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# Root exudates drive soil-microbe-nutrient feedbacks in response to plant growth

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## Abstract

Although interactions between plants and microbes at the plant–soil interface are known to be important for plant nutrient acquisition, relatively little is known about how root exudates contribute to nutrient exchange over the course of plant development. In this study, root exudates from slow- and fast-growing stages of *Arabidopsis thaliana* plants were collected, chemically analysed and then applied to a sandy nutrient-depleted soil. We then tracked the impacts of these exudates on soil bacterial communities, soil nutrients (ammonium, nitrate, available phosphorus and potassium) and plant growth. Both pools of exudates shifted bacterial community structure. GeoChip analyses revealed increases in the functional gene potential of both exudate-treated soils, with similar responses observed for slow-growing and fast-growing plant exudate treatments. The fast-growing stage root exudates induced higher nutrient mineralization and enhanced plant growth as compared to treatments with slow-growing stage exudates and the control. These results suggest that plants may adjust their exudation patterns over the course of their different growth phases to help tailor microbial recruitment to meet increased nutrient demands during periods demanding faster growth.

KEYWORDS

GeoChip, plant development, plant–soil feedback, root exudates, soil bacterial community

  

## 1 | INTRODUCTION

Plants adapt and respond to biotic and abiotic factors in their environment via diverse strategies, including the secretion of compounds from roots into the surrounding soil. These root exudates modulate plant performance by changing the soil physico-chemical environment around the root, promoting nutrient acquisition and attracting specific beneficial microbial groups, collectively termed plant–soil feedbacks (PSFs) (Bais, Weir, Perry, Gilroy, & Vivanco, 2006; Fageria & Stone, 2006; Marschner, Römheld, & Cakmak, 1987). As such, rhizosphere microbial communities can directly influence plant performance both positively or negatively (Kulmatiski, Beard, Stevens, & Cobbold, 2008). Patterns of exudation change throughout a plant's life cycle (Chaparro et al., 2013), leading to changes in the structure, diversity and function of the associated rhizosphere microbiome (Chaparro et al., 2013; Chaparro, Badri, & Vivanco, 2014). Notably, phenolic acids and triterpene in root exudates play major roles in regulating rhizosphere bacterial community structure (D. V. Badri, Chaparro, Zhang, Shen, & Vivanco, 2013; Huang et al., 2019). Soil microbes carry out critical functions of decomposition and energy transformation processes (Berendsen, Pieterse, & Bakker, 2012), and shifts in the microbiome over the course of the plant life cycle may be linked to changes in plant nutrient demands. Several studies have shown that plants can improve the availability of mineral nutrients directly by releasing organic acids to solubilize phosphorus (P) and nitrification inhibitors to direct available nitrogen (N) (Jones, 1998; Subbarao et al., 2009). For example, higher N demands in the fast-growing stages of plant growth development (Kelly, Bacon, & Wells, 1995; Malagoli et al., 2004; Rossato, Laine, & Ourry, 2001) are correlated with higher densities of N-fixing microbes in rhizosphere soil relative to slow-growing stages (Chaparro et al., 2014; Lee, Castro, & Yoshida, 1977; Philippot, Raaijmakers, Lemanceau, & van der Putten, 2013). Thus, it is important to consider PSF's from both directions, not only considering plant impacts on the soil microbiome, but also microbiome feedbacks on plant growth and nutrition.

Modification of nutrient availability via soil microbiome processes can be a key component of PSFs, as plant traits influence the composition and function of soil microbial communities (Grigulis et al., 2013; Laughlin, 2011;Orwin et al., 2010; Vries et al., 2012). Such effects are linked to the quality and/or quantity of available nutrient resources in the soil (Bardgett & Wardle, 2010). Plant–microbe interactions, which range from mutualistic to neutral or parasitic in nature, can shift with varying nutrient conditions (Hoeksema et al., 2010), plant type and plant density (Casper & Castelli, 2007). For example, low nutrient conditions with slow plant growth lead to low N content in plant tissues and detritus, which promotes soil food webs with higher abundances of fungi and slower rates of nutrient cycling (Orwin et al., 2010; Vries et al., 2012). On the other hand, fast-growing plants, with high N-content tissue, release higher-quality resources into the soil, thereby promoting a bacterial-based food web, which is related to a more rapid cycling of nutrients (Orwin et al., 2010; Vries et al., 2012). Individual plants also vary in their growth rates over the course of their development, with most plants first displaying rather slow growth, followed by a period of more rapid growth (Couch et al., 2017; Leite, Ciampitti, Mariano, Vieira-Megda, & Trivelin, 2016). However, we currently have a poor understanding of how different plant growth stages influence PSF's.

The overall aim of the present study was to test whether PSF responses differ across plant developmental stages. We examined the effects of root exudates collected from different plant growth stages on subsequent available soil nutrients and microbial communities, as well as the feedback consequences for plant growth. We hypothesized that root exudates would impact soil microbial community structure and increase the diversity of microbial functional genes related to nutrient cycling. We further hypothesized that root exudates, collected from slow-growing versus fast-growing stage plants, would have differential impacts on soil microbial community structure and functional gene capacity, with exudates from the fast growth stage having a greater impact on the availability of soil nutrients and associated increases in plant growth. To test these hypotheses, we first collected and analysed root exudates from the slow-growing and fast-growing stages of *Arabidopsis* plants under sterile hydroponic conditions. Collected exudates were then applied to soil to determine the impacts on available nutrients and plant growth. Soil microbial community taxonomic and functional structures were also evaluated by bacterial 16S rRNA gene tag sequencing and GeoChip functional gene arrays, respectively (Figure 1), to track microbial community responses to exudate treatments.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material and growth conditions

*Arabidopsis thaliana* (wild-type Columbia (Col-0)) seeds were purchased from Lehle Seeds (Round Rock). For surface sterilization and germination, seeds were rinsed with Clorox bleach (Sodium Hypochlorite, 8.25%) for 1 min, washed with distilled water three times, plated on full strength MS (supplemented with 3% sucrose) agar (1.5%) square plates and then put vertically in a growth chamber (Percival Scientific) at 25 ± 2°C with a photoperiod of 16 hr light/8 hr dark for germination.

### 2.2 | Collection and characterization of root exudates from slow and fast plant growth stages

In the first part of our study, *Arabidopsis* root exudates were collected from slow and fast growth stages, as defined by previous experimental and modelled studies of *Arabidopsis* development (Mündermann, Erasmus, Lane, Coen, & Prusinkiewicz, 2005). The *Arabidopsis* growth curve based on internode lengths fit a "sigmoid curve," consisting of slow-growing, logarithmic, linear and senescence phases. The slow-growing phase was approximately 8–10 days after sowing, and the linear (fast-growing) phase was best captured 22–24 days after sowing (Mündermann et al., 2005). We, therefore, collected exudates

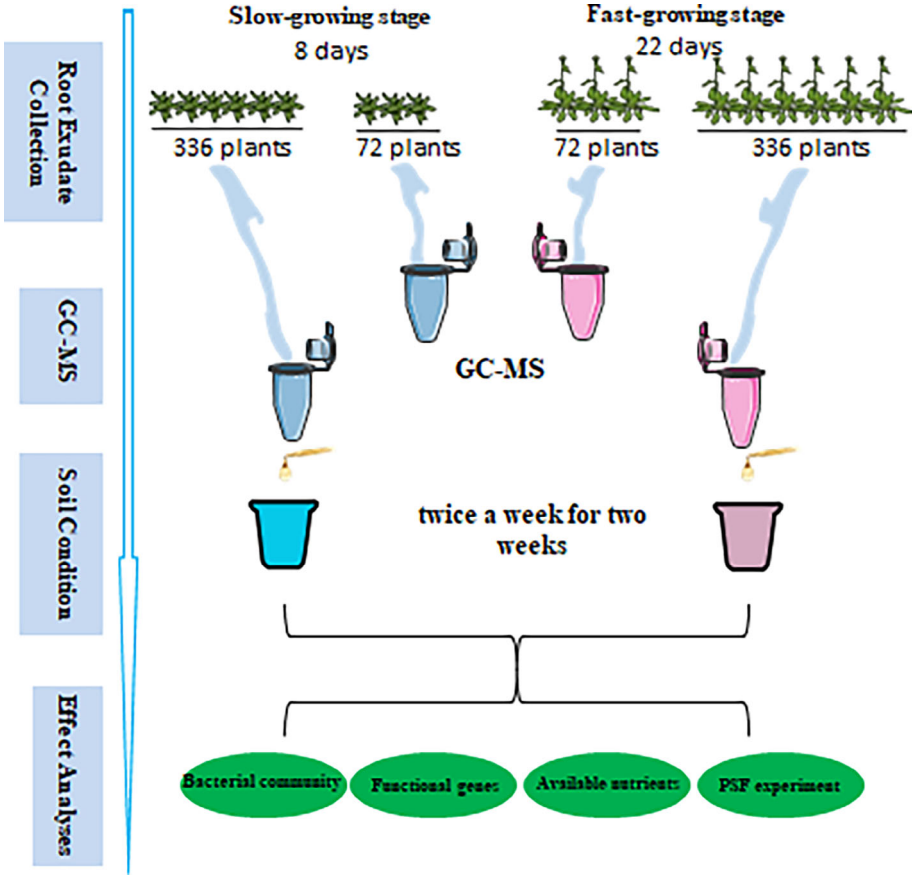


**FIGURE 1** Schematic representation of the experimental design. *Arabidopsis* plants at two growing stages were used to collect root exudates hydroponically. One portion of the collected exudates was analysed with GC–MS, the other was used for soil conditioning. Soils were conditioned with the two different exudate treatments twice a week for 2 weeks. Soil DNA was then extracted and analysed using 16S rRNA amplicon sequencing and GeoChip microarray technologies to assay soil community bacterial composition and functional genes, respectively. Finally, plant-growth feedback effects in the exudate-conditioned soils were investigated in a pot experiment

