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Small-scale heterogeneity of soil properties in farmland affected fava beans growth through rhizosphere differential metabolites and microorganisms

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Abstract

Background Soil heterogeneity has been acknowledged to influence plant growth, with the small-scale soil heterogeneity always being overlooked in practice. It remains unclear how rhizosphere soil biotics and abiotics respond to soil heterogeneity and how rhizosphere interactions influence crop growth.

Results In this study, we planted fava beans in a farmland around an e-waste dismantling site, and a distinct boundary (row spacing is 30 cm) was observed in the field during the flowering stage, which divided fava beans phenotypes into two distinct groups (Big vs Little) based on the differences in biomass and height. Soil total concentrations of As, B, Co, Cr, Cu, Pb, Sr, Zn, Ni, Cd and soil pH significantly differed in the rhizosphere of fava beans in the two adjacent rows, which were located on either side of the boundary, with a row-spacing of 30 cm. Random Forest analysis demonstrated that these differentiated soil properties (soil pH, total As, B, Cd, Co, Cr, Cu, Mo, Ni and Zn) substantially influenced fava beans growth (height and biomass). Metagenomic sequencing showed that microbial taxa were significantly enriched their abundance in rhizosphere soils between the two groups of fava beans, with eukaryotic taxa being more sensitively affected. A total of 20 metabolites including coniferyl alcohol, jasmonic acid, resveratrol, and L-aspartic acid, etc. were significantly correlated with fava beans growth. These metabolites were significantly enriched in 15 metabolic pathways (nucleotide metabolism, pyrimidine metabolism, purine metabolism, biosynthesis of plant secondary metabolites, lysine biosynthesis, etc.). Furthermore, 11 microbial genera involved in these metabolic pathways, and these genera were differentially enriched between the two groups and significantly correlated with fava beans growth.

Conclusions Overall, the integrated analysis of multi-omics revealed that soil properties heterogeneity at small-scale altered the rhizosphere differential microorganisms and metabolites, which functionally influenced fava beans growth and tolerance to environmental stress. Notably, even soil heterogeneity at such a small spatial scale can cause significant differences in plant growth, and the comprehensive explorations utilizing multi-omics techniques provide novel insights to the field management, which is crucial for the survival and sustainable development of humanity.

Keywords Fava beans, Soil heterogeneity, Small-scale, Multi-omics, Elements

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Background

With the continued growth of the global population, the demand for food is projected to double by 2050, which will present unprecedented challenges for the sustainable development of human society [1]. Increasing the productivity of existing arable land has been recognized as an effective strategy to mitigate food shortages [2]. Crop yield is determined by a variety of factors, including soil nutrient concentrations, physicochemical properties, contaminants and microbiome [3–6]. However, soil properties including physical, chemical, and biological properties exhibit spatial heterogeneity at different spatial scales [7–9]. Soil heterogeneity could be attributed to various factors. At broader scales, factors such as climate, geographic location, light, and vegetation cover could substantially affect soil heterogeneity [10, 11]. Additionally, human activities have been identified as major drivers to soil heterogeneity at moderate to small scale [12]. For instance, e-waste dismantling operations have been associated with elevated levels of Cd, Cu, and Pb in soils compared to areas without such activities, as observed in Zhejiang Province, China [13].

Soil heterogeneity could potentially impact crop growth and yield at different spatial scales. A large-scale study in northern China revealed a significant positive correlation between potato yield and a soil quality index, with soil pH identified as the primary limiting factor [14]. At the 1-km scale, soil moisture heterogeneity was shown to be the main driver of cowpea (*Vigna unguiculata* (L.)) yield [15]. Moreover, rhizosphere metabolic processes had significance effects on soil properties, microbial community assembly, nutrient availability and plant growth. For instance, elevated ethylene emissions from the legume peanut rhizosphere were found to increase the relative abundance of specific actinobacterial species, and thereby reshaping the whole rhizosphere microbial network, which might be able to improve availability of essential nutrient for plant growth [3]. Similarly, volatile organic compounds (VOCs) emitted by potato plants had been shown to promote the growth of neighboring tomato plants by modulating the tomato rhizosphere microbiota [16]. In another case, methyl ferulate exuded by tobacco roots significantly suppressed *P. nicotianae* and recruited disease-suppressive rhizosphere microbes, thereby contributing to the control of tobacco black shank disease and improving disease resistance [17]. However, the biochemical mechanisms in the differences of plant growth, which triggered by soil heterogeneity, were still poorly clarified, possibly due to the complex constitutes and interactions exist in the rhizosphere.

In recent years, omics technologies have significantly advanced our understanding of the underlying mechanisms of changes in rhizosphere metabolites and soil microbiomes, which functionally contribute to plant growth [18, 19]. Under Cu stress, significant reductions in the metabolism of purine and nucleotide, which rhizosphere metabolites involved in, were identified as the reason for the inhibition of rice growth [20]. Changes in the structure of rhizosphere microbial community, along with the regulation of gene expression related to glutathione, phytochelatin, and membrane transporter pathways, were identified as key detoxification mechanisms in rice following Si addition [21]. A previous study demonstrated that heterogeneity in microelements existed in soils located across two provinces in China, where Mg and EC were positively correlated with monoterpene biosynthesis and growth of *Citrus reticulata* 'Chachi', as revealed by metagenomic and transcriptomic analyses [22]. Integrating of multi-omics data can facilitate a comprehensive understanding of the functional roles of rhizosphere microorganisms and metabolites, thereby providing novel insights into the mechanisms underlying crop growth.

Over the past decades, long-term e-waste dismantling activities conducted in unregulated workshops have frequently occurred in the coastal regions of southeastern China, leading to the accumulation of heavy metals and organic pollutants in agricultural soils [23, 24]. These anthropogenic activities might result in small-scale heterogeneity of soil pollutants, which potentially affects plant growth [25]. Fava beans are important local crop with high tolerance to environmental stresses, low pollutant accumulation capacity (Table. S1), and strong nitrogen fixation [26]. However, few studies have investigated the effects of the small-scale soil heterogeneity on fava beans growth and their stress tolerance.

In this study, a field experiment was conducted in farmland adjacent to an e-waste dismantling site in Taizhou City, Zhejiang Province, China, where fava beans were planted. During the flowering stage of plant growth, we observed a clear boundary dividing the phenotypes of fava beans into two distinct groups: the Big group (plants with relatively heavier biomass and higher height) and the Little group (plants with relatively lighter biomass and lower height). These two groups were located on either side of the boundary, with a row spacing of 30 cm. Therefore, the soil properties, consisting of elements, nutrients, enzymatic activities, and physicochemical properties of the rhizosphere soils in the two adjacent rows were determined. The biomass, height and antioxidant enzyme activities of fava beans in the two adjacent rows were also measured. Metagenomics and metabolomics analyses were conducted to investigate differences in rhizosphere soil microbiome, metabolite profiles, and

associated metabolic pathways between the two groups of fava beans in adjacent rows. A combined multi-omics approach was used to uncover the mechanistic effects of small-scale soil heterogeneity on fava beans growth. We hypothesized that the distinct phenotypes of fava beans were caused by soil heterogeneity. Furthermore, we hypothesized that soil heterogeneity significantly altered rhizosphere soil microbiome and metabolites, which functionally contribute to fava beans growth.

