communities, the bacterial *16S* ribosomal RNA gene and fungal *ITS* genes were PCR-amplified using the following

degenerate primers: V3-V4 (341f-806bR) primers for 16S (5'-3', forward: CCTACGGGAGGCAGCAG; reverse: GGACTACHVGGGTWTCTAAT) (Takahashi et al. 2014) and ITS1f-ITS2 for fungal ITS (5'-3', forward: CTTGGTCA TTTAGAGGAAGTAA; reverse: GCTGCGTTCTTCAT CGATGC) (Ihrmark et al. 2012). As a check for possible DNA contamination in the above protocols, ZymoBIOMICS microbial community standards were included in the workflow as both positive and negative controls. Following DNA amplification using AccuStart II PCR ToughMix (55 °C/33 PCR cycles) and library preparation, Illumina MiSeq sequencing was used for paired-end sequencing of the amplicons with read lengths of 300bp.

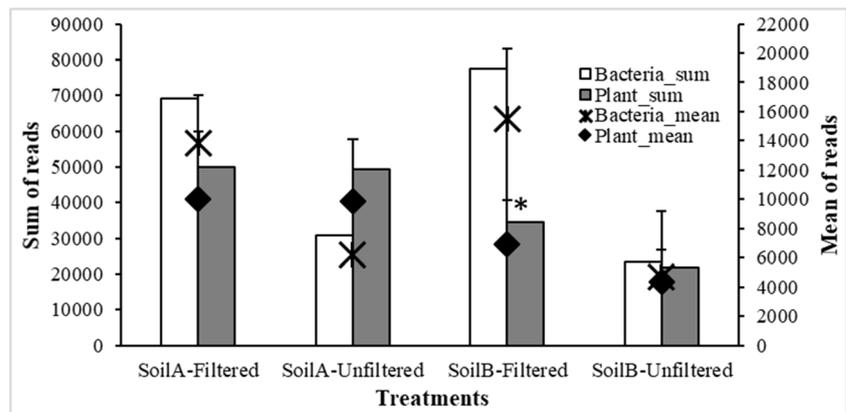
DADA2 and Phyloseq packages were used to analyze the sequenced reads (McMurdie and Holmes 2013; Callahan et al. 2016). Briefly, forward- and reverse reads were trimmed based on Phred read quality score threshold of 30 allowing 290bp and 250bp of forward and reverse reads, respectively for reads overlap. *ITS* reads were not trimmed to avoid losing relevant information. *ITS* databases like UNITE provide a good coverage of fungal taxa although many of the sequences may not be identified to specific taxa in comparison to the *LSU* (large subunit) marker gene which in turn may affect alpha diversity (Xue et al. 2019). After de-noising sequencing errors using predicted and observed error rates, the forward and reverse reads were merged to infer sequence variants. Chimeric sequences were removed using the consensus method and the SILVA reference (release 132) was used for taxonomic assignment. The read counts per sequence variant were normalized using *rarefy\_even\_depth* of Phyloseq R package to account for sequencing depth bias. However, for differential abundance computation using DESeq2, raw reads were converted into a DESeq object using *phyloseq\_to\_deseq2* function and DESeq2 was used for the normalization of library depth. Statistical significance of the pre-treatment effects was tested with an analysis of deviance using the *manyglm* function of *mvabund* package, based on 5000 bootstrap iterations (Wang et al. 2012), whereas the difference in reads assignment was tested with simple ANOVA in R studio version 3.6

## Results

### Sequenced reads assignment and sequencing depth

The proportions of sequenced reads from plant organelles and microbial *16S* or *ITS* were investigated. Plant reads were identified and sorted out as belonging to the order “Chloroplast” and/or family, “Mitochondria”. In the filtered samples, more reads were sequenced from bacterial *16S* amplicons than from plant organelles, unlike in unfiltered samples (Fig. 1). Fungal *ITS* sequencing, as expected, did not pick up plant sequences (data not shown), but could be used to assess the impact of the

**Fig. 1** Total number (sum–left axis) and mean number (average–right axis) of *16S* sequenced reads mapping to bacterial and plant DNA in filtered and unfiltered samples of Soil\_A and Soil\_B. \*Denotes significant difference at  $\alpha = 0.01$  (ANOVA). Error bars are standard deviations



protocol on off-target microbes. Furthermore, the numbers of sequenced reads were generally higher in the filtered than unfiltered samples (Table 1), probably due to random sequencing bias. The proportion of *16S* reads assigned to bacterial species (Amplicon Sequence Variants, ASVs) were significantly higher in filtered samples than in the unfiltered, whereas there was no effect on the *ITS* reads (Table 1). The significantly higher percentage of assigned *ITS* reads compared to the *16S* may be linked to the use of untrimmed *ITS* sequences in the analysis or the better coverage of fungal diversity in the UNITE database by our sequencing depth than bacterial diversity. To eliminate possible sequencing depth biases, only normalized assigned counts were used for downstream analyses.