during these time periods to capture distinct developmental stages of *Arabidopsis* using an established procedure for exudate collection adapted from Dayakar V Badri et al. (2009) and Chaparro et al., (2013). Briefly, pre-cultured 7-day-old seedlings were transferred to sterilized Magenta boxes (each box containing 10 ml of liquid MS with 1% sucrose and one seedling). All boxes were placed on an orbital shaker at 90 rpm under 25 ± 2°C and illuminated by cool white fluorescent light (45 μmol $m^{-2}$ $s^{-1}$) with a photoperiod of 16 hr light/8 hr dark. Nutrient solutions were replaced every week by transferring the plants to new Magenta boxes with 10 ml of fresh liquid MS with 1% sucrose. Prior to exudate collection, plants were transferred to new Magenta boxes with 10 ml of sterile water after removing the surface-adhering exudates by washing mildly with sterile water. The root exudates of each stage were collected over a 2-day period of continuous secretion. Immediately after each collection event, root exudates were filtered through a 0.45 μm (Millipore, MA) to remove root sheathing and root border-like cells and divided into two portions. In total, 4,080 ml of exudates from 408 individual plants were collected for each stage. One 720 ml portion, containing root exudates from 72 individual plants (24 plants for each replication, three replicates), was frozen, subsequently freeze-dried (Labconco, MO) and stored at −20°C for gas chromatography–mass spectrometry (GC–MS) analysis (Figure 1). Three pooled exudate samples were then created for each plant growth stage by combining the exudates of 24 plants. This sampling strategy was adopted to provide sufficient material for GC–MS analysis and ensure three highly representative biological replicates (Chaparro et al., 2013; Chaparro et al., 2014). The other 3,360 ml portion (from 336 individual plants with 112 plants per replicates) was immediately frozen, freeze-dried and finally concentrated five times (2 ml of final volume from one plant), and stored at 4°C for later experimental procedures (see below).

To characterize the chemical composition of root exudates collected from slow-growing and fast-growing stages, freeze-dried root exudates were processed as described by Chaparro et al., (2013) and Badri et al., (2013) and subsequently subjected to gas chromatography–mass spectrometry (GC–MS) analyses at the Genome Center Core Services, University of California Davis. Briefly, extracts were dried under $N_2$ gas and then methoximated and trimethylsilylated. The derivatives were analysed by an Agilent 6890 gas chromatograph (Santa Clara, CA) containing a 30-m-long, 0.25-mm inner diameter rtx5Sil-MS column with an additional 10 m integrated guard column, and the GC–MS conditions described by Badri et al., (2013). The GC injection temperature was 50°C ramped to 250°C by 12°C $s^{-1}$, and the column temperature was first set at 50°C for 1 min, then ramped at 20°C $min^{-1}$ to 330°C and finally held constant for 5 min. The mass spectrometer was used with unit mass resolution at 17 spectra $s^{-1}$ from 80 to 500 Da at −70 eV ionization energy and 1800 V detector voltage with a 230°C transfer line and a 250°C ion source. Metabolites were automatically detected with the BinBase algorithm (Fiehn et al., 2008) and unambiguously identified in comparison with the retention index and mass spectrum of each analyte against the Fiehn mass spectral library. All compounds appeared

consistently across all samples and were, therefore, likely to be of biological origin. The peak area of each compound was normalized based on the internal standard and used for further comparison.

## 2.3 | Soil conditioning with root exudates from different plant growth stages

The soil used in this experiment was collected in July 2014 from the Michigan Extension Station (N42° 05′ 34″, W86° 21′ 19″ W, elevation 630 ft), Benton Harbor, MI. The soil is a sandy loam with nutrient depletion (Table S1). Before being conditioned by root exudates, the soil was brought to 40% of water holding capacity and then stored at 4°C. Cubical pots (125 $cm^3$) were lined with Whatman No. 3 mm filter paper to prevent soil loss as described by Badri et al., (2013). Each pot was then filled with 40 g soil, and all the pots were incubated in a growth chamber at 25 ± 2°C for 2 weeks prior to the application of the root exudates. Seedlings from the soil's resident seedbank were continuously removed during this 2-week pre-culture period. Collected exudates were applied to the soil without sowing of *Arabidopsis* plants, resulting in three treatments; soil supplemented with slow-growing stage root exudates, soil supplemented with fast-growing stage root exudates and soil given a water control (nine pots in total). Each pot received 4 ml of solution per dose, which is equivalent to the root exudates collected from two plants, added every 3 days, over a 2-week period. Plant root exudate concentrations were different between the slow-growing and fast-growing treatments, reflecting the enhanced exudate production and greater root biomass of the fast-growing stage. Thus, the dosage of exudates applied from fast-growing stage plants was higher than that of the slow-growing plant stage, which we consider to be most representative of the exudate-induced impacts on soils during natural *Arabidopsis* plant development. A total of 27 soil samples (≈ 10 g each) were collected at the end of the experimental period for each treatment. A portion of each sample was stored at −80°C prior to DNA extraction and analysis of available nutrients. The remainder was stored at 4°C for the subsequent experiment designed to determine feedbacks on *Arabidopsis* growth (see below).

## 2.4 | Soil DNA extraction, 16S rRNA gene amplification and total bacterial quantification

For each of the treatments, nine replicate potted soils were conditioned and used as sources of DNA for preparation of amplicon sequencing libraries and quantitative real-time PCR (qPCR). Based on previous reports, nine replicates provide sufficient representativeness and statistical power for downstream community analyses (Chaparro et al., 2014; Yuan et al., 2015). Total genomic DNA was extracted using the PowerSoil DNA Isolation Kit (Mo Bio), according to the manufacturer's instructions using 0.5 g of soil for each replicate collected after root exudate or control treatments (see above). DNA purity and concentration were determined using a Nanodrop ND-1000 spectrophotometer (Thermo, Waltham, MA). The V4 region of the bacterial 16S rRNA gene was PCR-amplified (read1: 5′-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3′, read2: 5′-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3′ and index: 5′-ATTAGAWACCCBDGTAGTCCGG CTGACTGACT-3′) from the isolated DNA using individually barcoded dual-indexed primers, as designed by Kozich, Westcott, Baxter, Highlander, and Schloss (2013). PCR amplification was performed under the following conditions: the reaction mixture (25 μl) contained 10 μl of 2.5× HotMasterMix (contains DNA Polymerase, dNTPs and buffer), 1 μl of each primer (10 μmol/μl), 1 μl template DNA (10 ng/μl) and 12 μl $ddH_2O$; PCR included 30 s at 95°C, followed by 30 cycles of 95°C for 20 s, 55°C for 15 s and 72°C for 50 s, with a final extension of 15 s at 72°C in an Applied Biosystems thermal cycler (GeneAmpPCR system 2700). PCR products of appropriate size were excised from a 1.5% agarose gel, following gel electrophoresis, and purified using AMPure beads (Agencourt) prior to Illumina MiSeq sequencing at the Roy J. Carver Biotechnology Center, University of Illinois, Urbana-Champaign.

We used quantitative PCR to quantify total soil bacteria after conditioning with root exudates using primers targeting bacterial 16S rRNA genes. All qPCR amplifications were performed on the Applied Biosystem 7,500 Real-Time PCR System (ABI) using SYBR Premix Ex Taq (TliRnaseH Plus) (TaKaRa Biotechnology Co., Ltd). Protocols for qPCR were performed as previously described using the primers 347F: 5′-GGAGGCAGCAGTRRGGAAT-3′ and Bact531R: 5′-CTNYGTMTTACCGCGGCTGC-3′ (Yuan et al., 2016). Quantitative PCR was performed for the three technical replicates for every sample, and amplification specificity was verified by melt-curve analysis and agarose gel electrophoresis. Amplification efficiency and $R^2$ values were 101.94% and 0.9993, respectively.