Methods

Field experiment design and management

The experimental farmland, contaminated by e-waste dismantling, was located in Taizhou City, Zhejiang Province. Fava beans were planted in rows spaced 30 cm apart. Field management and fertilization were the same as the local measures. During the flowering stage of fava beans growth, we observed a distinct boundary in the field, and on both sides of the boundary, there were significant differences in phenotype of fava beans (Fig. S1).

Plant and soil sampling

Fava beans plants with significant phenotypes in the two adjacent rows were randomly sampled for analysis, and their corresponding rhizosphere soils were simultaneously collected (Fig. S1). The whole plants were measured for biomass and height, and also divided into parts of roots, stems and leaves for antioxidant enzyme assays. The rhizosphere soil of each plant was determined for soil properties and elements concentration, metagenome and metabolome analyses. The plant samples used for antioxidant enzyme determination, and the soil samples used for omics analyses were immediately placed in insulated box with ice and transferred to a -80 °C freezer within 6 h. To ensure sampling representativeness, all samples were randomly collected in quintuplicate.

Soil samples preparation before determination.

50.0 g of each soil sample was immediately divided for soil moisture determination, dissolved organic carbon (DOC) and NH_4^+–N and NO_3^-–N extraction. The remaining soil was air dried, ground and sieved to obtain different sizes fractions for subsequent analyses: < 2 mm for pH, < 0.85 mm for available phosphorus (AP), < 0.425 mm for soil sucrase (S-SC), urease (S-UE), and acid phosphatase (S-ACP) activities, and < 0.149 mm for elements and soil organic matter (SOM) determination.

Fava beans phenotypes and antioxidant enzyme activity determination

Five fava bean plants in each row were carefully excavated during the flowering stage to measure their phenotype traits (biomass and height) for estimating their growth status. Based on these phenotype traits, fava beans were classified into two phenotypic groups: the Big group consists of individuals with higher biomass and height, and the Little group consists of individuals with lower biomass and height. The antioxidant enzyme activities (superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD)) in fava bean tissues (roots, stems, and leaves) were determined by using the commercial kits (Solarbio Science & Technology Co., Beijing, China).

Soil abiotic properties determination

Soil pH was measured using a pH meter with a soil-to-water ratio of 1:2.5. Soil moisture was determined by calculating the weight loss after oven-drying at 105 °C until a constant weight was reached. Dissolved organic carbon (DOC), NH_4^+–N and NO_3^-–N were measured as described in [27]. Briefly, DOC, NH_4^+–N and NO_3^-–N were extracted by 0.1 M KCl, followed by measurement using a TOC analyzer (Multi N/C 3100, Analytik Jena, Germany) for DOC, and a continuous flow analyzer (AA3, SEAL Analytical, Germany) for NH_4^+–N and NO_3^-–N. Available phosphorus (AP) was extracted by 0.5 M $NaHCO_3$ and determined using the molybdenum-antimony colourimetric method at a wavelength of 700 nm [26]. Soil organic matter (SOM) was analyzed by the $K_2Cr_2O_7$–H_2SO_4 oxidation–reduction colorimetric method at a wavelength of 590 nm [28]. Soil sucrase (S_SC), soil urease (S_UE) and soil acid phosphatase (S_ACP) were determined by using the commercial kits (Solarbio Science & Technology Co., Beijing, China).

Soil elements concentrations were determined using inductively coupled plasma emission mass spectrometry (ICP-MS) following microwave-assisted acid digestion [29]. Briefly, all soil samples were ground into fine powder (particle size less than 0.149 mm), and 0.1000 g soil of each sample was subjected microwave digestion with 6 ml HNO_3 and 2 ml HCl for total concentration of soil elements (As, B, Co, Cr, Cu, Pb, Sr, Zn, Ni, Cd, Mg, Al, Mo, Nd, Mn, Fe) determination by inductively coupled plasma emission mass spectrometry (ICP-MS, NexION 300X, PerkinElmer, USA). These elements had been identified as the characteristic elements in the dismantling of e-waste, and potentially affect crop growth [30–32].

Soil metagenomic sequencing and analysis

Microbial genomic DNA were extracted from rhizosphere soils using the Mag-Bind® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA), followed by determination of DNA concentration and purity using TBS-380 and NanoDrop 2000, respectively. Paired-end sequencing was conducted on the DNBSEQ-T7 platform (Majorbio BioPharm Technology Co., Ltd., Shanghai, China) using the DNBSEQ-T7RS Reagent Kit (FCL PE150).

After quality control, metagenomic sequences were assembled into contigs using MEGAHIT [33]. The predicted open reading frames (ORFs) from each assembled contig were performed using Prodigal [34] with a length greater than 100 bp, and translated into amino acid sequences to perform gene prediction via the NCBI pipeline. The retrieved genes were clustered using CD-HIT [35] with 90% sequence identity and 90% coverage. The longest sequence in each cluster was selected as the representative sequence for constructing a non-redundant gene catalogue. Representative sequences from the non-redundant gene catalogue were aligned against the NR database (Version 20230830) using DIAMOND (identity > 0, alignment length > 0, *e* value < 1e-5) to obtain taxonomic annotations. Functional annotation was achieved by aligning the same sequences to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Version 20230830) under the same parameters [36].

The taxonomic and functional profiles of rhizosphere microbial communities associated with fava beans of different phenotypes were visualized using a circus plot, where reads per kilobase per million (RPKM) was used to represent gene abundance [37]. Linear discriminant analysis effect size (LEfSe) was used to identify microorganism with significantly different abundance between the two groups (Big vs Little) of fava beans (LDA > 2, $P < 0.05$), where RPKM was used as gene abundance.

Metabolite profiling and analysis

Non-targeted LC-MS/MS metabolomic analysis of rhizosphere soils was conducted according to the method described in a previous study [38]. Briefly, a 50 mg of rhizosphere soil was mixed with 400 µL of extraction solution (methanol:water = 4:1, v/v) containing 0.02 mg/mL of the internal standard L-2-chlorophenylalanine, along with a 6 mm stainless steel grinding bead, in a 2 mL centrifuge tube. The mixture was used for metabolite determination. LC–MS/MS analysis was carried out using a Thermo UHPLC-Q Exactive HF-X system equipped with an ACQUITY HSS T3 column (100 mm × 2.1 mm i.d., 1.8 µm; Waters, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Further details regarding LC–MS/MS instrumental parameters are provided in the Supporting Information.

Raw data generated from the UHPLC-MS/MS analysis were imported into the Progenesis QI software (Waters Corporation, Milford, USA) for metabolomic data processing. Metabolic features were annotated by matching their mass spectra to reference spectra in curated biochemical databases, based on accurate mass, MS/MS fragment spectra, and isotope ratio difference. The annotated metabolites were subsequently mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to retrieve information on their chemical structures and biological functions. KEGG further classifies these metabolites according to their involvements in specific biological functions and pathways.

Principal component analysis (PCA) was performed to assess the overall differences in rhizosphere metabolites between the two groups of fava beans (Big vs Little). Significantly different metabolites were identified using Orthogonal partial least squares discriminant analysis (OPLS-DA), based on the criteria (variable importance in the projection (VIP) > 1 and $P < 0.05$). Differences in metabolites expression between the two groups were visualized by volcano plots. The significantly differential rhizosphere metabolites (DMEs) that could be annotated into metabolic pathways by the KEGG database were collected for further analyses (Table. S2).