Principal coordinate analysis (PCoA) of distance relationships among samples with Bray-Curtis metrics based on the normalized read counts showed that there is a significantly higher variation among the unfiltered samples than the filtered samples for *16S* sequences (Supplementary Fig. S2a, b). Furthermore, analyses of deviance showed that for the *16S* normalized read counts, both soil type and micropore filtration pre-treatment, significantly affected the amplicon sequence variants (ASVs) (Supplementary Table ST1), but no effect was detected on fungal (*ITS*) ASVs (data not shown).

Rarefaction curves showed indeed that filtered samples had more sequencing depth and species abundance than the unfiltered samples for bacterial (*16S*) but not fungal (*ITS*) read counts (Supplementary Fig. S2c, d).

### Root microbial abundance

Microbial abundance plots showed that filtered samples in both soil types recorded significantly more bacterial ASVs with less variations than unfiltered samples (Fig. 2a, b). But there was no effect on fungal abundance (Fig. 2c). However, these abundance plots did not show a significant difference in microbial abundance between Soil\_A and Soil\_B. Nonetheless, there was more overlap of assigned ASVs between treatments than between soils, suggesting that the pre-treatment did not negatively impact on the identification of microbes; rather, it improved the number of ASVs that could be identified (Fig. 2d, e).

### Identification and differential abundance of root microbial composition

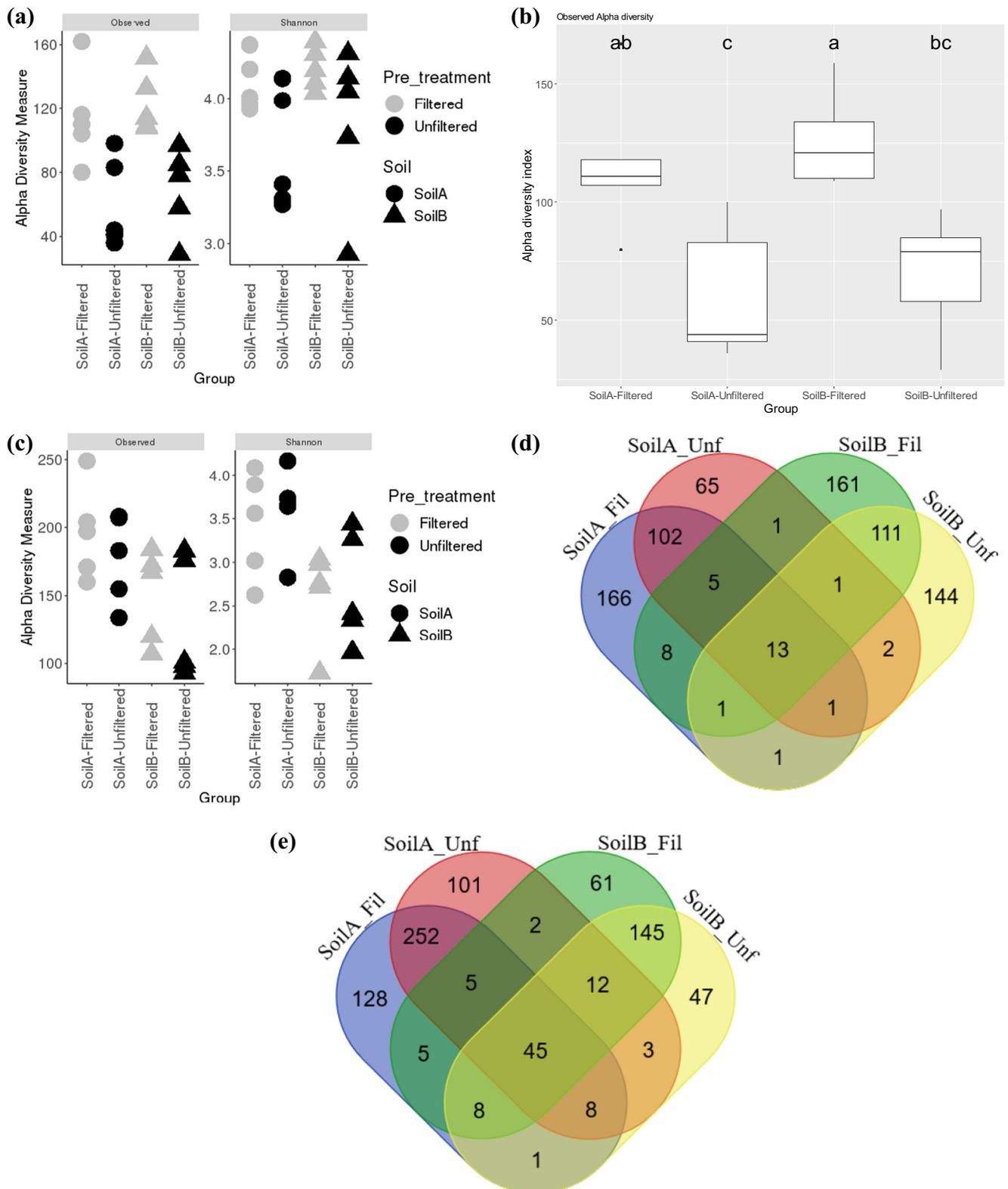
We compared the treatment approaches in terms of differential microbial abundance so as to ascertain the best approach for