## 2.5 | DNA sequence processing

Illumina MiSeq raw sequences were processed and analysed using the Quantitative Insights into Microbial Ecology (QIIME) software package (version 1.8.0) (Caporaso et al., 2010). Pair-end sequences were truncated to 180 bp with the FASTX-Toolkit (Gordon & Hannon, 2010) to increase overall read quality. The trimmed forward and reverse read pairs were then joined, de-multiplexed and quality controlled using the default arguments in the join_paired_ends.py and split_libraries_fastq.py, respectively. The remaining sequences were clustered into operational taxonomic units (OTUs) at a level of 97% sequence identity using a UCLUST-based (Edgar, 2010) open reference OTU picking workflow against the Greengenes 13_8 reference database (McDonald et al., 2012). The OTUs identified as chimeras via UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011), as well as singletons, were removed from further downstream analyses. Representative sequences were taxonomically assigned using an RDP naïve Bayesian rRNA Classifier (Wang, Garrity, Tiedje, & Cole, 2007) with a confidence threshold of 80%. All OTUs annotated as chloroplasts and mitochondria were removed from the OTU table, and all samples were then rarefied to 6,183 sequences to even the sampling effort. The

relative abundance of a given phylogenetic group was set as the number of sequences affiliated with that group divided by the total number of sequences per sample. In total, Illumina MiSeq sequencing generated 6,183–19,045 high-quality 16S rRNA gene sequences per sample classified into a total of 5,560 OTUs at the 97% sequence similarity level. Where noted, high abundance (Relative abundance >0.1%) core identified genera were also investigated using previously described methods (Bowen et al., 2017).

## 2.6 | GeoChip analysis

Soil microbial functional genes were analysed with GeoChip 5.0, and analyses were performed by the Institute for Environmental Genomics and Department of Microbiology and Plant Biology at the University of Oklahoma (Norman, OK). Briefly, purified soil gDNA (500 ng) was fluorescently labelled and then hybridized to GeoChip 5.0 microarrays at 67°C, in a rotator revolving at 20 rpm, for 24 hr in a hybridization oven (Agilent Technologies, Cornelius, OR). After hybridization, the arrays were washed at room temperature with the Agilent hybridization buffer, and scanned with a NimbleGen MS200 Microarray Scanner (Roche NimbleGen, Inc., Madison, WI). Probe signal intensities were quality filtered and normalized using previously described methods (Li et al., 2017). A total of nine samples were analysed in triplicate per treatment.

## 2.7 | Soil nutrient availability after exudate conditioning

In order to investigate soil nutrients required for plant growth, we analysed ammonium ($NH_4^+$), nitrate ($NO_3^-$), available phosphorus (AP) and available potassium (AK) contents after exudate conditioning (before plant growth). For soil analyses, 5 g of soil of each replicate was collected and analysed at the Soil, Water and Plant Testing Laboratory at Colorado State University. Briefly, $NO_3^-$ and $NH_4^+$ contents were determined using a Continuous Flow Analyzer (Mbuthia et al., 2015). Soil AK was determined by flame photometry after extraction with ammonium acetate solution, and AP was determined using the molybdenum blue method after extraction with sodium bicarbonate (Saber, Guirguis, & Zanati, 1973; Silva, Koralage, Weerasinghe, & Silva, 2015).

## 2.8 | Impacts of soil exudate conditioning on plant growth

The preparation of sterile *Arabidopsis thaliana* seedlings was as described above. 7-day-old sterile seedlings were transplanted into pots containing soils that had been subjected to one of the two exudate treatments or the water control (nine pots per treatment). Pots were then incubated in a growth chamber (Percival Scientific) with a photoperiod of 16 hr of light/8 hr of dark at 25 ± 2°C for 3 weeks and watered frequently. After 3 weeks of growth, the *Arabidopsis* plants were harvested to measure the fresh shoot weight. After plant harvest, new *Arabidopsis* plants were re-planted in the same soil for a total of two generations, resulting in 18 replicate plants per soil condition.

## 2.9 | Cumulative impact of root exudates from different growth stages on soil nutrient availability and plant growth

A second pot experiment using exudate-conditioned soils was then designed to reflect natural plant growth conditions by treating plants with root exudates from different growth stages and to examine the impacts of root exudates applied in a reverse chronosequence. Each treatment consisted of nine pots, using the conditioned soils generated from the plant growth and soil nutrient availability analyses described above. To this end, we again collected root exudates, but from a total of three growth stages: slow-growing stage (S), pre-fast-growing stage (P) and fast-growing stage (F). Root exudates were collected as described above, but with the addition of the intermediate exudate collection time frame, which was 15–17 days after sowing. The root exudates were then concentrated to 2 ml/plant. As described above, 40 g soil was added to each pot and pre-cultured in a growth chamber at 25 + 2°C for 2 weeks. This experiment consisted of seven treatments as outlined in Table 1. All exudate treatments were applied in 4 ml doses, every 3 days, for a 2-week period, and sterile *Arabidopsis* seedlings sown one per pot with nine replicate pots per treatment.

## 2.10 | Statistical analysis

One-way ANOVA with the Duncan's multiple range test was performed for multiple comparisons to determine the significant

**TABLE 1** Treatments to evaluate cumulative impacts of root exudates.

| Code | Treatment | Duration |
|---|---|---|
| C | Water | 2 weeks |
| S | Slow-growing stage exudates | 2 weeks |
| SP | Slow-growing stage exudates for 2 weeks, followed pre-fast-growing stage exudates for 2 weeks | 4 weeks |
| SPF | Slow-growing stage exudates for 2 weeks, followed by pre-fast-growing stage exudates for another 2 weeks, then fast-growing stage exudates for an additional 2 weeks | 6 weeks |
| F | Fast-growing stage exudates for 2 weeks | 2 weeks |
| FP | Fast-growing stage exudates for 2 weeks, followed by pre-fast-growing stage exudates for another 2 weeks | 4 weeks |
| FPS | Fast-growing stage exudates for 2 weeks, followed by pre-fast-growing stage exudates for another 2 weeks, and then slow-growing stage exudates for 2 weeks | 6 weeks |

differences of the following: total soil bacteria copy number, Shannon diversity indices of microbial functional genes, normalized abundance of key N and P cycling genes, plant available nutrient contents and plant biomass. Two-tailed, unpaired *t*-tests were performed to compare groups with respect to Shannon diversity and evenness indices of root exudates. All statistical tests performed in this study were considered significant at $p < .05$ using SPSS version 17.0 software (SPSS Inc., Chicago, IL). The R platform (version 3.5.3) was used to run the following analyses and to generate plots using the "ggplot2" package. Calculations of Shannon diversity and evenness indices based on the 16S OTU table, root exudate profiles and GeoChip data were conducted using the "vegan" package (Dombrowski et al., 2017). To compare the differences in bacterial community structure, root exudate profiles and GeoChip data across all samples, unweighted UniFrac principal coordinate analysis (PCoA, based on the Bray–Curtis distance metric) and principal components analysis (PCA, based on the Euclidean distance metric) were performed, respectively, and the differences in exudates or community profiles among treatments were tested using PERMANOVA (Permutational multivariate analyses of variance, adonis, transformed data by Bray–Curtis, permutation = 999) with the "vegan" package. In addition, after "normalize" transformation of the obtained OTU table, multiple regression tree (MRT) (De'Ath, 2001) analysis, based on the Bray–Curtis distance matrix, was conducted to evaluate root exudate effects on the bacterial communities using "vegan," "mvpart" and "MVPARTwrap" package). Testing of linear discriminant analysis effect size (LEfSe) was performed to identify significant differences in bacterial taxa across the three treatments using Kruskal–Wallis (KW) sum-rank test. Linear discriminant analysis (LDA, with a score of ≥2) was then performed to estimate the effect size of each differentially abundant taxon (Segata et al., 2011). The correlation between root exudate profiles and bacterial phylogeny, and between root exudate profiles and functional gene profiles, was estimated using a Mantel test (type = Spearman) in the "vegan" package. In addition, Pearson correlation analyses were calculated in the "corrplot" package and used to reveal correlations between (a) the abundances of selected microbial phyla and root exudate categories, (b) the abundances of selected root exudate categories and functional gene profiles (categories and genes) and (c) available nutrient contents in soils and plant biomass. Redundancy analyses (RDA) were performed to determine properties significantly driving microbial community structure, and then a partial variation partitioning analysis (VPA) was performed in the "vegan" package to calculate the proportion of contribution to the community structure for each property.