Linking multi-omics with soil properties and fava beans phenotypes

The workflow of the integrated multi-omics analysis in relation to fava bean phenotypes was presented in Fig. S2. Significantly correlated differential metabolites (SCDMEs) were identified using the Mantel-test (Mantel's $P < 0.05$). The VIP values and relative abundance of these metabolites (SCDMEs) were visualized in bubble plot and heatmaps. Significantly enriched metabolic pathways (SEMPs), in which the SCDMEs were involved, were identified through enrichment analysis, and pathways with $P < 0.05$ were considered significantly enriched.

Genes involved in the SEMPs were extracted to construct a new functional gene set. This gene set was then aligned to the NR database to identify the rhizosphere microbial genera (MGs) associated with the SEMPs. Significantly different microbial genera (SDMGs) between the two groups (Big vs. Little) were identified using a Wilcoxon rank-sum test with false discovery rate (FDR) correction, where the RPKM was used as genes abundance. Statistically significant differences were defined at $P < 0.05$. Among the SDMGs, microbial genera significantly correlated with fava beans phenotypes (SCDMGs) were selected using the Mantel test (Mantel's $P < 0.05$).

Spearman correlation analysis was performed to assess the associations among fava beans phenotypes, rhizosphere soil properties, significantly correlated differential metabolites (SCDMEs), and significantly correlated differential microbial genera (SCDMGs). Correlations with $P < 0.05$ were considered statistically significant.

Structural equation modeling (SEM)

SEM was used to explore the mechanisms underlying the differences in the phenotypes of fava beans, regarding to the rhizosphere soil elements (As, B, Co, Cr, Cu, Pb, Sr, Zn, Ni, Cd), soil pH, rhizosphere differential soil

microorganisms (11 SCDMGs) and metabolites (21 SCDMEs). All variables were standardized using Z transformation (mean = 0, standard deviation = 1) to improve data normality. Dimensionality reduction of the differential indices was performed via principal component analysis (PCA), with the first principal component representing the majority of variance. Pairwise correlations among these variables were computed using the Mantel test (R packages: *Ecodist*) [39]. The resulting covariance matrix was imported into AMOS 17.0 (SPSS, Chicago, IL, USA) for SEM construction and analysis. Maximum likelihood estimation was used to fit the model to the covariance structure. A priori and theoretical modeling was adjusted according to the criterion of low chi-square (χ^2), non-significant probability level ($P > 0.05$), goodness of fit index (GFI > 0.90), Akaike information criterion (AIC) and root mean square error of approximation (RMSEA < 0.05) to ensure the final model was well fitted [40].

Statistical analysis

Statistical analyses were performed on the R platform. A two-tailed Wilcoxon rank-sum test was used to assess the difference in each variable between the two groups (Big vs Little) of fava beans. Random Forest analysis (R package: *rfPermute*) [41] was used to identify the key factors that explaining variance in biomass and height of fava beans. The importance of each variable was computed for the average of 5000 trees and determined by an increase in node purity (IncNodePurity). Mantel-test (R package: *Ecodist*) [39] was applied to explore the potential associations between the phenotypes of fava beans and the significantly differential metabolites (SDMEs) and microbial genera (SDMGs). Spearman correlation analysis (R packages: *psych, pheatmap*) [42, 43] was employed to identify the relationship between the phenotypes of fava beans and the significantly correlated rhizosphere microbial metabolites (SCDMEGs), microbial genera (SCDMGs) and soil properties.

Results

Differences in fava beans growth between the two groups

Significant differences in biomass and height were detected between the two groups of fava beans (Big vs Little). As shown in Fig. 1, the biomass of fava beans in the Big group (with an average of 238.0 g/plant) was significantly greater than that in the Little group (with an average of 182.0 g/plant) (Fig. 1(1)A; $P < 0.05$), and the height of fava beans in the Big group (with an average of 86.0 cm/plant) was also significantly greater than that in the Little group (with an average of 66.0 cm/plant) (Fig. 1(1)B; $P < 0.05$). However, there were no significant differences in the activities of enzymes (including POD, SOD and CAT) of fava beans tissues between the two groups (Fig. S3; $P > 0.05$).

Differences in rhizosphere soil properties between the two groups

Significant differences in rhizosphere soil pH and elemental concentrations were observed between the two groups. Soil pH was significantly lower in the Big group (with average value of 5.8) compared to the Little group (with average value of 6.3) (Fig. 1(2)A; $P < 0.05$). Soil total concentrations of As, B, Co, Cr, Cu, Pb, Sr, Zn, Ni, and Cd were significantly lower in the Big group (Fig. 1(2); $P < 0.05$). In contrast, no significant differences were detected between the two groups for soil total concentrations of Mg, Al, Mo, Nd, Mn and Fe, as well as for soil enzyme activities (S_ACP, S_UE, S_SC), NH_4^+–N, NO_3^-–N, AP, SOM, DOC, and moisture content (Fig. S4; $P > 0.05$).

Random Forest analysis identified soil total concentrations of Mo, Cu and Cd as key factors significantly associating with fava beans biomass (Fig. 1(3)A; $P < 0.05$). Additionally, soil pH and total concentrations of Zn, Ni, Cr, Co, B and As were identified as major predictors for fava beans height (Fig. 1(3)B; $P < 0.05$).

Differences in rhizosphere microbial communities and functions between the two groups

The taxonomic composition of rhizosphere soil microorganisms was mainly abundant in *Pseudomonadota*, *Actinomycetota*, *Acidobacteriota*, *Chloroflexota*, and *Candidatus_Rokubacteria* at the phylum level, collectively accounting for over 60% of the total relative abundance of microbial communities (Fig. S5(A)). The functional pathways mainly associated with biosynthesis of secondary metabolites and microbial metabolism in diverse environments, and two-component system and carbon metabolism, etc., which account for more than 30% of the total relative abundance (Fig. S5B).

The rhizosphere microorganisms with differential abundances between the two groups were primarily identified among eukaryotes (Fig. 2), 22 eukaryotic taxa were significant differently enriched between the two groups (Fig. 2A). The relative abundances of the orders *Diversisporales* and *Magnaporthales* were significantly higher in the Big group, whereas the orders *Coniochaetales*, *Spizellomycetales* and *Peridiniales* were more abundant in the Little group (Fig. 2A). For archaea, 3 taxa exhibited differences in their relative abundance between the two groups (Fig. 2B). The relative abundances of the class *Archaeoglobi*, the order *Natrialbales*, and the family *Natrialbaceae* were significantly higher in the Little

