Plant Soil
https://doi.org/10.1007/s11104-023-06409-5

RESEARCH ARTICLE

Adding plant metabolites improve plant phosphorus uptake by altering the rhizosphere bacterial community structure

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Received: 13 June 2023 / Accepted: 20 November 2023

Abstract

Background and aims Plant-derived metabolites play a crucial role in mediating plant–microbe interactions affecting plant development, health and ability to withstand biotic and abiotic stresses. However, how the key plant metabolites, e.g., flavonoids and fatty acids secreted by roots, regulate soil microbial communities to promote plant phosphorus (P) nutrition, growth and development remains unclear.

Methods We determined whether the addition of different concentrations (0, 50 or 500 μmol kg^{-1}) of myristic acid (fatty acid), quercetin, naringenin or luteolin (flavonoids) to the soil to improved soil organic P utilization efficiency and enhanced plant growth by changing microbial community.

Results Flavonoids could directly regulate rhizosphere bacterial community structure, with a significant increase in the relative abundance of Micrococcaceae and Nocardioidaceae by the addition of 50 μmol kg^{-1} of naringenin, luteolin, 500 μmol kg^{-1} of quercetin, naringenin or luteolin. The addition of myristic acid had weaker impact on bacterial community structure. The altered bacterial community structure lead to the increased alkaline phosphatase activity in the rhizosphere to promote the mineralization of organic P, which could facilitate plant growth and P uptake to different extents.

Conclusion Our results indicate that the addition of flavonoids enhanced organic P mineralization by selecting individuals which secreted more phosphatases. These findings can provide guidance for effective manipulation of composition of plant-microbial communities to increase plant P nutrition and/or efficiency of use of P fertilizers.

Keywords Alkaline phosphatase activity · Fatty acid · Flavonoids · Mineralization of organic P · Rhizosphere bacterial community

Responsible Editor: Jan Jansa.

Siji Wang and Shilong Duan contributed equally to this work.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11104-023-06409-5.

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Published online: 06 December 2023

Introduction

Plants are hosts for complex microbial communities, which naturally colonize all plant parts, including roots, stems and leaves (Ofek-Lalzar et al. 2014; Bai et al. 2015; Vogel et al. 2016). Importantly, plants can benefit from these microbes directly or indirectly, with mechanisms including nutritional assistance (i.e., facilitation of nutrient uptake from the soil), disease suppression and increase in tolerance to abiotic and biotic stresses (Mendes et al. 2011; Berendsen et al. 2012; Zolla et al. 2013). Despite the importance of plant-associated microbes, how plants influence microbial assemblage and how the changes in microbial diversity benefit plant growth and development remain poorly understood (Garbeva et al. 2004; Igwe and Vannette 2019). Thanks to the rapid developments in omics technologies, such as metagenomics, metatranscriptomics and metabolomics, the complex interactions between plants and their associated microbes are gradually being revealed (Caporaso et al. 2010; Ofek-Lalzar et al. 2014).

The rhizosphere is a critical interface between plants and soil as well as the site for many globally important fluxes, including those of nutrients and water. A wide variety of microbes inhabit and have functional roles in the rhizosphere, varying in number and diversity from a few thousand to millions (Berendsen et al. 2012). These microbes are regulated by compounds in root exudates and establish complex interactions with plants to coevolve in nature (diCenzo et al. 2020). Root exudates typically comprise a diverse set of micromolecular primary metabolites, such as sugars, amino acids and carboxylates as well as various macromolecular secondary metabolites, including alkaloids, terpenoids, phenylpropanoids and polyphenolic compounds (Badri and Vivanco 2009; Hartmann et al. 2008; Huang et al. 2014; Lu et al. 2021). Besides providing carbon (C) and nitrogen (N) substrates for microbial growth, root exudates have a multitude of effects on rhizosphere microbes by acting as signaling molecules, attractants, stimulants, but also as inhibitors or repellents (Baetz and Martinoia 2014). A growing body of research has shown that plant metabolites can significantly affect the growth and activity of soil bacteria and change the composition of the rhizosphere microbial community. For example, a recent study indicated that a limited distribution of daidzein within a few millimeters of the root surface, has marked effect on the assemblage of rhizosphere bacterial community. Specifically, daidzein possesses dual functions of attracting and repelling bacteria. When the concentration of daidzein was large in the rhizosphere, there was an increase in relative abundance of Comamonadaceae and a decrease in relative abundance of rhizobia, with an overall reduced bacterial α-diversity (Okutani et al. 2020).

Arbuscular mycorrhizal (AM) fungi are the most abundant and widely distributed fungi in the soil and can form symbiotic relationship with more than two thirds of terrestrial plants to help them acquire nutrients from the soil (Brundrett and Tedersoo 2018), especially phosphorus (P). The distribution and community composition of AM fungi in the soil have a major role in facilitating plant growth and impacting the function of terrestrial ecosystems (Hazard et al. 2013). In recent years, some studies have provided new ideas for the growth, development and community regulation of AM fungi. For example, the transfer of fatty acids synthesized in the host plants to AM fungi is thought to be critical for sustaining mycorrhizal symbiosis (Jiang et al. 2017; Luginbuehl et al. 2017), because AM fungi lack genes for their biosynthesis of long-chain fatty acids (Tisserant et al. 2013). Sugiura et al. (2020) discovered the external supply of myristate, a common fatty acid in root exudates detected by Li et al. (2017), can serve as a C and energy source for AM fungi in pure culture conditions, inducing extensive hyphal growth and forming infection-competent secondary spores. But in the soil, how myristate interacts with soil minerals and even other soil microbes to affect the growth and development of AM fungi is unclear. Flavonoids, a typical plant metabolite, have been reported to stimulate AM fungal spore germination and hyphal growth (Tian et al. 2021). A previous study on alfalfa (*Medicago sativa*) showed that quercetin, which is naturally released from alfalfa seeds, increased spore germination, hyphal elongation, and branching of *Glomus* species in vitro (Tsai and Phillips 1991). Specific flavonoid compounds also enhanced the level of mycorrhizal colonization (Scervino et al. 2005). In addition, the same flavonoid compounds exhibited different effects on growth parameters of different AM fungi from the genus *Glomus* and *Gigaspora* (Scervino et al. 2006).

In addition to regulate the growth and development of AM fungi, fatty acids and flavonoids are attracting attention as signal molecules to regulate soil bacterial community structure and function. Myristic acid secreted by tobacco roots can act as chemical signal that significantly induces the chemotactic response and swarming motility of *Ralstonia solanacearum* and stimulate the formation of biofilms of *R. solanacearum*. The addition of exogenous myristic acid strongly attracted *R. solanacearum* to colonize root surfaces (Li et al. 2017). Flavonoids broadly contribute to the diversity of the Arabidopsis rhizosphere microbiome and preferentially attract Aeromonadaceae. A culturable *Aeromonas* sp. H1 isolated from the rhizosphere promoted smooth swimming by transcriptionally enhancing flagellum biogenesis and inhibiting fumarate reduction under the mediation of flavonoids (He et al. 2022). Schütz et al. (2021) found that the exogenous addition of quercetin resulted in significant changes in bacterial community structure, with smaller relative abundances of Chloroflexi, larger percentages of Proteobacteria and Bdellovibrionota.

The plant metabolites secreted by roots which play important roles in regulating rhizosphere microbial community structure have been reported. Firstly, it is important to regulate the symbiosis between plants and AM fungi. Kameoka et al. (2019) observed that palmitoleic acid induced hyphal branching and stimulated the formation of secondary spores. Sugiura et al. (2020) conducted a co-culture experiment using various fatty acids and showed that myristate significantly promoted the growth of AM fungi. Rillig et al (2023) proposed that that the application of myristate increased the biomass of AM fungi in the soil and promoted the symbiotic relationship between AM fungi and plants. Secondly, some plant metabolites have been shown to regulate plant-associated microbial communities (Jacoby et al. 2020). For example, flavonoids regulate specific microbial communities in the rhizosphere (Chen et al. 2019). Flavonoids (apigenin and luteolin) increase the relative abundance of Oxalobacteraceae in the rhizosphere soil (Yu et al. 2021), and the exogenous addition of naringenin promoted rhizoma formation (Abdel-Lateif et al. 2013). The α-diversity of quercetin-treated soil bacteria was reduced and the relative abundance of Pseudarthrobacter was increased in the soil and plant roots (Schütz et al. 2021). Therefore, we selected myristic acid, quercetin, naringenin and luteolin to regulate the soil AM fungi and bacteria, thereby influencing the mobilization of soil P and plants P uptake.

Collectively, despite the importance of plant specialized metabolites (i.e., fatty acids and flavonoids) in the composition and function of microbial communities in the rhizosphere, little is known about the further effects of these metabolites in the soil. In addition, it is unclear whether these substances can improve plant P nutrition and promote growth and development by regulating soil microbial communities. In the present study, we determined whether the addition of different concentrations of myristic acid (fatty acids), quercetin, naringenin or luteolin (flavonoids) to the soil would improve soil organic P utilization efficiency and enhance plant growth by changing the microbial community. We hypothesized that: (1) the addition of fatty acids or flavonoids would alter the community structure of rhizosphere soil bacteria and AM fungi; (2) the alteration of the rhizosphere soil bacterial and AM fungal community structure would accelerate the turnover of P in the soil; and (3) the addition of these substances ultimately enhanced plant growth and P uptake efficiency.