## 3 | RESULTS

### 3.1 | Composition of root exudates from slow and fast plant growth stages

A total of 534 compounds were detected in the root exudates recovered from the different plant growth stages, of which 202 were identified by GC–MS and placed into compound classes (Supplementary files). PCA ordination showed that the root exudate profiles from slow and fast growth stages were significantly separated from each other (*adonis*, $r^2 = 0.84$, $p < .001$; Figure 2a), with the first two principal components explaining 93.1% (90.8 and 2.3%) of the total variation. The types of chemicals were the same across the two growth stages, but distinct differences between the abundance of some compounds were detected. Furthermore, the chemical diversity and evenness of the root exudate profiles from the fast growth stage were higher than those recovered from the slow growth stage plants (Figure 2b). Identified compounds were categorized into sugars (26), sugar acids (7), sugar alcohols (25), organic acids (60), nucleotides (12), amino acids (35), amides/amines (17) and others (20). Fast-growing stage plants secreted a higher quantity of sugars, sugar alcohols, organic acids, amino acids, amides/amines and unknown compounds than plants in the slow-growing stage (Figure 2c), with the greatest difference found for the amino acids category. The relative abundances of sugars, sugar alcohols and organic acids were higher during the slow-growing stage as compared with the fast-growing stage (Figure 2d).

### 3.2 | Impact of root exudates on soil microbial communities

The qPCR data estimated the total population of soil bacteria in the control (water) treatment to be a $\log_{10}$ copies/g soil count of 9.6. Bacterial density of the soils treated with exudates from the fast growth stage was not significantly different from the control (9.51 $\log_{10}$ copies/g soil), but bacterial density was significantly lower in the soils receiving exudates from the slow plant growth stage (9.20 $\log_{10}$ copies/g soil; Table S2).

Proteobacteria, Acidobacteria and Actinobacteria were the three most dominant phyla across all samples. Together, these phyla accounted for 48.43–64.86% of the total bacterial sequences recovered (27.85, 15.82 and 14.97%, respectively) (Figure 3a). The other major phyla (average relative abundance >1%) were Bacteroidetes (9.74%), Firmicutes (6.82%), Cyanobacteria (5.56%), Verrucomicrobia (4.02%), Thaumarchaeota (2.95%), Planctomycetes (1.68%) and Gemmatimonadetes (2.10%). The relative abundances of certain phyla differed between the two root exudate treatments (LDA scores >2, Kruskal–Wallis). Root exudates from the fast plant growth stage enriched the relative abundance of Actinobacteria and Firmicutes, while those from the slow growth stage promoted the relative abundance of Acidobacteria, Bacteroidetes, Planctomycetes and Thaumarchaeota (Figure S1). To assess the effects of root exudates from different developmental stages on soil microbiomes, PCoA ordination plots based on the weighted Unifrac and Bray–Curtis distances were performed and revealed three clearly separated groups (Figure 3b). The soil microbiome from each treatment was significantly (*adonis*, weighted Unifrac: $r^2 = 0.798$, $p < .001$; Bray–Curtis: $r^2 = 0.662$, $p < .001$) different from the others. Multivariate regression trees (MRT) revealed that the soil bacterial community of the slow-growing stage treatment was more similar to the control than the fast-growing stage exudate treatment and that it was significantly

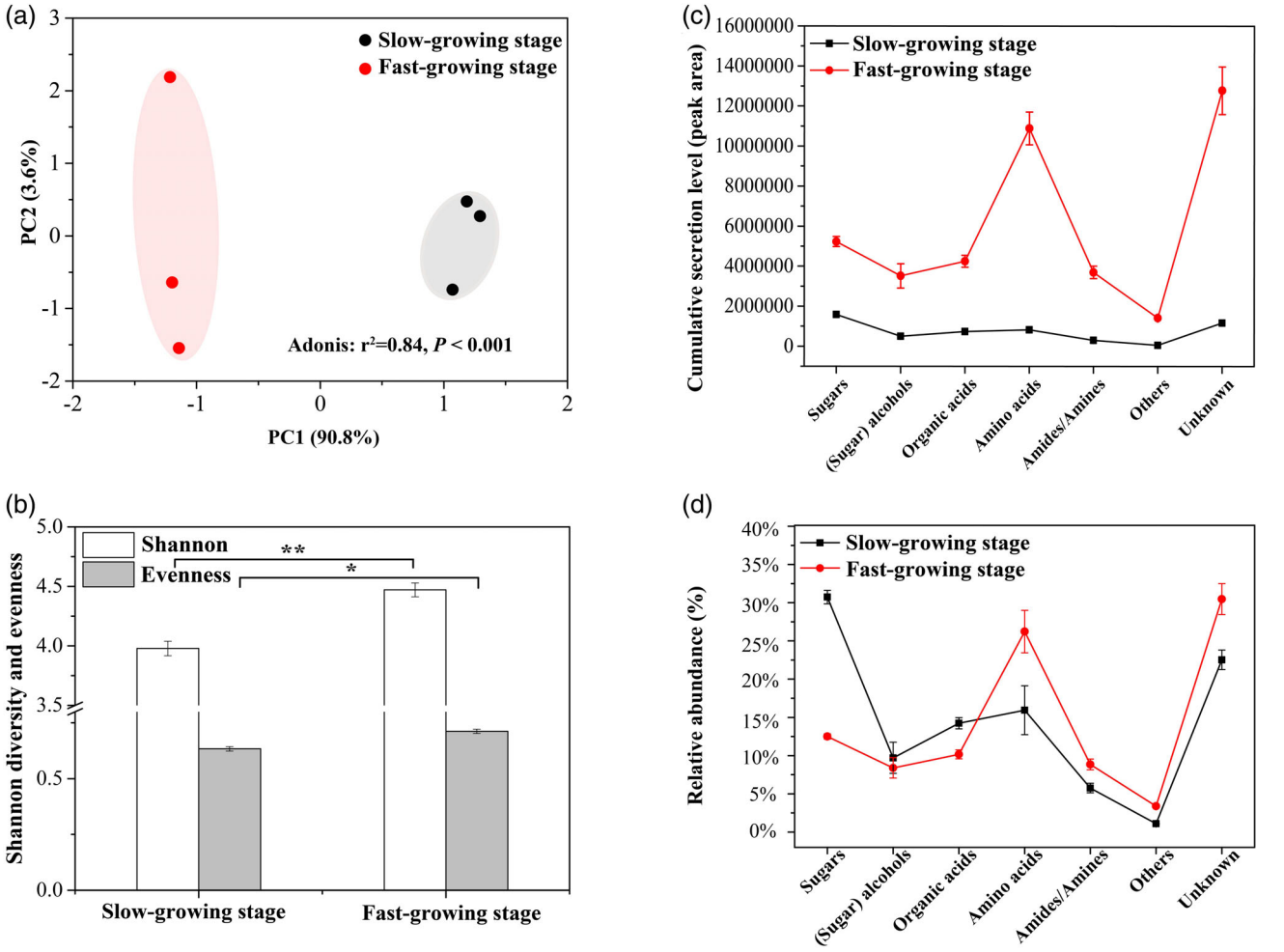


**FIGURE 2** (a) PCA analyses of root exudates of both slow-growing stage and fast-growing plant stages analysed by GC–MS. The exudate profiles from the two growth stages were significantly different (adonis, $r^2 = 0.84$, $p < .001$, $n = 3$). (b) Shannon diversity and evenness of root exudates profiles. "*" and "**" mean significant differences ($p < .05$ and $p < .01$, respectively, two-tailed and unpaired t-tests) of evenness between slow-growing and fast-growing stages ($n = 3$). (c) Cumulative secretion level of compounds in each category from the slow and fast growth stages. (d) Relative abundance of compounds in each category from the slow and fast growth stages [Colour figure can be viewed at wileyonlinelibrary.com]

separated from fast-growing stage treatment (Figure 3c). This indicates that root exudates from the fast-growing stage plants exerted a stronger influence on the soil microbiome than those from the slow-growing stage plants. Correlation analysis between compositions of microbial community and exudates indicated that the relative abundance of Bacteroidetes, Firmicutes and Actinobacteria was positively correlated to exudate categories in both stages, while Acidobacteria, Planctomycetes, Verrucomicrobia, Gemmatimonadetes and Proteobacteria negatively correlated to most of the categories of exudates (Figure 3a). The soil subjected to exudates from the fast growth stage exudate hosted a community with lower *alpha*-diversity than the other two treatments, whose values for *alpha*-diversity were very similar (Table S3).