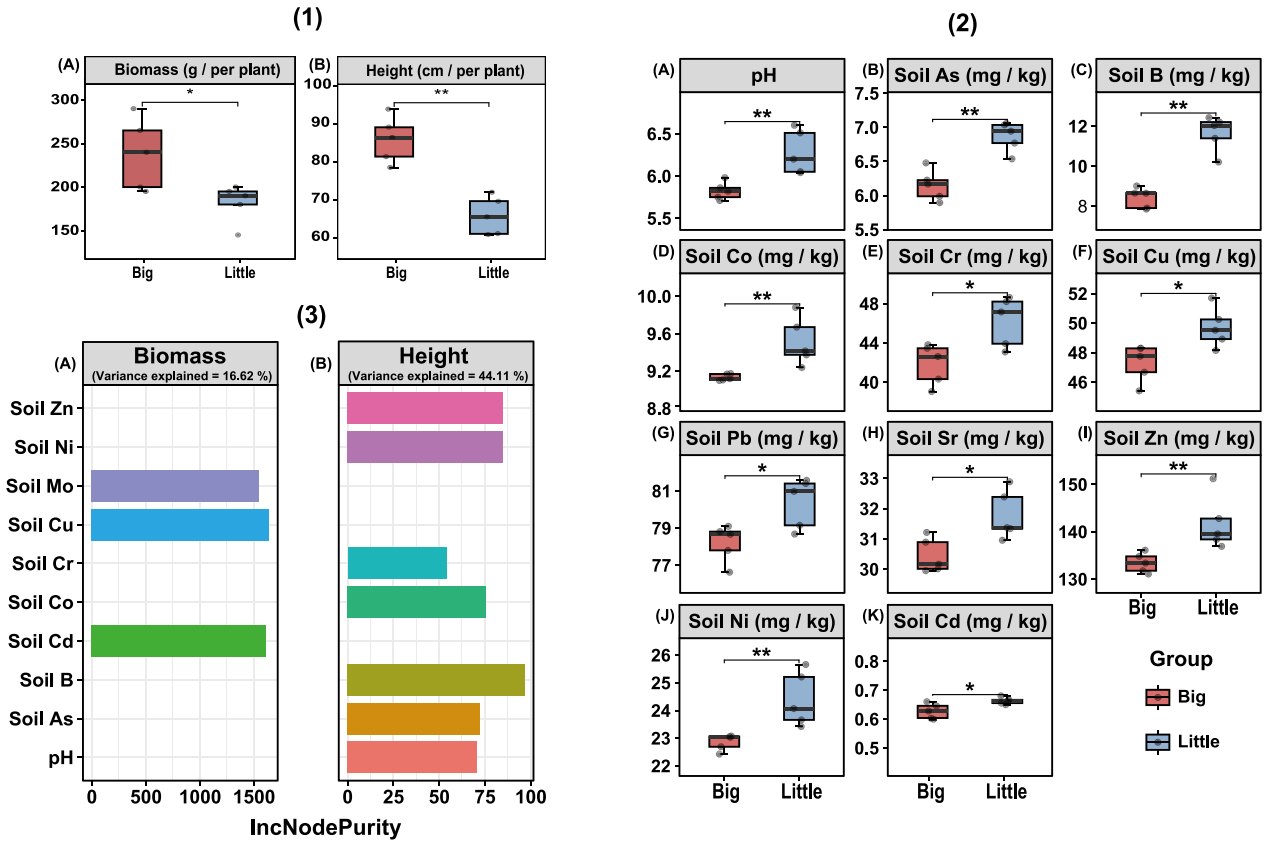

Fig. 1 Fava beans phenotypes, rhizosphere elemental concentrations and their statistical associations. (1) Biomass (**A**) and Height (**B**) of fava beans. (2) Soil pH (**A**) and total concentrations of As (**B**), B (**C**), Co (**D**), Cr (**E**), Cu (**F**), Pb (**G**), Sr (**H**), Zn (**I**), Ni (**J**), Cd (**K**). (3) Random Forest analysis identifying key soil factors associated with fava bean biomass (**A**) and height (**B**). Significant difference of each variable between the two groups (Big vs Little) was measured by Wilcoxon rank-sum test analysis. Asterisks indicate significance: $P<0.05$ (*); $P<0.01$ (**); $P<0.001$ (***). The Big group consists of individuals with higher biomass and height, and the Little group consists of individuals with lower biomass and height

group (Fig. 2B). Regarding viruses, a total of 9 taxa were enriched in terms of relative abundance between the two groups (Fig. 2C). In particular, the relative abundance of the family *Mesyanzhinovviridae* was significantly higher in the Big group (Fig. 2C). For bacteria, 9 bacterial taxa displayed significantly differences in relative abundance between the two groups (Fig. 2D). The class *Flavobacteriia*, the orders *Flavobacteriales* and *Nevskiales*, and the family *Flavobacteriaceae* exhibited significantly higher relative abundance in the Little group (Fig. 2D). A total of 8 microbial functions significantly differed between the two groups (Fig. S6).

Differences in rhizosphere metabolites between the two groups

Overall, most of the differential metabolites exhibited higher relative abundances in the Big group. According to the functional classification at KEGG level 2, the majority of these metabolites were associated with metabolic pathways, including biosynthesis of other secondary metabolites, amino acid metabolism, chemical structure transformation maps, lipid metabolism, and xenobiotics biodegradation and metabolism (Fig. 3A). PCA revealed distinct separation of rhizosphere metabolites profiles between the two groups (Fig. 3B). Compared to the Little group, a total of 462 rhizosphere metabolites were significantly upregulated, and 35 were significantly downregulated in the Big group (Fig. 3C; $P<0.05$). Among these metabolites, 88 differential metabolites (DMEs) were successfully annotated to KEGG metabolic pathways. Of these, 80 differential metabolites including choline, flavin mononucleotide, coniferyl alcohol, Alpha-d-glucose, L-aspartic acid, L-Proline, thymine and etc. were upregulated in the Big group (Fig. S7; Table S2; $P<0.05$). In contrast, 8 differential metabolites including (S)-10,16-Dihydroxyhexadecanoic acid, galactinol, cellobiose, dihydrolipoic acid, etc. were upregulated in the Little group (Fig. S7; Table S2; $P<0.05$).