Materials and methods

Plant and soil

Maize (*Zea mays* L., cv. Zhengdan 958) was used as the experimental plant. The seeds were soaked with 10% (v/v) H_2O_2 to sterilize for 15 min, and then rinsed with sterilized deionized water 10 times. The seeds were subsequently placed in the dark to germinate for 48 h at 27℃.

An alkaline (pH_{water} 7.8; water:soil = 5:1) soil (Inceptisol according to the USDA Soil Taxonomy) collected from Changping (116°12′08″ E, 40°12′34″ N), Beijing, China was used. The properties of the soil were as follows: 1.35 g total N kg^{-1}, 21.53 g organic C kg^{-1}, 4.18 mg Olsen-P kg^{-1} (extracted by 0.5 M $NaHCO_3$), and 214.2 mg exchangeable K kg^{-1} (extracted by CH_3COONH_4). The soil was sieved to 2 mm and air dried. The following nutrients were added per kg of soil: 200 mg N ($(NH_4)_2SO_4$), 10 mg P (KH_2PO_4), 200 mg K (K_2SO_4), 50 mg Mg

($MgSO_4 \cdot 7H_2O$), 5 mg Zn ($ZnSO_4 \cdot 7H_2O$), 5 mg Mn ($MnSO_4 \cdot H_2O$), 2 mg Cu ($CuSO_4 \cdot 5H_2O$) and 75 mg organic P in the form of inositol hexakisphosphate (Phytin, with 19.6% P content, CAS 3615-82-5, Tokyo Chemical Industry, Tokyo, Japan).

Experimental design

A total of 2 kg of soil was added to the pot with the diameter of 19 cm in the top and 12 cm in the bottom, and the height of 14 cm. First, 1.8 kg of soil was added into the pot and then 360 mL of deionized water was added to moisten the soil to 70% of field capacity. Subsequently, approximately 45 g (zeolite:river sand, 1:3) of two different AM fungal inoculants (i.e., *Rhizophagus intraradices* EY108 and *Funneliformis mosseae* MD118), were placed on the soil surface. The purpose of our experiment was to explore the regulation of different plant metabolites (fatty acids and flavonoids) on soil microbial community (including bacteria and AM fungi). Considering that the number of indigenous AM fungal spores is normally small in the soil and the colonization efficiency of AM fungi to roots is generally poor or inconsistent, we added two widely available AM fungal species, i.e., *Rhizophagus intraradices* and *Funneliformis mosseae*, to confer the maize roots are well colonized by AM fungi. Three germinated seeds were placed on the top of the inoculants and then the left 0.2 kg of soil was added to cover the seeds. After one week, each pot was thinned to leave one seedling with the homogenous growth. During the growth of maize, soil moisture was kept at 18–20% (w/w, c. 70% of field moisture capacity) as determined gravimetrically by weighing the pots every 2 days and adding deionized water when necessary. The maize was grown in the greenhouse at China Agricultural University, Beijing (116°16′ 49″ E, 40°1′ 41″ N) from January 13 to March 14, 2021. The growing temperature of maize was 28/22℃ (day/night), and the photosynthetically active radiation was 273 μmol/m^2/s.

In this experiment, four different compounds were added to the soil: myristic acid (CAS 544–63-8), quercetin (CAS 117–39-5), naringenin (CAS 480–41-1) or luteolin (CAS 491–70-3). They were all purchased from Shanghai Yuanye Bio-Technology Co., Ltd. For each compound, two concentrations were considered (50 μmol kg^{-1} and 500 μmol kg^{-1}). The control treatment had no exogenic compound added. The choice of the concentrations of the compounds were based on the C content of the plant metabolites. In pot experiments, we found that the addition of fructose (20 μmol kg^{-1} and 4 mmol kg^{-1}) in hyphal exudates significantly improved the activity of soil phosphatase in the hyphosphere, which was associated with the increase of bacterial population and the change of the bacterial community structure (Zhang et al. 2020). In the rhizosphere, we used glucose, fructose and sucrose to simulate root exudates to study their effects on rhizosphere soil microbes. The results showed that the abundance of Saccharimonadales increased as well as the activity of alkaline phosphatase in maize rhizosphere increased when large concentrations of C (2 mmol kg^{-1} C) were added to the soil. Therefore, we choose the concentrations of myristic acid, quercetin, naringin and luteolin based on the C concentrations of substances such as glucose and fructose. All substances were mixed thoroughly with the soil before potting and each treatment had four replicates for a total of 36 pots. The experiment was arranged in a randomized block design.

Sample collection and the determination of shoot biomass, P content, mycorrhizal colonization and hyphal length density

Plants were harvested 61 days after sowing (at V8, eighth-leaf stage). Soil was divided into rhizosphere and bulk soil. The maize roots were shaken gently to separate the soil attached to the roots and collected as the bulk soil. The rhizosphere soil (about 1 mm thick cylinder of soil around roots) was collected by brushing off the soil closely adhering to the whole root (Chu et al. 2020). The soil samples were sieved to 2 mm, mixed uniformly and divided into four parts, which were stored at -80℃, -20℃, 4℃ and room temperature for further analysis. The plants were separated into shoot and root samples. The shoots were oven-dried at 105℃ for 30 min, and then at 70℃ to constant weight to determine shoot dry weight. Subsequently, the samples were placed in a mill for grinding to a powder. Determination of shoot and root P content was conducted as detailed in the study by Thomas et al. (1967). Specifically, the powder was digested in 5 mL of 98% H_2SO_4 mixed with 2 mL of 30% H_2O_2 in a glass tube placed in a digestion oven at 280 °C for 30 min. Then, the temperature was increased to 360 °C until the solution

became clear. Subsequently, the P concentration was quantified using the vanadate-molybdate-yellow colorimetry method. The roots were cleaned with deionized water, patted dry and divided into three parts. The oven-dried roots were used to determine the root P concentration. The roots were stored at -20℃ and subsequently used for the determination of mycorrhizal colonization according to Trouvelot et al. (1986). Briefly, the roots were soaked in 10% KOH (w:v) at 90 °C for 40 min, washed with deionized water 3 to 5 times and acidified with 2% HCl for 5 min. Subsequently, trypan blue was added and stained in a 90 °C water bath for 30 min. After removing from the trypan blue solution, a mixed solution of lactic acid:glycerol (1:1, v:v) was added to destain the roots for 24 h at room temperature. Microscopic examination was performed to determine the mycorrhizal colonization. The roots stored at -80℃ was used to extract DNA for analysis of the relative abundance and community structure of AM fungi. In addition, the hyphal length density (HLD) in rhizosphere soil and bulk soil was determined by the membrane filter technique and the gridline intercept method following the description of Staddon et al. (1999). Briefly, the mycorrhizal hyphae were extracted from the air-dried soil by using the membrane filter technique and hyphal length was assessed using the gridline intercept method at 200×magnification and converted to HLD expressed as meters of hyphae g^{-1} soil.

Determination of Olsen P, Olsen organic P and alkaline and acid phosphatase activity

The Olsen P and Olsen organic P concentration of rhizosphere soil was determined as described by Olsen (1954) and Schoenau and Huang (1991): 2.5 g soil stored at room temperature was extracted with 50 mL 0.5 M $NaHCO_3$, and the concentration of Olsen P in the extract solution was determined using the method of molybdenum-antimony and colorimetric assay at 882 nm. Then, 20 mL of the extract solution was digested with 0.15 g $K_2S_2O_8$ and 1 mL H_2SO_4, and the concentration of total P was measured using the same method. The difference between total P and Olsen P allowed the calculation of Olsen organic P. The alkaline and acid phosphatase (ALP and ACP) activities in the rhizosphere soil were measured according to Tabatabai and Bremner (1969) and Neumann (2006) with modification: 0.1 g soil stored at -20℃, 1.6 mL of 200 mM $NaHCO_3$ (pH = 8.2, for ALP) or CH_3COONa (pH = 5.2, for ACP) and 0.4 mL *p*-Nitrophenyl phosphate (150 mM, Sangon Bioengineering Co., Ltd., Shanghai, China) solution were added to a 10 mL centrifuge tube in turn. It was mixed uniformly and incubated in a 30 °C water bath for 30 min. Then 2 mL 0.5 M NaOH was added to terminate the reaction. The concentration of the released *p*-nitrophenyl was determined by colorimetry at 405 nm. The phosphatase activity was expressed as 1 pmol of *p*-nitrophenol released from *p*-nitrophenyl phosphate per second (pKatal) g^{-1} dry weight (DW) soil.