Further examination of core genera, as defined by Bowen et al. (2017), indicated that of the core taxa, root exudates from the fast-growing stage plants increased the relative abundances of *Arthrobacter* and *Bacillus* compared the slow-growing stage exudate treatment and the control treatment. Root exudates from the slow-growing stage did support a higher relative abundance of *Gp16*, *Gp10*, *Lysobacter*, *Gp3*, *Aridibacter* and compared to the fast-growing exudate and control treatments (Figure S2, Figure S3). Interestingly, the relative abundance of core microbiome taxa (taxa present in 100% of samples) was significantly higher in the fast-growing stage exudate treatment (80.45%) as compared to the slow-growing stage (72.36%) and control treatments (64.86%).

### 3.3 | Impact of root exudate application on the diversity and structure of microbial functional genes

A total of 27,358 functional gene probes were detected across all three treatments. The control microbial community had the lowest number of detected functional genes (14,943), while the other two root exudate treatments were rather similar, with 22,432 and 23,145

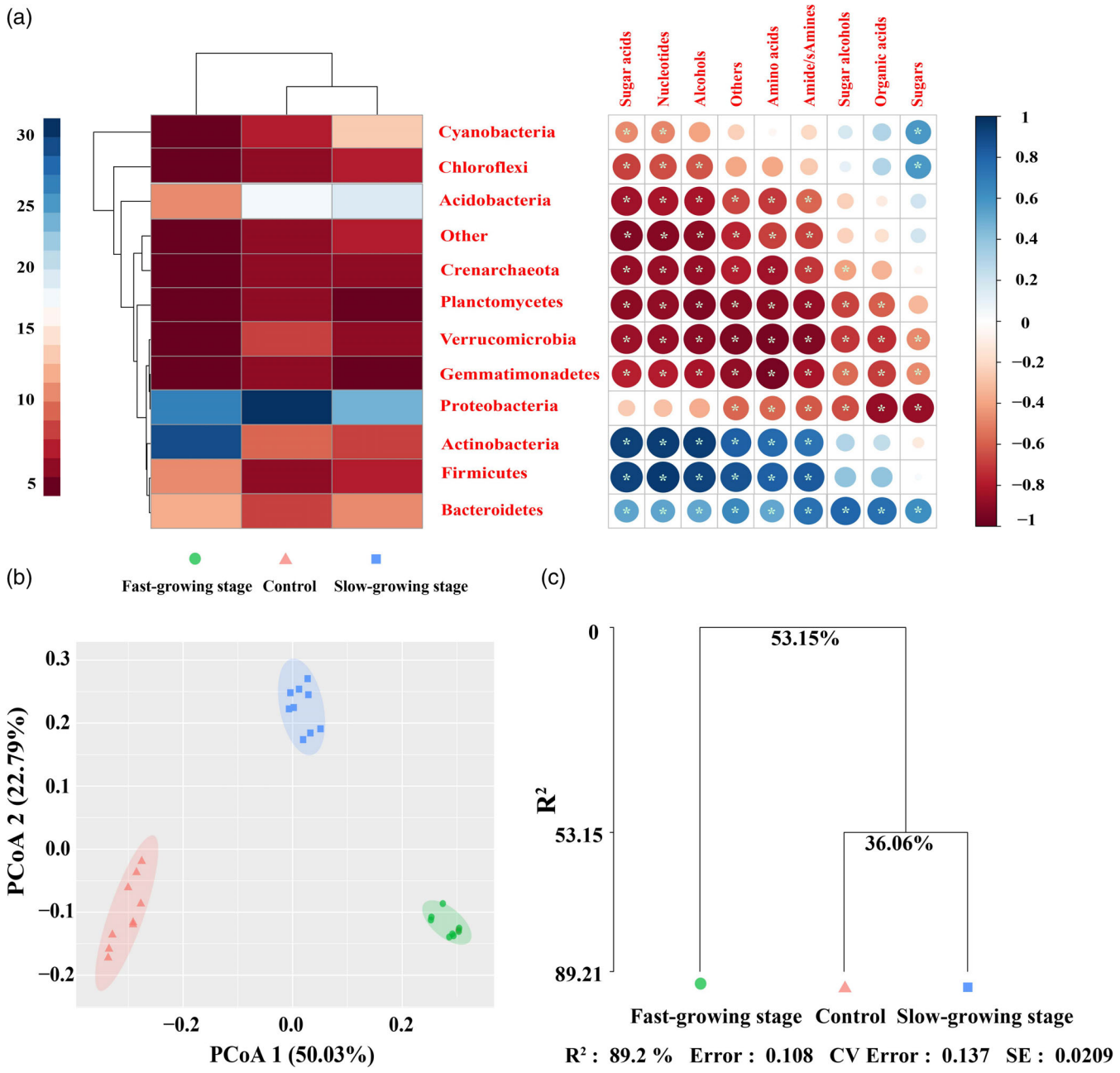


**FIGURE 3** (a) Relative abundance of major bacterial phyla detected in the bacterial communities of control and root exudate-conditioned soils, and correlation analysis of bacterial community compositions and exudate components. * indicates significant differences ($p < .05$). (b) Principal coordinates analysis (PCoA) of the pairwise community dissimilarity (Bray–Curtis index) of the microbial communities as analysed by MiSeq 16S rRNA gene tag sequencing. (c) Multivariate regression trees (MRT) analysis of the bacterial communities of control and root exudate-conditioned soils [Colour figure can be viewed at wileyonlinelibrary.com]

positive probes from the fast-growing and slow-growing exudate treatments, respectively (Figure 4a). Higher functional diversities (defined here as the number of gene probes) of nutrient cycling (N and P) genes were detected in the two exudate-conditioned soils as compared to the control treatment. The average detected signal intensity for probes in these genes categories was notably higher in the slow growth stage exudate samples (Figure S4), as compared to both the control and fast-growing stage samples. In addition, one-way ANOVA analysis revealed that root exudates caused a significant ($p < .01$) increase in functional gene Shannon diversity (Figure 4b) in both exudate treatments, as compared to the control.

Principal components analysis (PCA) of all detected functional genes revealed that samples from root exudate treatments harboured gene assemblages that were distinct (adonis, $p < .01$) from the water control (Figure 4c). The PCA profile indicated that the functional gene composition of the three treatments were significantly different from one another. The normalized signal intensity of functional genes involved N and P cycling showed significantly (ANOVA, $p < .05$)