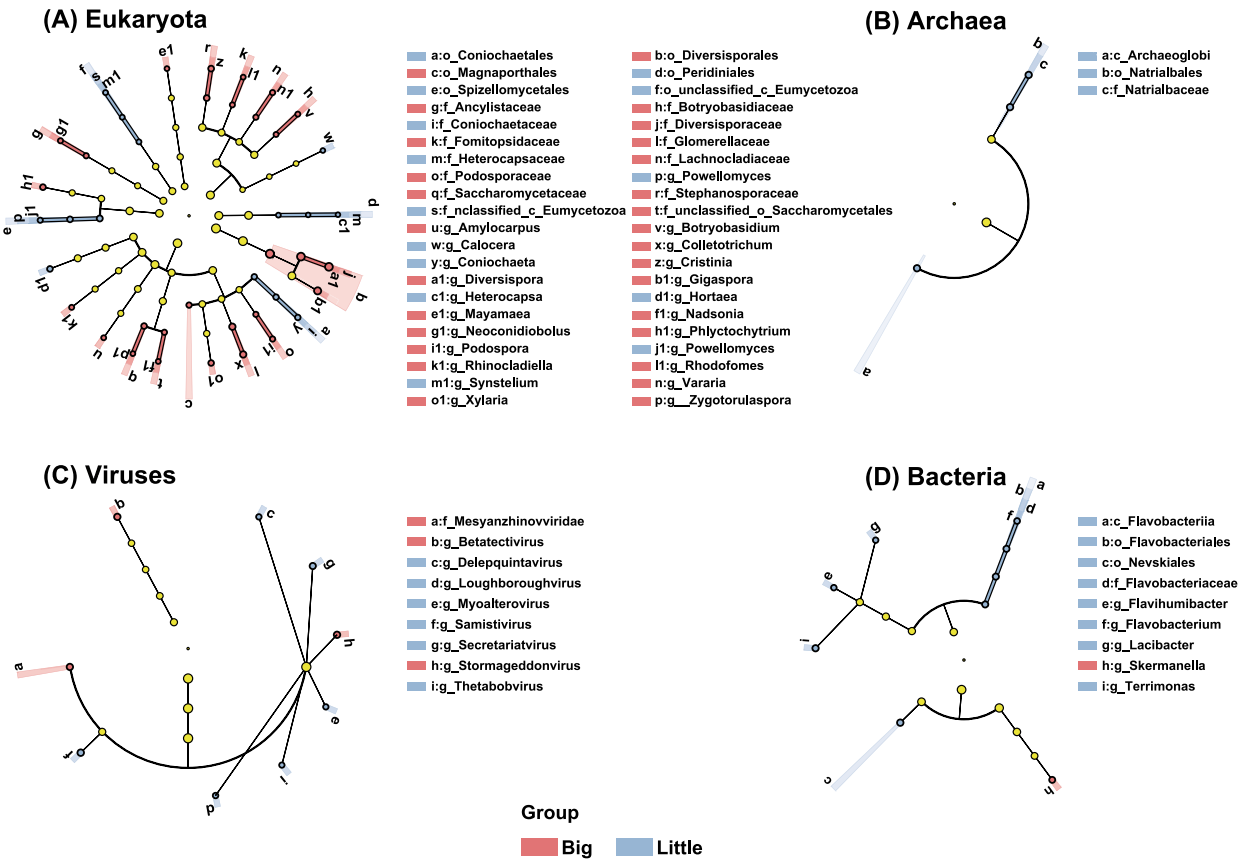

Fig. 2 Rhizosphere soil microbial taxa that susceptible to the two groups (Big vs Little) revealed by LEfSe analysis. The Big group consists of individuals with higher biomass and height, and the Little group consists of individuals with lower biomass and height. Differentially enriched taxa in eukaryotes (**A**), archaea (**B**), viruses (**C**), and bacteria (**D**). Colors indicate enrichment in either the Big (red) or Little (blue) group. The circular cladogram represents taxonomic levels from phylum to genus (inner to outer rings)

Associations among soil properties, phenotypes, rhizosphere metabolites and metagenome of fava beans

Among the 88 differential metabolites (DMEs), a total of 21 metabolites (SCDMEs) were identified as significantly correlated with the phenotypes of fava beans based on Mantel-test analysis (Fig. S8; Table. S3; $P < 0.05$). Of these SCDMEs, 20 metabolites, including flavin mononucleotide, jasmonic acid, L-aspartic acid, Alpha-d-glucose, traumatic acid, etc., were significantly enriched in the Big group, whereas only (S)-10,16-Dihydroxyhexadecanoic acid (C08285) was significantly depleted in the Big group (Fig. 4A; $P < 0.05$). Functional enrichment analysis revealed that SCDMEs were significantly enriched in 15 metabolic pathways (SEMPs), including biosynthesis of plant secondary metabolites, biosynthesis of phenylpropanoids, pyrimidine metabolism, alpha-Linolenic acid metabolism, purine metabolism, etc. (Fig. 4B; $P < 0.05$).

A total of 46 microbial genera (SDMGs) involving in the 15 significantly enriched metabolic pathways (SEMPs) were tracked and found to significantly differ in relative abundance between the two groups. Among the SDMGs, 28 genera, including *Acutalibacter*, *Psychromonas*, *Listeria*, *Thermostaphylospora*, *Frigoriglobus* and others were enriched in the Big group, while 18 genera, such as *Mycolicibacillus*, *Sphingobium*, *Mameliella* and others, were enriched in the Little group (Fig. S9; Table. S4; $P < 0.05$). Furthermore, 11 microbial genera (SCDMGs), including *Psychromonas*, *Listeria*, *Peptostreptococcus*, *Thioploca*, *Acutalibacter*, *Caldibacillus*, *Mycolicibacillus*, *Thermostaphylospora*, *Frigoriglobus*, *Mameliella*, and *Sphingobium,* were found to be significantly correlated with the fava bean phenotypes based on Mantel-test analysis (Fig. S10; Table. S5; $P < 0.05$).

SCDMEs and SCDMGs were strongly correlated with fava beans phenotypes and with the soil properties that previously identified as key factors through Random Forest analysis (Fig. 5A, B; $P < 0.05$). Among the SCDMEs, 20 metabolites, including flavin mononucleotide, jasmonic acid, L-aspartic acid, alpha-d-glucose, traumatic acid, etc., were positively related with biomass and height of fava beans, and negatively related to the soil properties