DNA extraction, 18S rRNA and 16S rRNA gene PCR and sequencing

The roots and rhizosphere soils stored at -80℃ were used to extract DNA using the DNAsecure Plant Kit (Catalogue number: DP320, Tiangen Biotech Co. Ltd., Beijing, China) and the FastDNA SPIN Kit for Soil (Catalogue number: 116560200, MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer instructions, respectively. TE eluate buffer and DES eluate buffer were added to elute the DNA to a final volume of 80 and 100 µL, respectively. Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies Inc., Wilmington, DE, USA) was used to determine the concentration and quality of DNA. Subsequently, the extracted DNA was stored at -20℃ until further processing. To identify the AM fungal taxa, PCR was performed by a nested approach, targeting a partial 18S rRNA gene fragment of AM fungi. Briefly, the first round of PCR was performed with primers NS1 and NS4 (Lee et al. 2008; Alguacil et al. 2011) and the second round of PCR was performed with primers AML1 and AML2 (Lee et al. 2008). Primers AML1 and AML2 were linked to the Illumina adaptors. Then, an 8-bp unique barcode sequence was inserted between the A adaptor and the AML1 primer sequence as an identifier for amplicons from different samples. To identify the general bacteria and assess their community structure, the PCR was performed with 519F/907R primers pairs (Stubner 2002), targeting the 16S rRNA gene of bacteria and sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing.

The PCR were performed in 25 µL volumes for 18S rRNA and 16S rRNA gene with 1 µL DNA, 5

μL of buffer (5×), 0.25 μL of Fast pfu DNA Polymerase (5U/μL), 2 μL (2.5 mM) of dNTPs, 1 μL of each primer (10 μM) and 14.75 μL ddH_2O. Products of the PCR were separated on a 1% agarose gel in 1×TAE buffer by electrophoresis and then purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. Two pools of PCR products were prepared for sequencing. The first contained 18S rRNA gene amplicons and the second contained 16S rRNA gene amplicons which were sequenced at the Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China) by the Illlumina MiSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles).

Bioinformatics processing

We processed the sequencing data by using Quantitative Insights Into Microbial Ecology (QIIME2, 2019.4) pipeline (Caporaso et al. 2010). In brief, original sequencing reads were assigned to their samples according to the barcode and identified as valid sequences. Low-quality sequences required further filtering (Gill et al. 2006; Chen and Jiang 2014): the length of the sequences less than 150 bp, average Phred scores of the sequences less than 20, sequences with ambiguous bases, and sequences with mononucleotide repeats more than 8 bp. Paired-end reads were assembled using FLASH (Magoc and Salzberg 2011). Unique sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by UCLUST (Edgar 2010) after chimera detection. A representative sequence from each OTU was selected by using default parameters. Taxonomic annotations were assigned to each OTU representative sequence by BLAST searching the representative sequences set against the MaarjAM database for 18S rRNA gene and the Greengenes database for 16S rRNA gene (Desantis et al. 2006). An OTUs table was then generated, recording the abundance of each OTU in each sample and the taxonomy of these OTUs. The AM fungal 18S rRNA and bacterial 16S rRNA sequences obtained from this study were deposited in the National Center for Biotechnology Information Sequence Reads Archive (SRA accession: PRJNA955890).

Statistical analyses

The IBM SPSS statistics 19 package (SPSS Inc., Chicago, IL, USA) was used to perform all data analysis in the experiment. Levene´s test was used to ascertain homogeneity of variance ($P \leq 0.05$). The data of mycorrhizal colonization was subjected to ANOVA after arcsine transformation. The shoot biomass, shoot and root P concentration, shoot P content, HLD, the concentration of Olsen P and Olsen organic P, ALP and ACP activities among the four substances (myristic acid, quercetin, naringenin or luteolin) or three concentrations (0, 50 or 500 μmol kg^{-1}) were analyzed by one-way ANOVA and evaluated by the Duncan test at $P \leq 0.05$. The correlation of HLD and shoot biomass with ALP and ACP activities in rhizosphere soil was analyzed by Pearson method. Spearman's method was used to analyze the correlation of the top 10 relative abundances at family level of rhizosphere species with ALP activity. "Venn Diagram" (Venn Diagram) analysis work by Shanghai Paisenuo Biotechnology Co., Ltd. completed. Heatmap was performed on Chiplot platform (https://www.chiplot.online), an online data analysis website. Significant differences ($P \leq 0.05$) between different substances were analyzed by using Duncan test.

A random forest analysis was performed to identify which bacteria represented in the top ten relative abundances at family level were the most important determinants in the alteration of soil bacterial community. We used the randomForest, rfPermute and ggplot2 package of R for analysis, and rank bacterial taxa relative abundance by their importance in contributing to the accuracy of concentration prediction by the models. This was performed using the importance() command from the randomForest package (Wang et al. 2022a, b), and bacterial taxa were ranked in descending order of importance to the accuracy of the model. Finally, we annotated the significance of each predicted bacterial taxa ($*P < 0.05$; $**P < 0.01$).

The SparCC algorithm was applied to construct the correlation network. We established four microbial symbiotic networks on four substances (i.e., myristic acid, quercetin, naringenin or luteolin), with eight samples (four each of 50 μmol kg^{-1} and 500 μmol kg^{-1}) forming a network. The "SpiecEasi" R package, which incorporates SparCC algorithms, was employed for conducting network reconstruction. OTUs exhibiting expression in a minimum of five samples and where the cumulative reads across all samples exceeded 100 were retained. Correlation coefficients (ρ) > 0.3 and corresponding P-values < 0.05 were used to construct networks. The

Fruchterman Reingold layout of network were generated by Gephi. The topology of the networks was obtained, including the number of edges and nodes, the mean path length, modularity and average degree.

Results

Shoot biomass, root and shoot P concentration, shoot P content, HLD of the rhizosphere soil and bulk soil and mycorrhizal colonization

Compared to the control treatment, the shoot biomass of maize was significantly increased by 36.37%, 60.29% or 40.73% in the 50 μmol kg^{-1} of myristic acid, naringenin or luteolin treatments, respectively (Fig. 1A). In the 500 μmol kg^{-1} of quercetin, naringenin or luteolin treatments, the shoot biomass of maize was significantly increased by 61.43%, 39.98% or 48.45%, respectively (Fig. 1A). In the control treatment, the root P concentration was 3.61 mg kg^{-1}. While in different concentrations of myristic acid, quercetin, naringenin or luteolin treatments, the root P concentration was 2.87–5.98 mg kg^{-1}. Compared to the control treatment, there was no significant difference in root P concentration in myristic acid, quercetin or naringenin treatments (Fig. 1B). There was no significant difference in shoot P concentration among different treatments (Fig. 1C). The shoot P content and shoot biomass showed the same trend in different treatments, but only significantly increased in the 50 μmol kg^{-1} of myristic acid or naringenin treatments by 33.47% or 40.47%, respectively (Fig. 1D). There was no significant difference in the 50 μmol kg^{-1} quercetin, luteolin, 500 μmol kg^{-1} of myristic acid, quercetin, naringenin or luteolin treatments compared with the control treatment.

In the rhizosphere soil, addition of 500 μmol kg^{-1} of quercetin or luteolin significantly increased the HLD by 57.32% or 48.78%, but there was no significant difference in the other two (myristic acid or naringenin) treatments compared with the control treatment (Fig. 1E). In the bulk soil, addition of 50 μmol kg^{-1} of quercetin, naringenin or 500 μmol kg^{-1} of myristic acid significantly decreased the HLD by 26.85%, 25.10% or 15.76%, respectively, but addition of 50 μmol kg^{-1} of luteolin significantly increased the HLD by 25.88% (Fig. S1). Mycorrhizal colonization was not significantly different among the four different substances (myristic acid, quercetin, naringenin or luteolin) or among the three concentrations (0, 50 or 500 μmol kg^{-1}, Table S1).

Olsen P and Olsen organic P content, alkaline and acid phosphatase activity of the rhizosphere soil

The 50 μmol kg^{-1} of quercetin or 500 μmol kg^{-1} naringenin treatments significantly increased Olsen P content in the rhizosphere soil by 27.55% or 25.89%, respectively. The content of Olsen P in the 50 μmol kg^{-1} quercetin treatment was significantly greater than that of the other three substances (myristic acid, naringenin or luteolin) at the same concentration (Fig. 2A). Olsen organic P content showed no significant difference among the four different substances (myristic acid, quercetin, naringenin or luteolin) or among the three concentrations (0, 50 or 500 μmol kg^{-1}, Fig. 2B).

Regardless of concentration, addition of myristic acid had no significant effect on ALP and ACP activities in rhizosphere soil. Compared with the control treatment, ALP and ACP activities were significantly increased in the 500 μmol kg^{-1} quercetin treatment by 86.83% and 47.45%, respectively. Similarly, in the 500 μmol kg^{-1} luteolin treatment, ALP and ACP activities were significantly increased by 121.60% and 47.99%, respectively (Fig. 2C, D). In addition, the 50 μmol kg^{-1} naringenin treatment significantly increased ALP activity by 85.33% and the 50 μmol kg^{-1} luteolin treatment significantly increased ACP activity by 36.44%. In the 50 μmol kg^{-1} naringenin or luteolin treatments, ALP activity of the rhizosphere soil was significantly greater than that in the 50 μmol kg^{-1} of myristic acid or quercetin treatments (Fig. 2C).