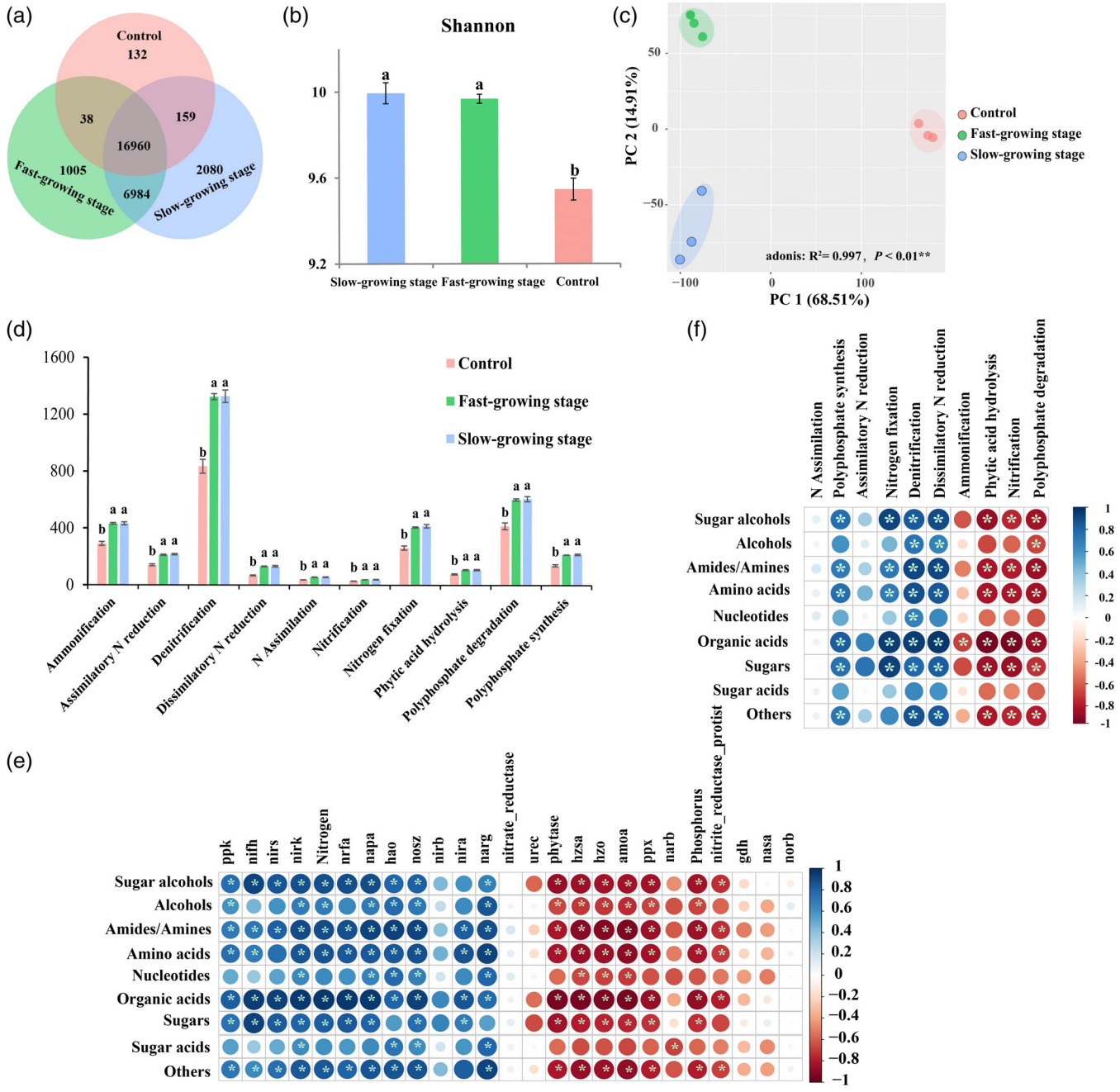


**FIGURE 4** (a) Venn diagram analysis of the functional genes in control and root exudate-conditioned soils. (b) Shannon diversity of soil bacterial functional genes detected by GeoChip analysis. (c) PCA analysis of soil bacterial functional genes of control and root exudate-conditioned soils. (d) Effects of root exudates on the normalized abundance of key N and P cycling genes. Different letters indicate significant differences ($p < .05$, one-way ANOVA, LSD post hoc test) among treatments (n = 3). (e) Correlation analysis of functional gene abundance and exudates components. * indicates significant correlation ($p < .05$, Pearson correlation). Circle size and colour shade represent the magnitude and direction of the *R* value of correlation analysis (Blue and red represent positive and negative correlations, respectively). (f) Correlation analysis of functional categories abundances with exudate components. * indicates significant correlation ($p < .05$, Pearson correlation). Circle size and colour shade represent the magnitude and direction of the *R* value of correlation analysis (Blue and red represent positive and negative correlation, respectively) [Colour figure can be viewed at wileyonlinelibrary.com]

higher gene abundances in both root exudate-conditioned treatments compared to the control treatment (Figure 4d).

Correlation analysis between compositions of microbial functional genes (involved in N and P cycling) and exudates indicated that the relative abundance of *ppk*, *nifh*, *nirs*, *nirk*, *nrfa napa*, *narg* and *nirb* gene targets were positively related to root exudate categories, while *phytase*, *hzsa*, *hzo*, *amoma* and *ppx* gene targets were negatively correlated to most of the categories of root exudates (Figure 4e,

Supplementary files). Thus, exudate conditioning significantly increased the genetic potential for most of the soil N cycling processes, including N fixation, denitrification and dissimilatory N reduction, and decreased the relative abundances of genes involved in soil P-cycling processes like phytic acid hydrolysis and polyphosphate degradation (Figure 4f, Supplementary files).

### 3.4 | Impact of root exudates on soil available nutrient content and plant growth

Soil $NO_3^-$, AP and AK contents were higher in the fast-growing stage root exudate-conditioned soil (Figure 5b-d), although the highest $NH_4^+$ content was found in the treatment conditioned with slow growth stage root exudates (Figure 5a).

Aboveground plant biomass was significantly ($p < .05$) higher in the fast-growing stage root exudate treatment, as compared to the control and the slow-growing stage exudate treatment (Figure 5e). We also observed a strong ($p < .05$) positive relationship between biomass and soil $NO_3^-$, AP and AK contents (Figure S5).

### 3.5 | Cumulative impact of root exudates from different growth stages on soil nutrient availability and plant growth

We continuously applied different stage root exudates to evaluate the cumulative effect on soil nutrient availability and plant growth (Figure 6). After measurement of the available nutrients present in each conditioned soil, we found that the root exudates of slow-growing stage and pre-fast-growing stage plants did not stimulate the availability of soil nutrients or plant growth, while this was the case for the fast-growing stage exudates (Figure 6a-d). There was, however, no similar cumulative effect on $NH_4^+$ content (Figure 6b). Fresh plant biomass data revealed that the fast-growing stage plant root exudates, whether applied alone or successively with exudates from other growth stages, stimulated plant growth as compared to the slow-growing stage and pre-fast-growing stage exudate treatments (Figure 6e). Interestingly, the most natural exudate sequence (SPF) did not result in the highest biomass or soil available nutrients as compared to, for instance, the FP and FPS treatments. These results indicate that the greatest plant growth-promoting effects could be attributed to fast-growing stage root exudates.

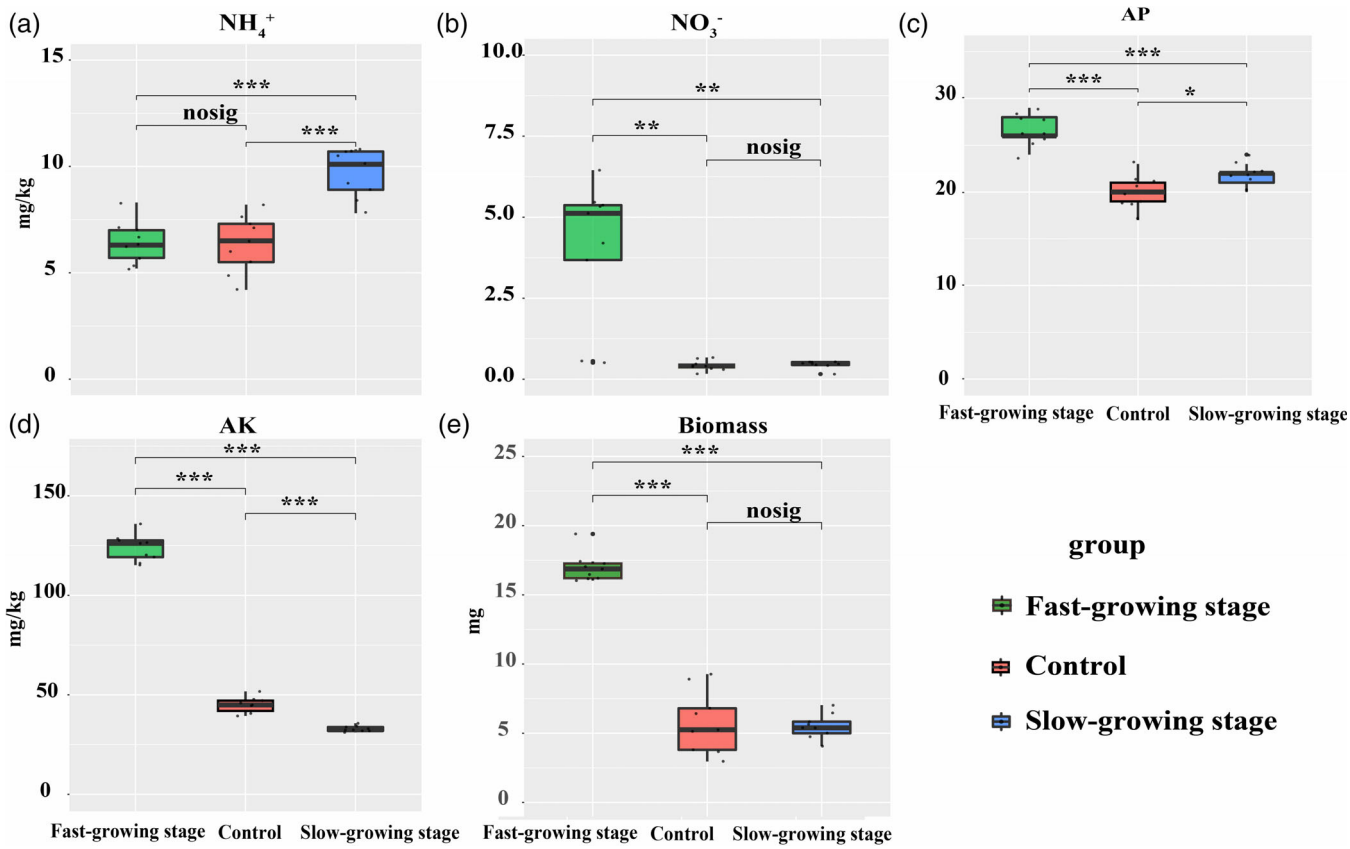


**FIGURE 5** Soil available nutrient contents and biomass of control or root exudate-conditioned soils. Available nutrients included nitrate, ammonium, phosphorus and potassium. "*" , "**" and "***" indicate significant differences ($p < .05$, $p < .01$ and $p < .001$, respectively (n = 3) [Colour figure can be viewed at wileyonlinelibrary.com]