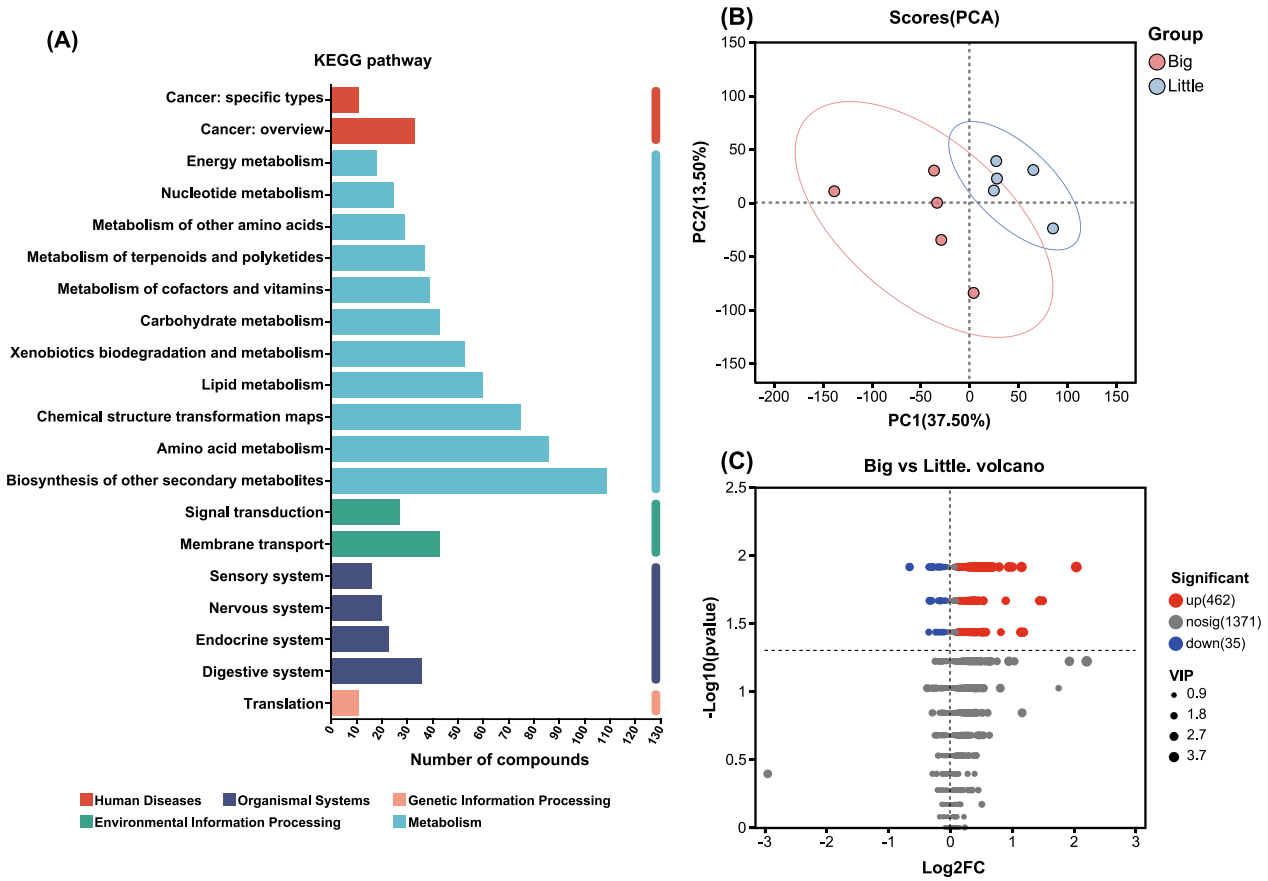

Fig. 3 Rhizosphere metabolites in the two groups (Big vs Little) of fava beans phenotypes. The Big group consists of individuals with higher biomass and height, and the Little group consists of individuals with lower biomass and height. **A**: Functional classification of identified metabolites based on KEGG Level 2 pathways. **B**: Principal component analysis (PCA) of rhizosphere metabolites between the two groups based on Bray–Curtis distance. **C**: Volcano plot showing significantly upregulated metabolites (red), downregulated metabolites (blue), and non-significantly changed metabolites (gray). In **A**, the x-axis indicates the number of identified metabolites per pathway; the y-axis represents KEGG classifications

(Fig. 5A). Notably, soil total concentration of B was the only soil property significantly correlated with all of the SCDMEs (Fig. 5A; $P < 0.05$). Regarding the rhizosphere microbial genera, *Psychromonas*, *Listeria*, *Peptostreptococcus*, *Thioploca*, *Acutalibacter*, *Caldibacillus*, *Thermostaphylospora*, and *Frigoriglobus* showed positive correlations with fava beans biomass and height, but negative correlations with the soil properties. In contrast, *Mameliella*, *Sphingobium,* and *Mycolicibacillus* exhibited the opposite pattern, being positively correlated with the soil properties and negatively correlated with fava beans biomass and height (Fig. 5B).

SEM revealed that the differential rhizosphere soil elements significantly affected fava beans phenotypes through alterations in the differential microbial taxa and metabolites in rhizosphere. In detail, these elements significantly affected fava beans phenotypes via soil pH, which was significantly driven by the differential microbial taxa (Fig. 6A). Standardized total effects analysis further demonstrated that the differential microbial taxa made the greatest contribution to the variance of fava beans phenotypes (Fig. 6B). These microbial taxa were significantly shaped by the differential soil elements, which altered their relative abundance. Through their biogeochemical activities, these microorganisms modulated soil pH, subsequently affected the relative abundance of metabolites, and ultimately influenced fava beans growth.

Discussion

In this study, small-scale (30 cm) heterogeneity of soil properties including soil total As, B, Cd, Co, Cr, Cu, Mo, Ni, Zn and pH triggered significant differences in phenotypes of fava beans through altering the rhizosphere soil microbiome and metabolites, which confirm our hypothesis. These findings highlight that small-scale agricultural soil heterogeneity deserves greater

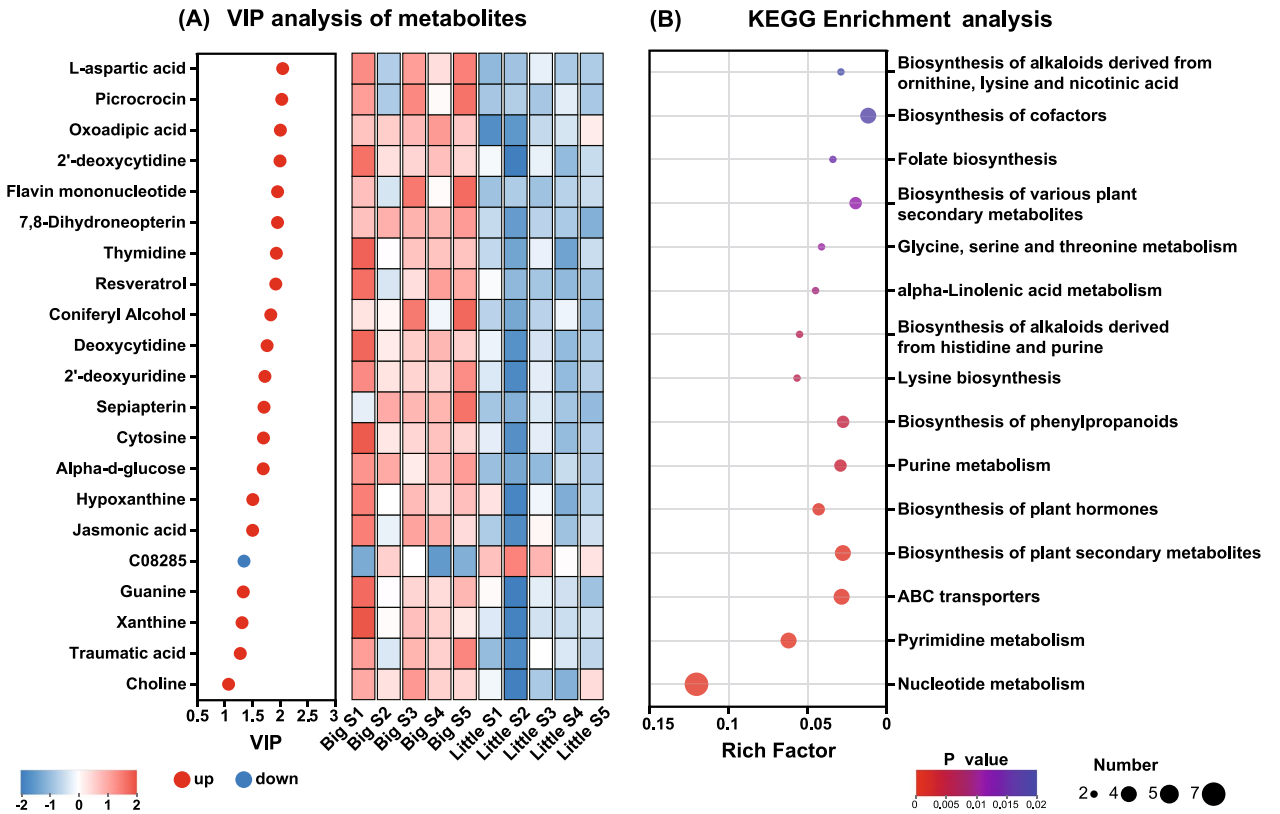

Fig. 4 Differential rhizosphere metabolites and their associated metabolic pathways between the two groups (Big vs Little) of fava beans phenotypes. The Big group consists of individuals with higher biomass and height, and the Little group consists of individuals with lower biomass and height. **A**: Differential metabolites identified by OPLS-DA, with variable importance in projection (VIP) values used to detect the differential metabolites between the two groups. The relative abundance of metabolites was in proportion to the heatmap gradient. **B**: KEGG metabolic pathways that differential metabolites enriched in

attention. Field-level interventions such as soil testing and formula planting may help reduce spatial variability and, in turn, optimize crop yield.