Correlation of shoot biomass and HLD with ALP and ACP activities in the rhizosphere soil

Linear regression analysis showed a significantly positive correlation between the shoot biomass of maize and ALP activity ($R^2=0.33$, $P=0.00023$, Fig. 3A) or ACP activity ($R^2=0.22$, $P=0.037$, Fig. 3B) in the rhizosphere soil. In addition, with the increase of ALP or ACP activities, HLD was also increased. HLD showed a significantly positive correlation with ALP and ACP ($R^2=0.15$, $P=0.021$ and $R^2=0.14$, $P=0.027$, respectively, Fig. 3C, D).

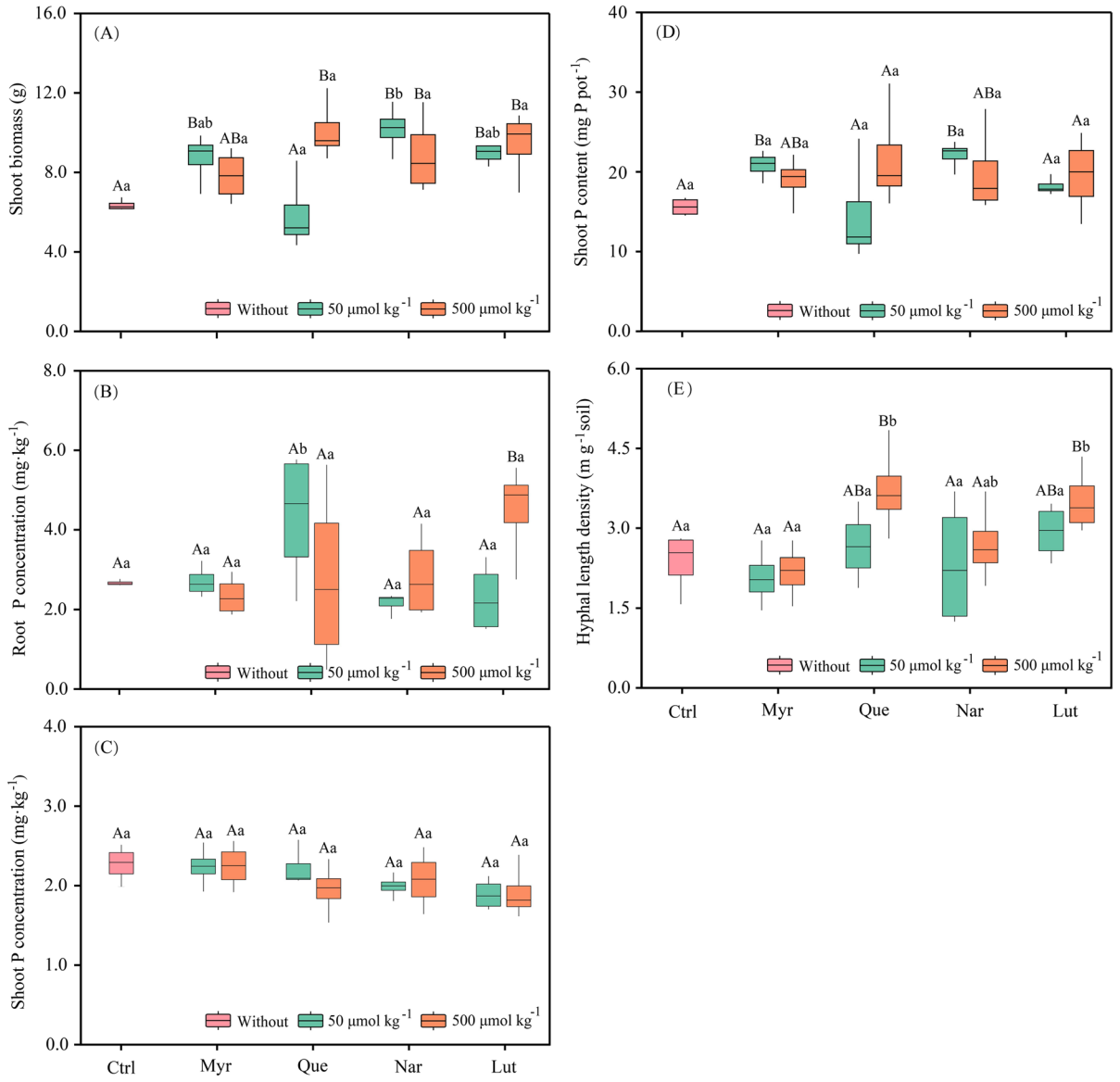

Fig. 1 The (**A**) shoot biomass, (**B**) root P concentration, (**C**) shoot P concentration and (**D**) shoot P content of maize and the (**E**) hyphal length density in the rhizosphere soil. Different capital letters indicate significant differences among the three concentrations for the same substance and different lowercase letters indicate significant differences among the four substances at the same concentration (Duncan test at $P \leq 0.05$). Boxplot shows median, first and third quartiles and whiskers are maximum and minimum values ($n = 4$). Ctrl, control; Myr, myristic acid; Que, quercetin; Nar, naringenin; Lut, luteolin

The community composition of AM fungi and bacteria in the rhizosphere soil

For AM fungi, there were no significant differences in the relative abundance at the genus level among the four different substances (myristic acid, quercetin, naringenin or luteolin) or among the three concentrations (0, 50 or 500 μmol kg^{-1}), and the relative abundance of *Glomus* was always the largest (more than 95%) among all the treatments, followed by *Paraglomus* (ranging from 0.03% to 4.10%, Table S2).

For bacteria, the histograms of relative abundances of different bacterial phyla showed that Proteobacteria and Actinobacteria were the most

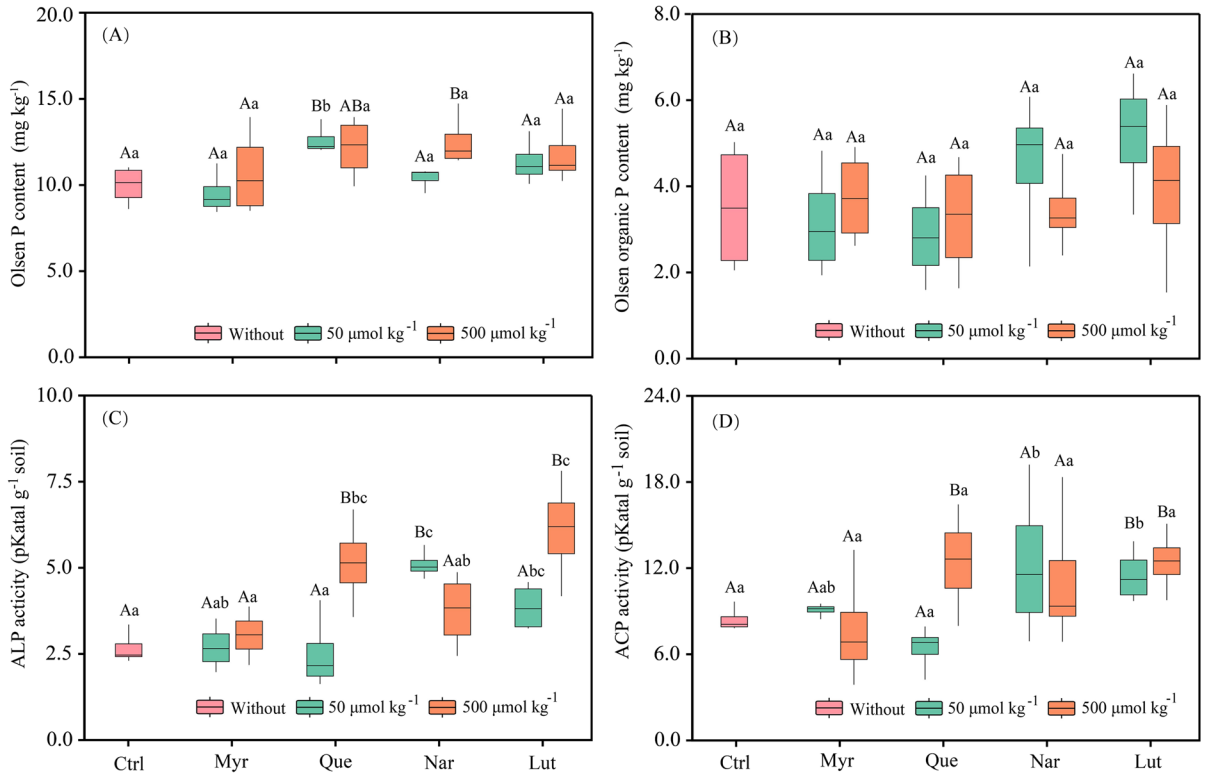

Fig. 2 The (**A**) Olsen P content and (**B**) Olsen organic P content (extracted by 0.5 M $NaHCO_3$), (**C**) alkaline phosphatase activity and (**D**) acid phosphatase activity of the rhizosphere soil. Different capital letters indicate significant differences among the three concentrations for the same substance and different lowercase letters indicate significant differences among the four substances at the same concentration (Duncan test at $P \leq 0.05$). Boxplot shows median, first and third quartiles and whiskers are maximum and minimum values ($n = 4$). Ctrl, control; Myr, myristic acid; Que, quercetin; Nar, naringenin; Lut, luteolin