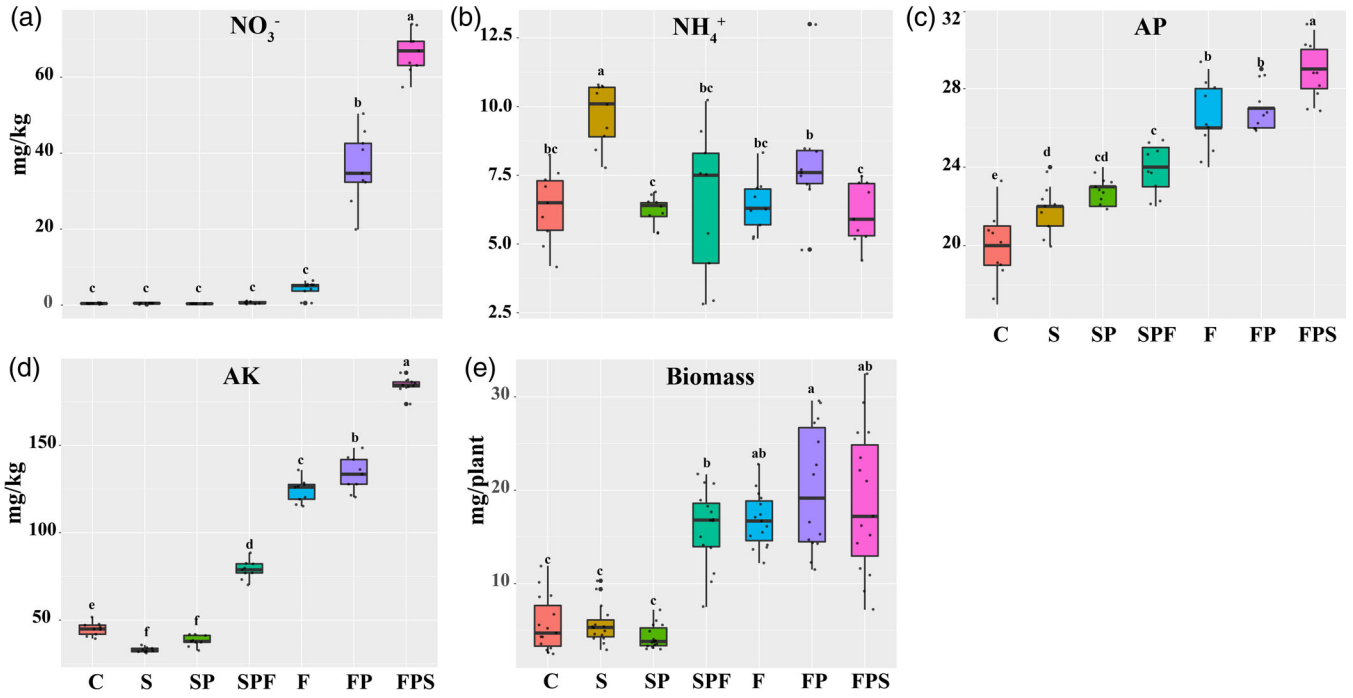


**FIGURE 6** Treatment design and cumulative effect of root exudates from different growth stage on soil available nutrient contents and biomass. (a) nitrate ($n$ = 3); (b) ammonium ($n$ = 3); (c) available phosphorus ($n$ = 3); (d) available potassium ($n$ = 3); (e) fresh biomass ($n$ = 6–9). Different letters indicate significant differences ($p$ < .05, one-way ANOVA, LSD post hoc test) among treatments [Colour figure can be viewed at wileyonlinelibrary.com]

### 3.6 | Linkages between microbial communities, root exudates, soil available nutrients and plant biomass

Mantel tests revealed a significant positive correlation between root exudate profiles and bacterial phylogeny (Spearman: $r$ = 0.72, $p$ = .001) and between root exudate profiles and functional gene profiles (Spearman: $r$ = 0.26, $p$ = .049). Redundancy analysis (RDA) identified a significant correlation ($p$ < .01) between microbial composition based on either functional genes or on 16S rRNA genes and a set of key soil nutrients, root exudate components and plant biomass (Figure 7a, b). The adopted models explained 59.01% and 78.92% of the total variation in the 16S rRNA gene data and functional gene composition, respectively.

The bacterial communities of soils receiving exudates from the fast growth stage were associated with higher abundances of organic acids, sugar acids, AP, AK and nitrate, while the communities of soils receiving exudates from the slow growth stage were related to higher abundances of amino acids, alcohols and ammonium. We also found that plant biomass correlated with the microbiome and all nutrients that were impacted by the exudates of the fast-growing stage. Similarly, RDA revealed a relationship between root exudates and soil available nutrient profiles and functional gene profiles (Figure 7b). All soil characteristics, as well as plant biomass, were correlated with root exudate conditioning and soil microbial functional potential. Partial CCA-based variation partitioning analysis (VPA) demonstrated that root exudates explained 27.1% of the variation in bacterial phylogenetic community structure, with soil available nutrients explaining 4.9% of this variation (Figure 7c). Root exudates explained 57.4% of the variation in functional gene composition, yet soil available nutrients had no discernable explanatory power (Figure 7d). These results suggest that exudate applications directly impacted microbial community taxa and functional gene composition. Although the root exudates from the two plant growth stages had disparate impacts on microbial community structure and available nutrient levels, they had similar effects in increasing detectable microbial functional gene diversity (Figure 7e).

## 4 | DISCUSSION

Numerous studies have indicated that rhizosphere soil microbial communities can shift over the course of plant development, an observation that has been attributed to changes in root exudate profiles over time (Chaparro et al., 2014; De-la-Peña et al., 2010; Kowalchuk, Buma, de Boer, Klinkhamer, & van Veen, 2002; Li, Rui, Mao, Yannarell, & Mackie, 2014). Our study supports this conclusion, and further revealed that the impacts of root exudates from the fast plant growth developmental stage were stronger than those collected during a period of slow plant growth. This might simply be attributed to a general increase in the quantity of exudates collected during the faster growth phase. However, we observed qualitative differences in exudate profiles recovered from different plant growth stages that could be linked to shifts in distinct microbial taxa within the soil