Small-scale soil heterogeneity affected fava beans growth

Soil heterogeneity is widespread phenomenon occurring across a broad range of spatial scales, from microhabitats to global distribution [6, 11]. In agricultural systems, field management practices, including ploughing, irrigation, fertilization, as well as geographical environment filtering can contribute to soil heterogeneity [12, 44]. Numerous studies have demonstrated that soil heterogeneity encompasses variations in nutrient availability and environmental stress, which could influence plant growth [7, 45, 46]. In our study, we observed notable differences in fava beans growth between the two adjacent rows separated by only 30 cm, suggesting that even small-scale heterogeneity could have measurable effects on crop performance.

Furthermore, we determined that soil pH and several concentrations of elements differed between the two groups, and these variables were also predicted as key drivers of fava beans growth by Random Forest analysis. Soil pH is widely recognized as a critical factor in plant growth, as it influences nutrient availability and biogeochemical cycling of soil elements [47, 48]. Previous studies have shown that increasing pH promoted the tea tree growth [49] and potato yield [14]. However, our results revealed the opposite phenomenon, the Big group of fava beans presented a relatively lower soil pH. As a leguminous species, fava beans are capable of biological nitrogen fixation, and larger fava beans may possess higher nitrogen-faxing capacity, as evidenced by elevated NH_4^+–N concentrations in their rhizosphere soils (Fig. S4). The conversion of atmospheric nitrogen into ammonia, followed by ammonium ion accumulation and nitrification, is known to contribute to rhizosphere acidification, potentially explaining the observed decrease in soil pH in the Big group [50].

In our study, fava beans grown in soils with elevated concentration of certain elements exhibited reduced biomass and height. Several studies have reported the

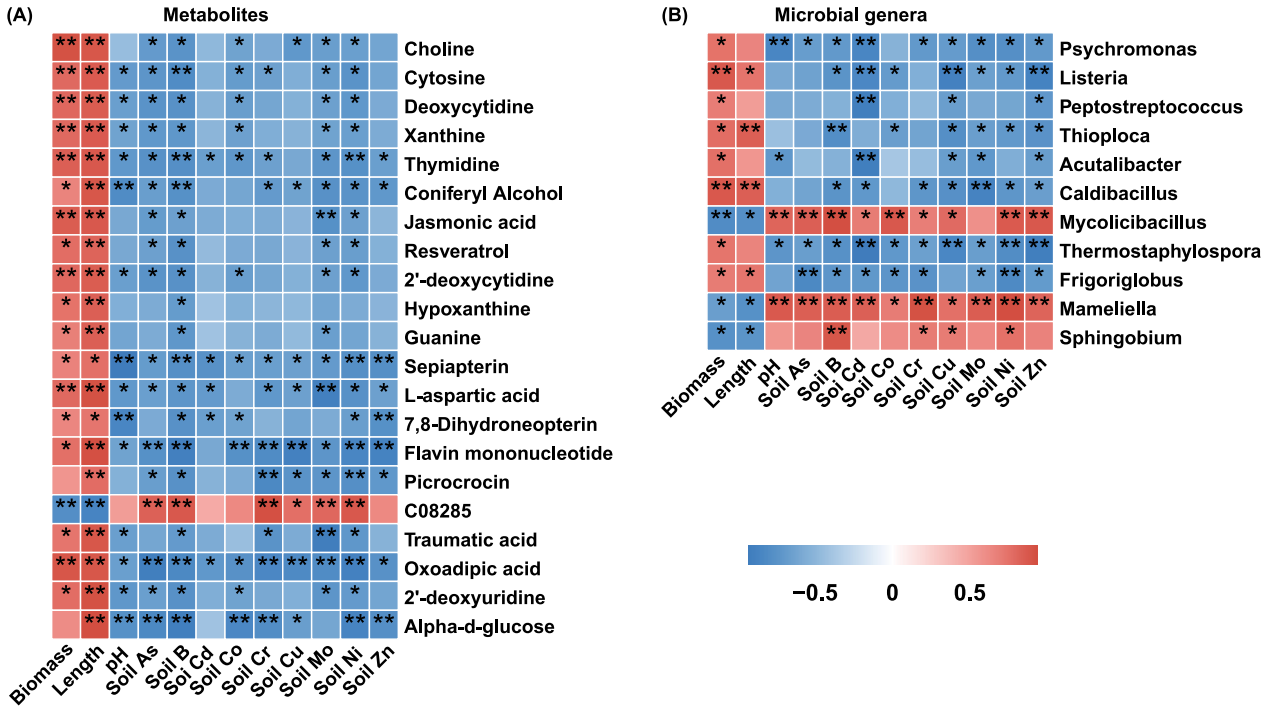

Fig. 5 Spearman correlation analysis between the soil properties, the phenotypes of fava beans, and the differential metabolites (**A**) and microbial genera (**B**). Statistically significant correlation was considered at $P < 0.05$. Asterisks indicate significance: $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***). Correlation coefficient was in proportion to the heatmap gradient

inhibitory effects of soil Cd, Cr and As on plant growth [51–53]. In particular, Cd and As in soil have been shown to disrupt photosynthesis and enzyme activity in rice, impair energy production and consequently lead to inhibited growth and lower yields [54]. Although Zn, Mo, Co, Cu, B and Ni are generally considered essential micronutrients for plant growth [55–57], their beneficial or inhibitory effects depend on their concentrations in soil and plant species [58, 59]. For instance, a previous study reported that Cu significantly reduced photosynthetic efficiency and inhibited rice growth when it exceeded 200 mg/kg in soil [60]. The quantitative requirement for B varies substantially across plant species [56], and similar dose-dependent and species-specific effects have also been observed for Zn, Mo, Co, and Ni with respect to crop growth [61–63].

Soil heterogeneity altered rhizosphere microorganisms

Previous studies have shown that soil microbial community composition can be influenced by environmental stress, plant cultivation, and soil physicochemical properties [64, 65]. Among these microorganisms, soil fungi are particularly sensitive to environmental stress and play essential roles in regulating plant growth [66]. In our study, LEfSe analysis revealed that significant shifts in the rhizosphere soil microbial community were predominantly observed within the eukaryotic taxa. Compared with bacteria, eukaryotic microorganisms are generally more responsive to environmental changes, due to their larger size and slower reproduction rates [27]. The composition of microbial communities could be specialized by environmental filtering (e.g., temperature, nutrient availability, and heavy metal contamination) [67]. In particular, the eukaryotic genus *Gigaspora* and the Bacterial genus *Skermanella* were enriched in the rhizosphere soil of the Big group. Both genera have been reported to be sensitive to soil Cd [68, 69]. The beneficial effects of soil microbial communities on plant growth, health, and crop yield and quality have also been well documented [70]. For instance, inoculation of *Cucumis sativus L.* seedlings with *Diversispora* has been shown to increase the accumulation of essential micronutrients, such as N, P, K and Ca, in cucumber roots [71].