abundant, followed by Acidobacteria, Firmicutes, Chloroflexi (Fig. 4A). At the phyla level, the 50 μmol kg^{-1} myristic acid treatment significantly increased the relative abundance of Cyanobacteria, while the 500 μmol kg^{-1} myristic acid treatment had no significant effect. Addition of 500 μmol kg^{-1} of quercetin to the soil significantly increased the relative abundance of Actinobacteria. Regardless of the concentration, addition of naringenin significantly increased the relative abundance of Actinobacteria, while in the luteolin treatment, the relative abundance of Actinobacteria was increased with the increase in the concentration of luteolin (Fig. 4A). The relative abundance of bacteria had the same trend at the family level. The histograms of relative abundances of different bacterial family showed that Xanthomonadaceae and Micrococcaceae were the most abundant, followed by Burkholderiaceae, Nocardioidaceae, Streptomycetaceae, Sphingomonadaceae, Bacillaceae, Subgroup_6, Rhizobiaceae, Microscillaceae. The relative abundance of Micrococcaceae increased with the increasing concentration of the four substances, among which the 500 μmol kg^{-1} naringenin treatment was the greatest (10.52%). In addition, the 500 μmol kg^{-1} quercetin, naringenin or luteolin treatments significantly increased the relative abundance of Nocardioidaceae by 75.39%, 30.55% or 68.74%, respectively, while there was no significant difference in the 500 μmol kg^{-1} of myristic acid treatment (Fig. 4B). Addition of different substances had different effects on the relative abundance of bacteria at the genus level. For example, Subgroup_6 and MB-A2-108, were the most abundant when no substance was added. Regardless of the concentration, the relative abundance of

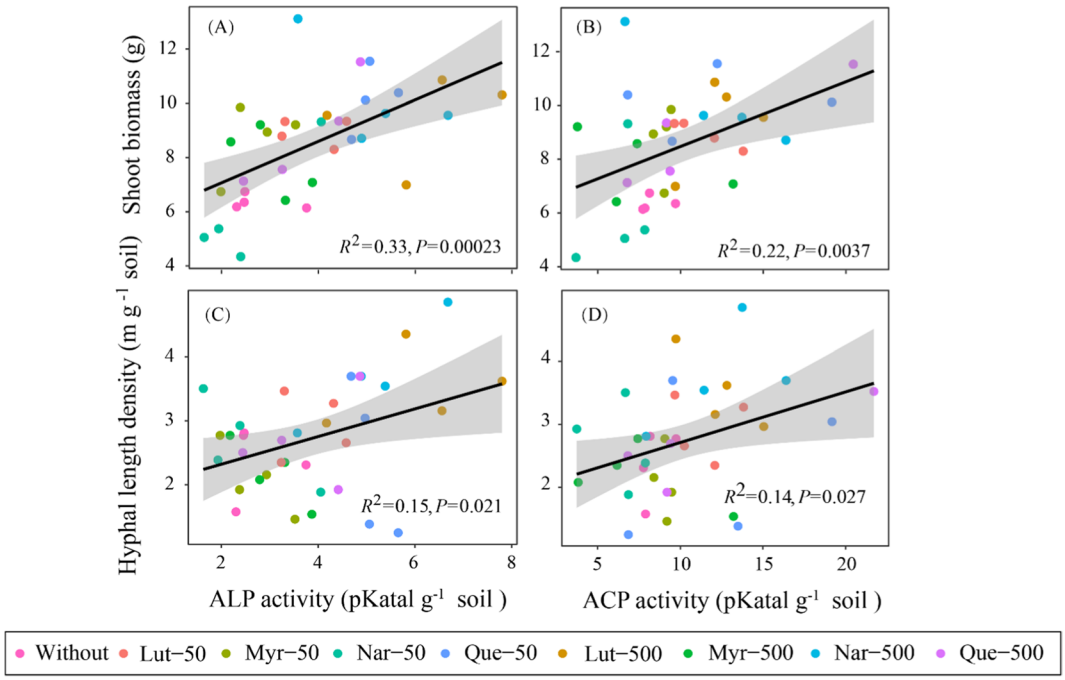

Fig. 3 Spearman correlation of shoot biomass and hyphal length density with ALP and ACP activities in rhizosphere soil when different concentrations (0, 50 or 500 μmol kg^{-1}) of the four substances (Myr, Que, Nar or Lut) were added to the soil. Myr-50, 50 μmol kg^{-1} myristic acid; Que-50, 50 μmol kg^{-1} quercetin; Nar-50, 50 μmol kg^{-1} naringenin; Lut-50, 50 μmol kg^{-1} luteolin; Myr-500, 500 μmol kg^{-1} myristic acid; Que-500, 500 μmol kg^{-1} quercetin; Nar-500, 500 μmol kg^{-1} naringenin; Lut-500, 500 μmol kg^{-1} luteolin

Nocardioides and Devosia were the greatest in the luteolin treatment, and the relative abundances of Subgroup_6 and Ramlibacter were the least in the naringenin or luteolin treatments (Fig. 4C).

Correlation between the relative abundance of top ten families with ALP activity

At the family level, the relative abundance of six bacterial taxa (i.e., Micrococcaceae, Burkholderiaceae, Nocardioidaceae, Streptomycetaceae, Subgroup_6 and Microscillaceae) among the top ten families showed significantly linear correlations with ALP activity of the rhizosphere soil (Fig. 5). Among these bacteria, the relative abundance of Micrococcaceae, Nocardioidaceae and Streptomycetaceae was significantly positively correlated with ALP activity of the rhizosphere soil (R^2=0.36, P=0.00015; R^2=0.4, P=0.000056; R^2=0.11, P=0.046, respectively, Fig. 5A, C, D), while the relative abundance of Burkholderiaceae, Subgroup_6 and Microscillaceae was significantly negatively correlated with ALP activity of rhizosphere soil (R^2=0.23, P=0.0031; R^2=0.33, P=0.00029; R^2=0.11, P=0.046, respectively, Fig. 5B, E, F). The relative abundance of the other four top ten bacterial families (i.e., Xanthomonadaceae, Bacillaceae, Sphingomonadacea and Rhizobiaceae) had no significant correlation with ALP activity of the rhizosphere soil (Fig. S2).

The key bacteria at the family level involved in the alteration of soil bacterial community

The relative abundance of bacteria at the family level under different concentrations (50 μmol kg^{-1} and 500 μmol kg^{-1}) of the same substance was combined and represented in a heatmap (Fig. 6A). Compared with the control treatment, the relative abundance of Micrococcaceae was significantly increased in all four treatments (myristic acid, quercetin, naringenin or luteolin), with the largest increase in the naringenin treatment. The relative abundance of Nocardioidaceae