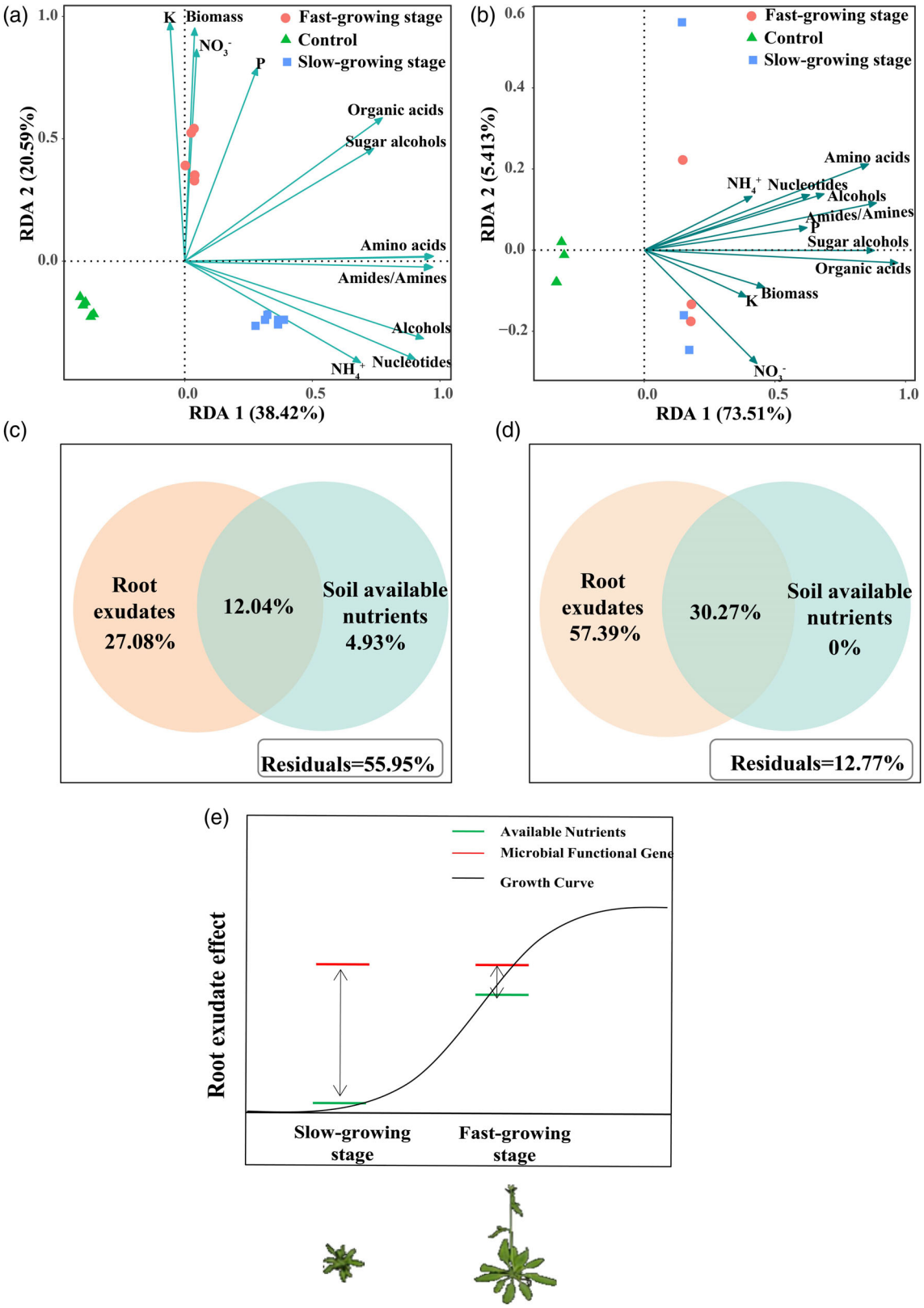


**FIGURE 7** Legend on next page.

community. These results thus support the notion that specific compounds within changing plant exudate profiles may drive soil microbial dynamics (Badri et al., 2013).

Mature ecosystems, typically, are comprised of individuals or groups that possess some level of functional redundancy (Allison & Martiny, 2008; Moya & Ferrer, 2016; Rosenfeld, 2002), which can be important to maintain function after a disturbance of microbial structure (Botton, Heusden, Parsons, Smidt, & Van Straalen, 2006; Wohl, Arora, & Gladstone, 2004). In this study, the taxonomic bacterial communities of soils conditioned by root exudates collected at different developmental stages were significantly different from each other, but the categories of soil microbial functional capacities evaluated by GeoChip analysis were similar. Both conditioned soils did, however, have functional capacities that were significantly different from the control soil (Figure 4d). This could reflect a level of microbial community functional redundancy that was stimulated by the root exudates. In addition, these results reflect the potential for root exudates to increase the functional potential of the soil microbial community, which may be to the plants' benefit. Exudate concentrations can play a major role in shaping microbial function gene abundances (Badri et al., 2013). In our study design, treatments with exudates from the slow growth stage received lower total exudate input as compared to the treatment with fast growth stage exudates, as we sought to reflect that natural dynamics of exudation (Figure 2). The exudate doses corresponded to approximately the input of two plants for each 40 g soil sample, irrespective of the plant stage. It is noteworthy, that this differential exudate dose did not result in different levels of functional gene abundances upon application to soil. Furthermore, the soil bacterial population significantly decreased when the soil was conditioned by root exudates collected from the slow growth stage. Thus, in this study, both the concentration of exudates and the type of exudates varied with growth stages, with ultimate impacts on microbial communities and soil microbial functional capacities being a product of these two factors.

It has been widely reported that plants release photosynthates to surrounding soils as a means of increasing nutrient availability either directly or via their associated microbiome (De Deyn, Cornelissen, & Bardgett, 2008; Lambers, Raven, Shaver, & Smith, 2008). However, the timing of such nutrient mining activities has generally remained unclear. Our results not only support the notion of a nutrient mining function for an exudate-stimulated soil microbial community, but also could indicate a co-adaptation process between the host plant and soil microbiome with exudates from the fast growth stage exhibiting the strongest impact on soil microbial communities and yielding the highest levels of available nutrients (Figure 5). These results were further supported by the cumulative application of exudates from different growth stages, which showed that only the exudates from fast growth stage plants provide increases in soil nutrient levels. Many studies of root-exudate-mediated microbial defence have reported good models of co-evolution between plants and soil microbes, such as the "cry for help" and "legacy" effects (Weller, Raaijmakers, Gardener, & Thomashow, 2002; Yuan et al., 2018), where plants select for microbial communities that help suppress plant pathogens. Our study revealed a co-evolution-like relationship between the plant and soil microbes that facilitates plant growth by optimizing the nutritional benefits provided by plant-associated microbes. Although the potential to mobilize soil nutrients is clearly already present in the soil microbiome, its stimulation by plant exudations patterns, specifically those from the fast plant growth stage, boost the functional capacity of the community. This paradigm is similar to the observed differences between slow-growing and fast-growing plant species, which exhibit different influences on soil microbial composition and soil nutrition availability (Bardgett & Wardle, 2010; Orwin et al., 2010; Vries et al., 2012). Potentially, the high level of soil nutrients and plant biomass in soils conditioned with fast growth stage root exudates was caused by higher quantities of nutrients in these high-dose exudates. However, we did not observe cumulative effects of exudate treatments that were mistimed. For instance, the SPF and FPS treatments yielded widely disparate results with respect to nutrient contents, despite receiving the same amount of total exudates.

In most soils, available mineral nutrients are not sufficient to meet optimal plant growth demands. Thus, plants have evolved systems to recruit symbiotic microbial partners that help make nutrients available (Adesemoye & Kloepper, 2009; Gyaneshwar, Kumar, Parekh, & Poole, 2002; Landeweert, Hoffland, Finlay, Kuyper, & van Breemen, 2001; Tinker, 1984). These relationships can be "fine-tuned" based on the plant's current nutrient demands and the soil nutrient availability. For example, N-fixing microbial associations increase when plants suffer from N deficiency (Chapman, Langley, Hart, & Koch, 2006), and arbuscular mycorrhizal fungi become more important and abundant when plants are under phosphorous limitation (Feddermann, Finlay, Boller, & Elfstrand, 2010; Hu et al., 2009). Our study revealed a similar pattern. The plant does not release a complex mixture of exudates when the nutrient uptake rates are probably relatively low, as is the case in the slow growth stage. The community does, however, undergo a shift in community structure and displays an increase in relative functional potential. Then, when entering fast growth phase, when plant nutrient demands are high, the plant can build upon these initial steps to, via enhanced exudate production,

**FIGURE 7** (a) Redundancy analysis (RDA) of abundant bacterial phyla, soil available nutrients, root exudate compounds and plant biomass from the different treatments. (b) Redundancy analysis (RDA) of bacterial functional gene categories, available nutrients, root exudate compounds and plant biomass from the different treatments. (c) Variation partitioning analysis (VPA) of the effects of soil available nutrients, root exudate compounds and interactions between them on bacterial community structure. (d) Variation partitioning analysis (VPA) of the effects of soil available nutrients, root exudate compounds and interactions between them on bacterial functional gene diversity. (e) Variations of soil available nutrition level and microbial functional gene relative abundances in the soils conditioned with exudates from the slow-growing and fast-growing stages of plant growth [Colour figure can be viewed at wileyonlinelibrary.com]

help drive differential release of mineral- and organic matter-bound nutrients (Figure S6).

Previous studies have been unable to separate the soil microbial functions, such as nutrient availability and functional capacities in one system. In this study, we speculate that the available nutrient content is a reflection of soil microbial function, and functional gene abundance as functional capacity. Interestingly, the microbial community functional potentials were similar regardless of the growth stage from which exudates were collected. Cumulative application of root exudates also supported the notion that slow-growing stage plant root exudates are not sufficient to activate the soil microbial function to yield higher soil nutrient levels. This could only be achieved via activation by fast-growing stage plant root exudates.

## 5 | CONCLUSIONS

Our results revealed that root exudates collected from fast growth stage plants and slow growth stage plants had similar effects on soil microbial functional capacities, but very different impacts microbial community structure and plant available soil nutrients. We further confirmed that these effects are dosage independent and likely depend on both the composition and relative concentrations of exudates. Based on the results presented here, we speculate that plants, via root exudate secretion, increase the functional potential of the soil microbial community, and then further stimulate beneficial functions demanded during later faster-growing stages through an elevated and more complex profile of root exudates.

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### CONFLICT OF INTEREST

The authors declare that they have no competing interests.

### AUTHOR CONTRIBUTIONS

Mengli Zhao, Jun Yuan and Jun Zhao: performed all experiments; Jun Yuan and Qirong Shen designed the study, and wrote the majority of the manuscript; Lauren Hale, Qiwei Huang and George A. Kowalchuk: provided critical comments on the study, and helped write the paper; Jun Yuan, Jun Zhao and Tao Wen: analysed the data; Jorge M. Vivanco and Jizhong Zhou: participated in the design of the study, provided comments and edited the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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