Soil rhizosphere metabolites associated with fava beans growth

Rhizosphere soil metabolites and their associated metabolic pathways, which are potentially linked to fava beans growth, significantly differed between the two groups. Notably, purines, pyrimidines, and nucleotide

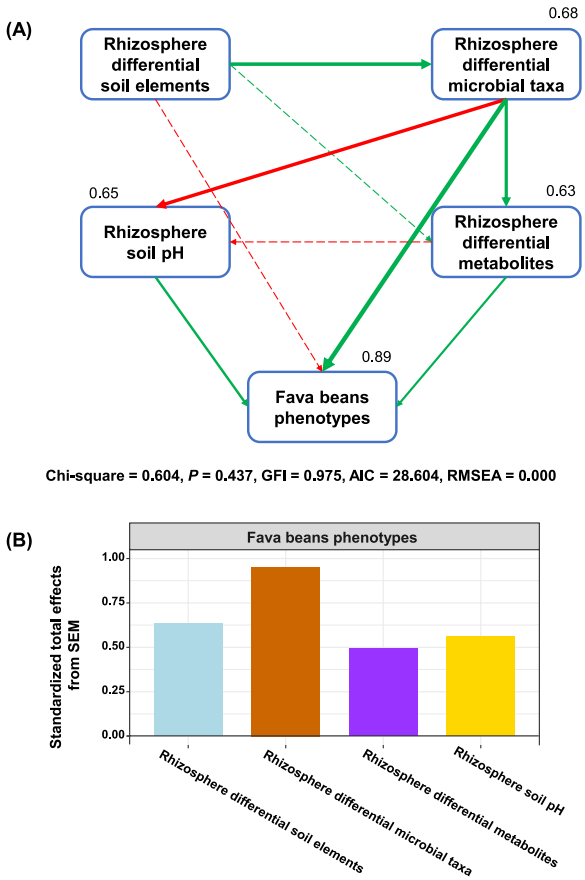

Fig. 6 Potential regulatory mechanisms linking soil properties heterogeneity to fava beans phenotypes. **A**: Structural equation model (SEM) was constructed based on variables of rhizosphere soil elements, soil pH, relative abundance of metabolites and microbial genera, and fava beans phenotypes. The degree of path coefficient is proportional to the width of the arrow line, and the solid line means that a significant correlation was observed between the two variables ($P < 0.05$). The r^2 value represents the rate of variance explained for each variable. **B**: The standardized total effects (direct plus indirect effects) for each influential variable calculated by the SEM. Low chi-square, non-significant probability level ($P > 0.05$), high goodness of fit index (GFI > 0.90), low Akaike information criterion (AIC) and low root mean square error of approximation (RMSEA < 0.05) indicated that our data well matched the hypothesis model

metabolism were significantly enriched in the rhizosphere soil of the Big group. These enrichments suggest favorable growth condition, as purines and pyrimidines are the major building blocks of nucleic acid synthesis and serve as precursors for numerous primary and secondary metabolites [72]. Moreover, plant secondary metabolites could help plants cope with various abiotic stresses [73, 74]. For example, coniferyl alcohol and resveratrol are involved in the biosynthesis of phenylpropanoids. Coniferyl alcohol is one of the three primary precursors for lignin biosynthesis [75], which is essential for water and solute transport in plants [76]. Jasmonic acid participates in the biosynthesis of various plant secondary metabolites, and had been shown to regulate plant growth and response to environmental stress [77, 78]. Moreover, amino acid synthesis is crucial for plant growth and energy supply [79, 80]. L-aspartic acid is a precursor for the synthesis of essential amino acids, such as lysine and threonine [81]. It also plays a role in the biosynthesis of plant hormones, which are key regulators of physiological and molecular responses, transport, and metabolism, etc., thereby promoting plant survival under unfavourable conditions [82, 83]. A previous study demonstrated that L-aspartic acid addition could enhance poplar trees growth by increasing photosynthetic efficiency and hormone levels [84].

Multi-omics revealed mechanisms for fava beans growth in response to soil heterogeneity

Spatial heterogeneity in soil pollutants has been shown to alter the soil microbiome [10, 85] and influence plants growth by modulating the rhizosphere soil microbial structure and metabolic profile [22, 86]. For example, a previous study showed that the excessive Cd in soil altered the metabolite profile and inhibited the growth of *Eclipta alba* (L.) [87]. In this study, elemental heterogeneity within 30 cm led to distinct rhizosphere metabolite profiles and microbial taxa between the two fava bean groups, which significantly impacted fava beans growth, as the SEM quantitively depicted. Most of the metabolites showed higher relative abundances in the Big group, and their metabolic pathways were found to mediate plant growth and stress responses. A previous study reported that Cu addition reduced purine metabolism in rice rhizosphere soil, disrupted nucleotide metabolism and ultimately inhibited plant growth [20]. In our research, metabolites such as resveratrol, jasmonic acid, and coniferyl alcohol were positively correlated with fava bean phenotypes and negatively correlated with the differential soil elements. Resveratrol was shown to alleviate photosynthesis reduction and boron accumulation in *Capsicum annuum L.* under excessive boron exposure [86]. Jasmonic acid could mitigate Cd-toxicity in chickpea plants through limiting Cd uptake and managing oxidative stress [88]. These findings suggest that pollutant-induced growth inhibition in fava beans is, at least in part, mediated through alterations in rhizosphere metabolites. Furthermore, the combined analyses of metagenomics and metabolomics revealed that fava beans could also recruit specific microbial taxa to the rhizosphere. These differential microbial taxa potentially promote plant growth and enhance stress

resilience through metabolic pathways in which key differential metabolites are enriched [89]. For instance, *Thermostaphylospora* was shown to promote the migration and transformation of As and reduce the toxicity of As [90].

Conclusions

In this study, small-scale soil heterogeneity was found to significantly influence fava bean growth, as evidenced by the distinct phenotypes observed in two adjacent rows under field conditions. Higher concentrations of elements (As, B, Cd, Co, Cr, Cu, Mo, Ni and Zn) and pH level were detected in the rhizosphere soil of the Little group, which implied the inhibitory effects on fava beans growth. Metagenomic analysis illustrated that soil heterogeneity differentiated rhizosphere microorganisms, with 11 differentially abundant microbial genera (SCDMGs) involving in 15 key metabolic pathways (SEMPs). Metabolomics demonstrated that these metabolic pathways were beneficial for fava beans growth and resistant to environmental stress through the 21 differential metabolites (SCDMEs) such as coniferyl alcohol, jasmonic acid, resveratrol, L-aspartic acid, etc. Overall, our findings highlight that even small-scale elemental heterogeneity in agricultural soils can markedly affect plant performance through rhizosphere plant–microbe–metabolite interactions. Our research proposed that more attention should be given to study the related consequences and mechanisms of small-scale heterogeneity in farmland to increase food production. Future research could investigate soil and plant properties at broader temporal scales to provide more precise observations and evidence.

Abbreviations

DMEs	Differential metabolites that can be annotated into functional pathway.
SCDMEs	DMEs that significantly correlated with phenotypes of fava beans
SEMPs	Metabolic pathways in which SCDMEs enriched.
MGs	Microbial genera involved in SEMPs.
SDMGs	MGs that with the differential relative abundance between the groups.
SCDMGs	SDMGs that significantly correlated with phenotypes of fava beans

Supplementary Information

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Supplementary file 1.

Supplementary file 2.

Supplementary file 3.

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Author contributions

ZBZ, LBW, and YFW designed the research. LBW, YFW, ZBZ, and TSJ managed the field trial stations. LBW, ZBZ, and TSJ sampled the soils and conducted the laboratory analyses and the raw data collection. LBW and ZBZ performed the data processes. LBW, YFW and ZBZ wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Raw data of the soil metagenome were deposited in the Sequence Read Archive of NCBI under the accession number PRJNA1175838. Metabolomics raw data was deposited in the OMIX, China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/omix/release/OMIX007833).

Declarations

Ethics approval and consent to participate

Not applicable as there were no human, animal or pathogen subjects involved.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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