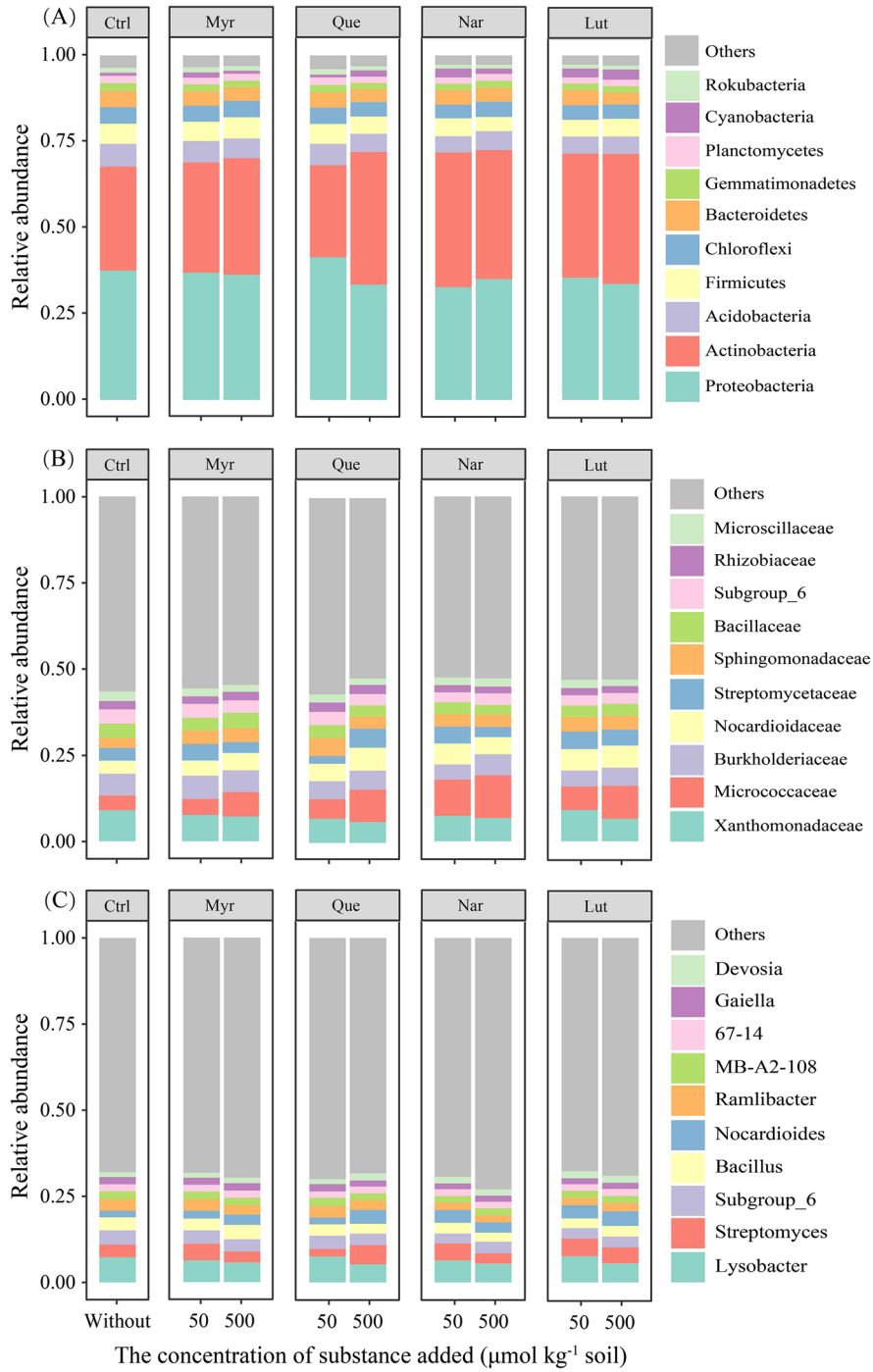

Fig. 4 The top ten relative abundance of different bacterial (**A**) phyla, (**B**) families and (**C**) genera in rhizosphere soil when different concentrations (0, 50 or 500 μmol kg^{-1}) of the four substances (Myr, Que, Nar or Lut) were added to the soil. Ctrl, control; Myr, myristic acid; Que, quercetin; Nar, naringenin; Lut, luteolin

was significantly increased only in the quercetin, naringenin or luteolin treatments, and there was no significant difference among these three treatments. In addition, the relative abundance of Bacillaceae was significantly reduced due to addition of quercetin, naringenin or luteolin to the soil (Fig. 6A). The random forest analysis showed that six of the top ten families, based on relative abundance, could be considered determinants in the alteration of soil bacterial community, i.e., Micrococcaceae, Bacillaceae, Rhizobiaceae,

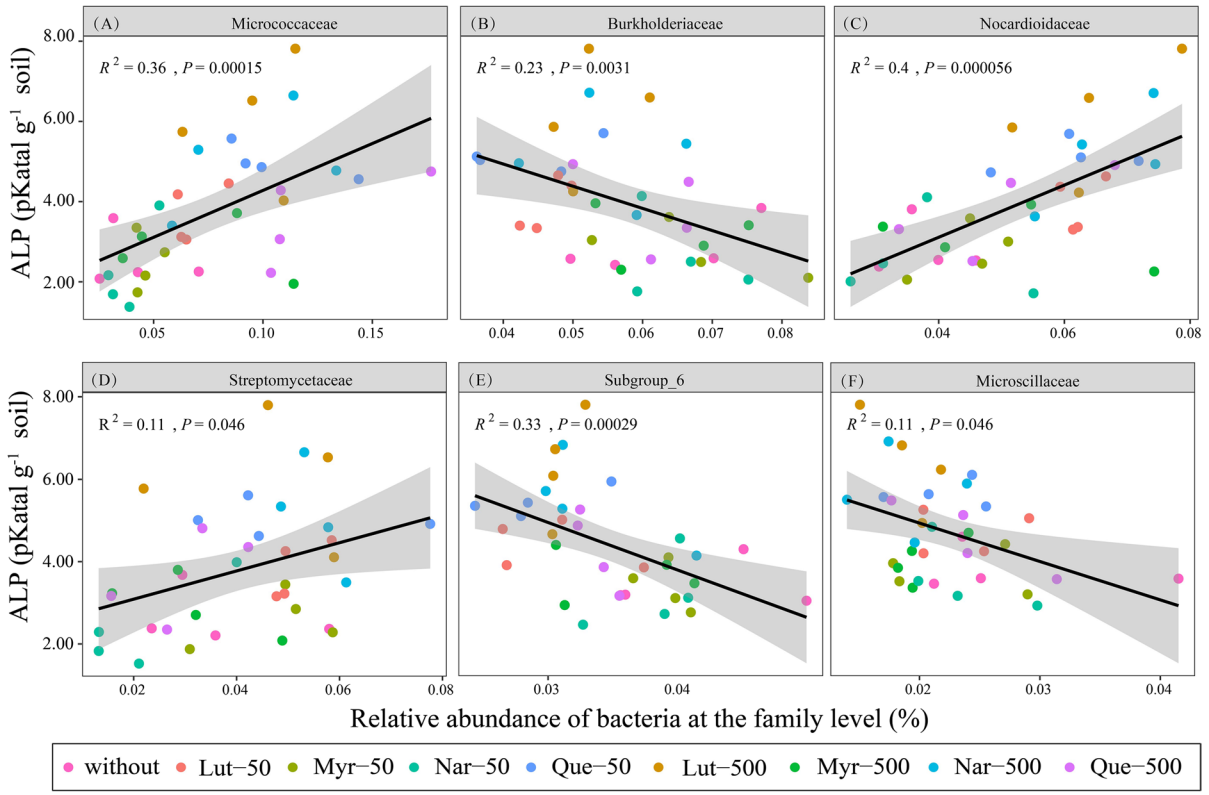

Fig. 5 The significant correlation of the relative abundance of six bacterial families among the top ten families with alkaline phosphatase (ALP) activity of rhizosphere soil when different concentrations of the four substances (Myr, Que, Nar or Lut) were added to the soil. Spearman's method was used to analyze the correlation of the relative abundances at family level of rhizosphere species with ALP activity. Myr-50, 50 μmol kg^{-1} myristic acid; Que-50, 50 μmol kg^{-1} quercetin; Nar-50, 50 μmol kg^{-1} naringenin; Lut-50, 50 μmol kg^{-1} luteolin; Myr-500, 500 μmol kg^{-1} myristic acid; Que-500, 500 μmol kg^{-1} quercetin; Nar-500, 500 μmol kg^{-1} naringenin; Lut-500, 500 μmol kg^{-1} luteolin

Subgroup_6, Streptomycetaceae and Nocardioidaceae (Fig. 6B).

Rhizosphere soil bacterial co-occurrence networks

We constructed a Venn diagram at the OTUs level to represent the intersections and overlaps among four substances, each with two different concentrations (50 μmol kg^{-1} and 500 μmol kg^{-1}) for a total of eight samples. The results showed that the addition of myristic acid, quercetin, naringin and luteolin had 5018 identical OTUs. There were 1314, 1528, 1541 or 1990 unique OTUs in the myristic acid, quercetin, naringin or luteolin treatments, respectively (Fig. S3).

Four co-occurrence networks were established based on SparCC correlation among four substances (Fig. 7). For example, in the myristic acid treatment, pairwise SparCC correlations between 93 OTUs were calculated to form 11 modules with the modularity of 0.955. The structure of co-occurrence networks for the myristic acid treatment was the most complex. The number of nodes in the quercetin treatment and myristic acid treatment were identical. However, the network connections observed in the quercetin treatment was only 38. Despite similar network connection numbers between quercetin and luteolin treatments, the quercetin treatment had more nodes than luteolin treatment (Table S3). The proportion of positive correlations with the luteolin (61.54%) treatment was greater than that in the myristic acid (61.02%), naringenin (56.52%) or quercetin (50.00%) treatments (Table S3).