**Table 1** Total counts of sequenced reads, non-normalized reads assigned to ASVs (Amplicon Sequence Variants) after processing and their percentages

Soil	Pre-treatment	16S			ITS		
		Sequenced reads	Assigned reads	Assigned reads (%)	Sequenced reads	Assigned reads	Assigned reads (%)
A	Filtered	220,788	64,966	29.4 <sup>b</sup>	356,385	261,114	73.3 <sup>c</sup>
	Unfiltered	169,000	29,661	17.6 <sup>a</sup>	282,477	210,221	74.4 <sup>c</sup>
B	Filtered	235,473	77,084	32.7 <sup>b</sup>	295,538	199,435	67.5 <sup>c</sup>
	Unfiltered	106,133	22,790	21.5 <sup>a</sup>	248,109	170,364	68.7 <sup>c</sup>

*16S* normalized assigned counts per sample: 2165; *ITS* normalized assigned counts per sample: 16,728

<sup>a,b,c</sup> Significant differences between assigned reads % using ANOVA test



**Fig. 2** Abundance plots of ASVs (microbes) in filtered and unfiltered samples for two soil types **a** bacterial alpha diversity (observed and Shannon measures) showing data points, **b** bacterial alpha diversity (observed) showing mean abundance at  $\alpha = 0.05$ , **c** fungal alpha diversity

(observed and Shannon measures); Venn diagrams showing the number of assigned ASVs for all treatment-soil combinations **d** 16S and **e** ITS. “Fil” implies filtered and “Unf” implies unfiltered

analyzing the differences in microbial abundance and composition between soils. In the root microbiome recruited in Soil\_A, 24 bacterial ASVs were differentially more abundant in filtered than in unfiltered samples at  $p < 0.01$ , while 8 ASVs were differentially more abundant even at  $p < 0.001$  with zero read counts in unfiltered samples (Supplementary Fig. S3.1). In Soil\_B, four bacterial ASVs were differentially more abundant in filtered samples versus the unfiltered treatment; and in one instance, an ASV was more abundant in unfiltered samples (Supplementary Fig. S3.1). Fungal abundance was, however, not differentially affected (data not shown).

A comparison of the fungal and bacterial phyla abundances between roots from the two soils shows some differences although further studies would be required to confirm these observations. Firstly, there are 24 folds more non-annotated fungi detected in Soil\_B than Soil\_A suggesting the existence of more fungal diversity in Soil\_B (Supplementary Fig. S3.2). Ascomycota was the most abundant fungal phylum in both soils, whereas Glomeromycota that includes arbuscular mycorrhizal fungi (AMF) was more abundant in roots of Soil\_A than Soil\_B. For bacterial abundance, Proteobacteria and Bacteroidetes were the most abundant phyla in roots from both soils (Supplementary Fig. S3.2). Generally, both soils showed a higher abundance of fungal phyla than bacteria.