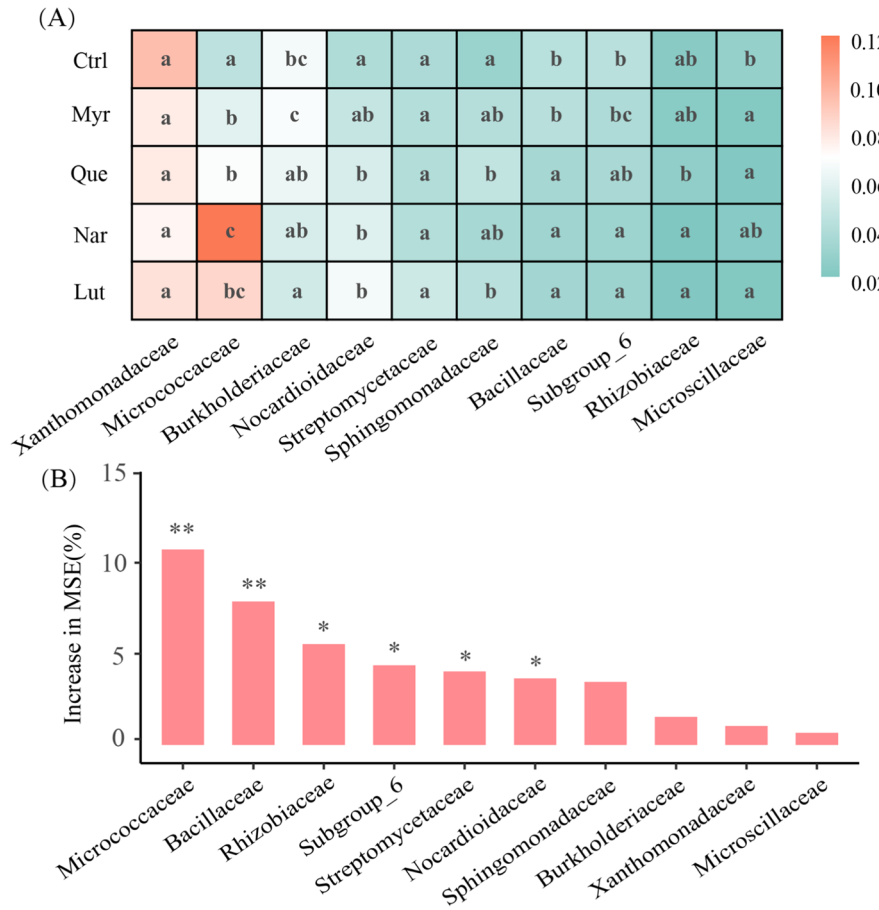

Fig. 6 The (**A**) heatmap showing the top ten relative abundance of bacteria at the family level in rhizosphere soil. Different letters (a, b and c) indicate significant differences between different treatments at the $P \leq 0.05$ level. The (**B**) significance ($P \leq 0.05$) of the top ten relative abundance of bacteria at the family level in rhizosphere soil predictors across all five treatments (Ctrl, Myr, Que, Nar or Lut), identified by random forest analysis. Bacterial taxa are ranked in descending order of importance to the accuracy of the model. Ctrl, control; Myr, myristic acid; Que, quercetin; Nar, naringenin; Lut, luteolin

Discussion

Plant–microbe interactions involve the exchange of various complex signals, nutrients and metabolites, which constantly occur and vary in space and time. Plant metabolites secreted by roots could be considered as the key substances that regulate plant–microbe interactions to shape the rhizosphere microbiome and affect their function, but such understanding is in its infancy (Korenblum et al. 2022). In the present study, we found that the rhizosphere bacterial community structure was significantly altered in the 50 μmol kg^{-1} of naringenin, luteolin, 500 μmol kg^{-1} of quercetin, naringenin or luteolin treatments, accompanied by a significant increase ALP activity and the relative abundance of Micrococcaceae and Nocardioidaceae in the rhizosphere soil. It is considered that their relative abundance is significantly positively correlated with ALP activity, and we speculate that they may secrete more ALP to facilitate the mineralization of organic P. The AM fungal community structure was not altered by these plant metabolites. Our results suggest that flavonoids can directly regulate rhizosphere bacterial community structure, and cause a shift in this structure towards species which specialize in mineralizing organic P. These plant metabolites ultimately facilitated plant growth and P uptake efficiency to different extents.

Addition of plant metabolites enhanced plant growth and P uptake

The addition of active substances to the soil have been widely shown to improve plant growth, increase crop production and quality, as well as

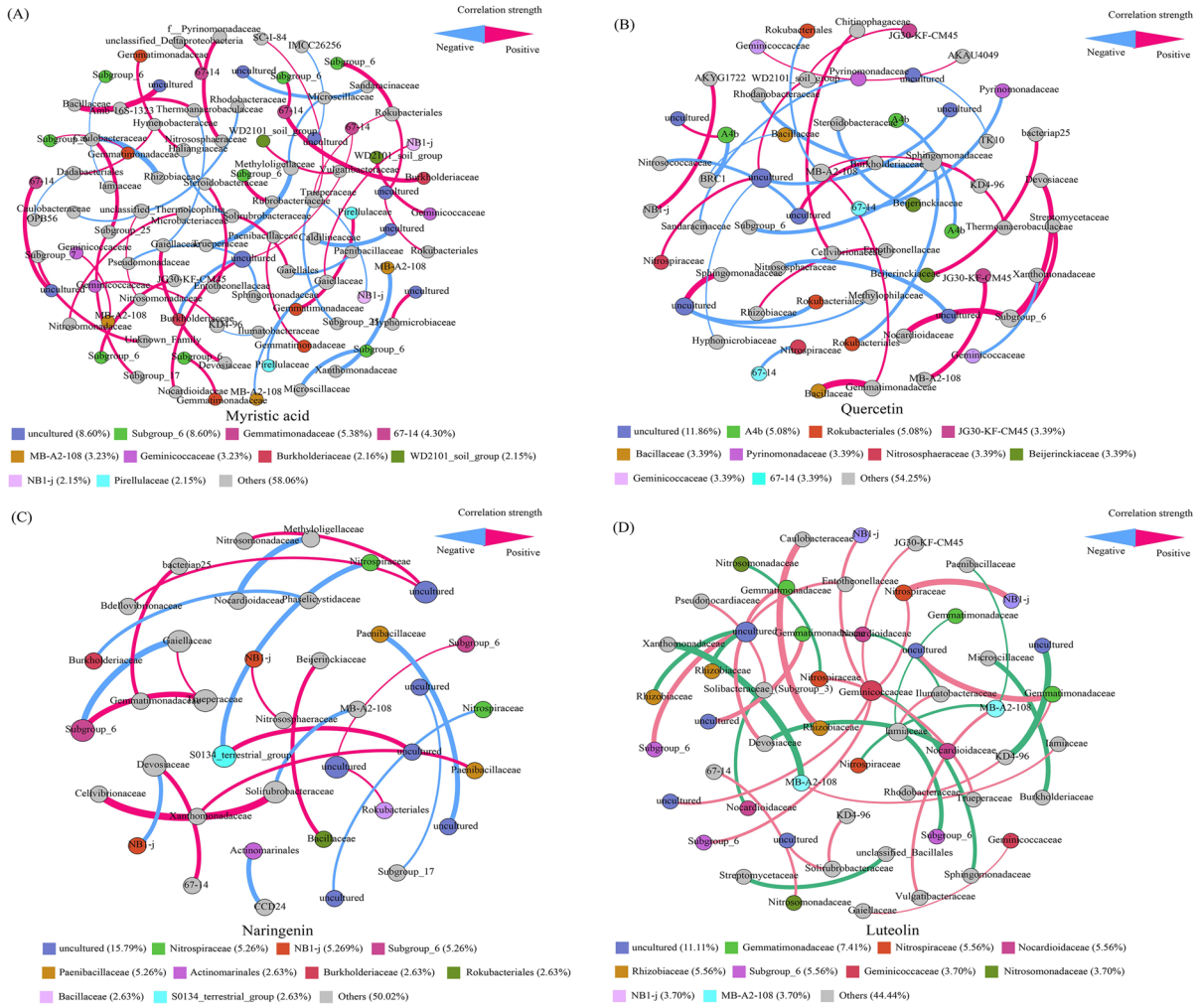

Fig. 7 Co-occurrence networks of bacterial OTUs based on correlation analysis in the treatments of (**A**) myristic acid, (**B**) quercetin, (**C**) naringenin or (**D**) luteolin. A connection stands for a strong (Correlation coefficients (ρ) > 0.3) and significant ($P < 0.05$) correlation. Each network is based on all OTUs at two different concentrations of the same substance (50 μmol kg^{-1} and 500 μmol kg^{-1}, $n = 8$)

alleviate plant stress (Baltazar et al. 2021; Graziani et al. 2022). For example, flavonoids are biostimulants, playing crucial roles in plant growth by inducing resistance against certain biotic and abiotic stresses, which have the ability to enhance microbial interactions and soil nutrient cycling in agricultural ecosystems due to their function as signaling molecules (Shah and Smith 2020). In the present study, both the growth and P uptake of maize were improved after adding myristic acid, quercetin, naringenin or luteolin to the soil and showed the same trend (Fig. 1A, B). Most plants have two pathways for absorbing inorganic nutrient: directly through the root epidermis and root hairs (direct pathway) and indirectly via AM fungal transfer from the extraradical hyphae into root cortical cells (mycorrhizal pathway) (Smith et al. 2011). The plant growth promoting rhizobacteria living in the rhizosphere interact intimately with roots to help plants obtain P through the direct pathway (Oliveira et al. 2009). While AM fungi can quickly extend in the soil and enlarge the range of plant P absorption from the root surface to up to 12 cm away (Li et al. 1991). When the mycorrhizal pathway is functioning,

AM fungi generally receive a large amount of C from plants to maintain mycorrhizal colonization and the growth and development of the extraradical hyphae due to the frequent exchange of C and P between AM fungi and plants (Duan et al. 2023). In this study, although AM fungal colonization was not increased, the HLD in the rhizosphere was significantly increased in the 500 μmol kg^{-1} quercetin or luteolin treatments (Fig. 1E, Table S1). These results indicated that the colonization by AM fungi was not the dominant mechanism in the improvement of plant growth and P nutrition after the addition of plant metabolites, but changes to HLD may play a role.