There were 84 bacterial ASVs more abundant in roots from Soil\_A when compared to Soil\_B, whereas 71 bacterial ASVs (including *Rhizobium*) were more abundant in Soil\_B roots compared to those in Soil\_A (Supplementary Fig. S3.3). Among the fungal ASVs, 92 genera were differentially more abundant in roots of Soil\_A than in Soil\_B, whereas 73 genera were differentially more abundant in roots of Soil\_B than in Soil\_A (Supplementary Fig. S3.4). Interestingly, differentially abundant AMF including *Funneliformis*, *Dominikia* and *Rhizophagus* were found only in roots of Soil\_A and not in Soil\_B.

## Discussion

Unravelling plant microbiome through DNA sequencing is challenging as a precaution is required to avoid plant DNA interference with microbial sequencing depth (Beckers et al. 2016; Fricker et al. 2019). Previous studies tackling this challenge compared several primers for their specificity in amplifying bacterial DNA (Beckers et al. 2016; Hanshew et al. 2013). The forward primers, 799F and its derivatives, emerged from these studies as the most specific for reducing plant plastid contamination. However, the coverage of the bacterial diversity by 799F (79.7%) is limited when compared with the coverage by 341F (91.2%) that we used in our study

(Beckers et al. 2016). Interestingly, the plant DNA sequence contamination range in Beckers et al. (2016) with 341F primer is between 40 and 91% depending on the reverse primer combination, while with our micropore-filtration protocol, the plant DNA contamination was reduced to 30–40% (Fig. 1). This improvement suggests that efforts in selecting applicable primer pair combinations can be further boosted when combined with our filtration protocol.

To our knowledge, the only publication of efforts to reduce plant organelle contaminants prior to DNA isolation and sequencing is a Nycodenz gradient-based separation technique (da Cunha 2016). The Nycodenz separation approach is intended to obtain uncontaminated bacterial cells, but fungal cells are also lost in the process. This means that an extra DNA isolation is required to capture the fungal component in microbiome studies. In our protocol, however, the same micropore filtrate was used to extract both bacterial and fungal DNA for sequencing. Importantly, our protocol did not alter the outcome of the fungal *ITS* sequencing (Supplementary Fig. S2, Table 1). Furthermore, the total number of sequenced reads (Table 1) and reads mapping to bacteria (Fig. 1) showed that our filtration protocol did not lead to any losses of microbial data. This is logical because the filtration system allows sufficient room for microbial cells to be collected in the filtrate while filtering out significant proportion of plant material.

For application purposes, in plant microbiome profiling, the results from our new protocol show the importance of microbial read depth in computing the composition and differential abundance of microbes, especially bacteria (Supplementary Fig. S3.1). For instance, eight bacterial species could be detected in roots of Soil\_A and four in roots of Soil\_B only by the filtration pre-treatment (Supplementary Fig. S3.1). Additionally, the plant system, *Zea mays*, on which we tested our protocol in two soils suggests that this methodology has a potential for use in investigating plant-microbe interactions in different soils, but this needs further studies in more plant systems and soil types. Among the observations from our limited test system are the abundance of *Rhizobium* species in the N-poor soil (Supplementary Fig. S3.3), and the presence of arbuscular mycorrhizal fungi (AMF) in the phosphate-poor soil (Supplementary Fig. S3.4), pointing to the potential for initiation of plant root interaction with microbes under nutrient deficiency (Cocking et al. 1994; Rosenblueth et al. 2018; Cotton et al. 2019; Kobae 2019).

In conclusion, we demonstrate here the importance of filtering-out plant cells before DNA extraction in microbiome studies, and the implementation of that in understanding plant response to sub-optimal soil fertility. The protocol, in our view, has a large application potential, for research in ecology, agriculture and biodiversity. A scaled-up production of

syringes with an inbuilt-filtration system as described here, would facilitate the routine implementation of this protocol in plant microbiome studies.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00374-021-01555-3>.

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**Author contribution** HJB, TM, and EBA designed the experiment and acquired the data; EBA and WT processed and analyzed the data; HJB, TM, and EBA wrote the manuscript; all authors gave final approval for publication.

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**Data availability** Sequence data is archived at NCBI Genbank (accession number [to be provided])

**Code availability** Not applicable

## Declarations

**Ethics approval** Not applicable

**Consent to participate** Not applicable

**Consent for publication** All authors have given their consent for this publication.

**Conflict of interest** The authors declare no competing interests.

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