Flavonoids in root exudates act as the key chemical signals that strengthen the associations between AM fungi and plants by stimulating AM fungal colonization and spore germination (Poulin et al. 1993; Larose et al. 2002; Cesco et al. 2012; Tian et al. 2021). Recently, Del Valle et al. (2020) found that organic C in soil can suppress the flavonoid signal by up to 70%, which shows the importance of biodegradation for the loss of flavonoid signal. In the present study, no significant difference was observed in the mycorrhizal colonization concerning the type and concentration of flavonoids. This outcome suggests several mechanisms could be involved. A large number of bacteria dwell in the soil, but fewer than 5% are active due to C limitation (Blagodatskaya and Kuzyakov 2013). Most of the remaining bacteria rapidly recover their activity and participate in the biogeochemical cycle of mineral elements in the soil with the input of activated C (Kuzyakov 2002). The flavonoids added to the soil may be degraded by bacteria as a C source before the germination of AM fungal spores, which may affect the regulation of AM fungi by flavonoids. In addition, the two concentrations employed in this study may not manifest conspicuous regulatory effects on AM fungi. This issue could potentially arise from the utilization of concentrations that are excessively high or excessively low. These results suggest that the concentration of flavonoids we added to the soil may have a suppressive effect on spore germination. In addition, Tian et al. (2021) found that spore germination of AM fungi and mycorrhizal colonization were significantly stimulated in the 1 mg L^{-1} or 10 mg L^{-1} of quercetin treatments. Our study found that no significant influence in the mycorrhizal colonization in quercetin treatment (50 μmol kg^{-1} or 500 μmol kg^{-1}). The inconsistency with the findings of Tian et al. (2021) may be due to a variety of factors, such as different means of addition of flavonoids and soil types.

Phosphorus containing organic compounds ubiquitous in the soil are complex forms not readily available to plant cells. Access to these requires phosphatase which release free P from organic compounds (Kageyama et al. 2011). Recently, several studies showed that plant biomass and P content increased with increased ALP activity in the rhizosphere soil (Wang et al. 2022a, b, 2023), which is consistent with our observations (Fig. 3A). In other words, increasing ALP activity ultimately promoted the P uptake efficiency and further improved the growth of plants in P-deficient soils. However, the process of ALP release from cells to the soil is complex and influenced by the environment during all stages of the transcriptional, translational and secretion.

Increased ALP activity was associated with the increased relative abundance of the family Micrococcaceae and Nocardioidaceae due to flavonoid addition

The addition of flavonoids had little effect on the alteration in bacterial community structure at the phyla and genus levels, but it did significantly increase the relative abundance of several bacteria (e.g., Micrococcaceae) at the family level (Fig. 4), which also demonstrates that plant metabolites play a crucial role in initiating and modulating dialogue between roots and soil bacteria (Badri and Vivanco 2009; Huang et al. 2014). The 7,4'-dihydroxyflavone secreted by root exhibits the capability to interact with a diverse range of soil bacteria, suggesting potential multifunctionality within the rhizosphere beyond its role in *nod* gene induction during legume-rhizobia symbiosis (Szoboszlay et al. 2016). In the present study, we found that ALP activity in rhizosphere soil was significantly increased with addition of 50 μmol kg^{-1} of naringenin, 500 μmol kg^{-1} of quercetin or luteolin (Fig. 2C). This may be related to the increase in the relative abundance of Micrococcaceae and Nocardioidaceae due a significant positive correlation between these two bacteria and ALP activity (Figs. 4B and 5B, D). Micrococcaceae and Nocardioidaceae belong to the Actinobacteria phylum, and

like other Actinomycetes, they are considered to be consumers of organic material in ecosystems, with the capacity to degrade and metabolize organic molecules by secreting a range of extracellular enzymes (Ludwig et al. 2012; Evtushenko and Ariskina 2015; Nouioui et al. 2018). For example, Micrococcaceae can colonize the surface of crop residues and accelerate their degradation in agroecosystems, significantly increasing the content of mineral nitrogen in the soil (Ortiz-Cornejo et al. 2017). In addition, Micrococcaceae has been shown to be a class of bacteria capable of solubilising mineral Pi (i.e., non-soluble P) by secreting organic acids (Mander et al. 2012; Bolo et al. 2021), whereas the function of Nocardioidaceae is largely unknown. Therefore, we speculated that the increase of ALP activity may be due to the secretion by Micrococcaceae and Nocardioidaceae. These phenomena meant that the metabolites produced by plants enhance the mineralization of organic P in the soil by altering the rhizosphere bacterial community. Some studies demonstrate that the alterations in the rhizosphere microbiome may in turn influence the metabolome of the plants. *Azospirillum* colonizing the rhizosphere have been shown to modify profiles of rice plant metabolites, with phenolic compounds such as flavonoids and hydroxycinnamic acid derivatives significantly increased, which in turn affected roots secretion (Chamam et al. 2013). In the present study, in addition to ALP activity, the activity of ACP mainly mediated by plants, was increased due to the addition of flavonoids, especially with the addition of 50 μmol kg^{-1} of luteolin, 500 μmol kg^{-1} of quercetin or luteolin (Fig. 2D). This may be due to the change of the rhizosphere bacteria community affecting the metabolism of the plant, which promotes the secretion of ACP, but the mechanism behind this requires further experimental verification.

The changes in the relative abundance of the family Micrococcaceae and Nocardioidaceae was the major determinant of improved plant growth and P nutrition

It has been shown that plant roots nurture a tremendous diversity of microbes via exudation of photosynthetically fixed C. In turn, probiotic members of the root microbiome promote plant growth and protect the host plant from nutrient stress. For example, under iron limitation, plant-secreted coumarin compounds (a phenolic secondary metabolite) are regulators of a beneficial plant-microbiota interaction. These specialized metabolites alter root microbiota composition and are required for microbiota-mediated plant iron uptake and immune regulation (Harbort et al. 2020; Stringlis et al. 2018). Based on bioinformatic analysis, we found six bacterial taxa (Burkholderiaceae, Micrococcaceae, Streptomycetaceae, Nocardioidaceae, Microscillaceac, and Subgroup_6) were likely related to the increase of ALP activity. Previous studies have revealed that Burkholderiaceae, Micrococcaceae and Streptomycetaceae were able to increase P (bio)availability from various P sources. For example, Burkholderiaceae activated non-soluble P in the soil through the secretion of organic acids and acid phosphatases, which promotes P uptake by plants (Caballero-Mellado et al. 2007; Peix et al. 2001). In addition, Micrococcaceae has been shown to be capable of solubilising non-soluble mineral P by secreting organic acids (Mander et al. 2012; Bolo et al. 2021). Similarly, our results suggest that the addition of flavonoids could act as mediators inducing changes in the structure of the microbial community in the rhizosphere, thereby influencing plant P nutrition and growth. In the present study, we further showed that the relative abundance of Micrococcaceae and Nocardioidaceae were significantly influenced by the addition of flavonoids (Fig. 6A). Interestingly, the increase in the relative abundance of Micrococcaceae and Nocardioidaceae in the rhizosphere soil was the important determinant for the alteration in soil bacterial structure (Fig. 6B). Actinomycetes taxa in microbial communities of the soil are often defined as rare species, and some studies suggest that the alterations in the relative abundance of these species can significantly affect the function of soil microbial communities in soil nutrient cycling (Chen et al. 2020; Liu et al. 2021). Finally, we constructed the co-occurrence networks using the OTUs to characterize the influence of different substances on structuring bacterial communities. Co-occurrence networks in the quercetin and luteolin treatments had the greater complexity among three different flavonoids (Fig. 7, Table S3).

In conclusion, our results indicated that the addition of flavonoids altered the rhizosphere soil bacterial community structure, strengthened the relationship between species and then enhanced organic P mineralization by potentially selecting individuals which secreted more phosphatases. Importantly, plant

growth and P uptake efficiency were improved as a consequence of this. Our findings may have important implications for understanding the microbial mechanisms underlying soil P cycling in many terrestrial ecosystems and could allow effective management of sustainable agroecosystems with fewer inorganic inputs. Agricultural by products are a potential resource which can be used as sources of flavonoids and can significantly improve the physical and chemical properties of soil when applied to the field. For example, application of low-cost plant-derived luteolin (e.g., peanut shells) could be used to regulate soil microbial communities and improve P cycling in ecosystems and increase agricultural production, is a potential application of our findings.

Acknowledgements This study was financially supported by the National Natural Science Foundation of China (42277112) and Shandong Natural Science Foundation (ZR2021MC042). Timothy S. George contribution through The James Hutton Institute was supported by funds from the Rural and Environment Science and Analytical Services Division of the Scottish Government.

Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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