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Regulation of root-associated microbiomes and root exudates by different tobacco species

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Abstract

Background The root-associated microbiomes are crucial in promoting plant growth and development through symbiotic interactions with their hosts. Plants may shape their microbiomes by secreting specific root exudates. However, the potential mechanisms how plant species determine root exudates and drive microbiome assembly have been little studied. In this study, three wild tobaccos and one cultivated tobacco were used to investigate the commonalities and differences of both root-associated microbiomes and root exudates.

Results Amplicon sequencing results suggested that tobacco species significantly affected microbial communities in both the rhizosphere and root endosphere, with the strongest impact on the fungal community in the root endosphere. The microbial networks of wild tobacco species were more stable than that of the cultivated tobacco, and fungal members played a more important role in the networks of wild tobacco species, while bacterial members did so in the cultivated tobacco. The rhizosphere bacteria of wild tobacco species showed a higher functional diversity than that of the cultivated tobacco, while the bacteria in the root endosphere presented a contrary pattern. Metabolomics analysis showed significant differences in the composition and abundance of root exudates among the four tobacco species, and the greatest difference was found between the three wild species and the cultivated one. Correlation analysis showed the strongest correlation between metabolites and rhizosphere bacteria, in which O-benzoic acid (2-methoxybenzoic acid) had the most positive correlations with rhizosphere bacteria, while β-ureidoisobutenoic acid had the most negative correlations with rhizosphere bacteria. The rhizosphere bacteria *Streptomyces*, *Hydrophilus* and *Roseobacter* had the strongest positive correlations with metabolites, and the rhizosphere bacterium *Nitrobacter* had the most negative correlations with metabolites.

Conclusion This study revealed the differences of microbial communities and root exudates in the rhizosphere and root endosphere of four tobacco species, which can further improve our understanding of plant–microbiome interactions during crop domestication.

Keywords Tobacco species, Rhizosphere, Root endosphere, Microbial community, Root exudate

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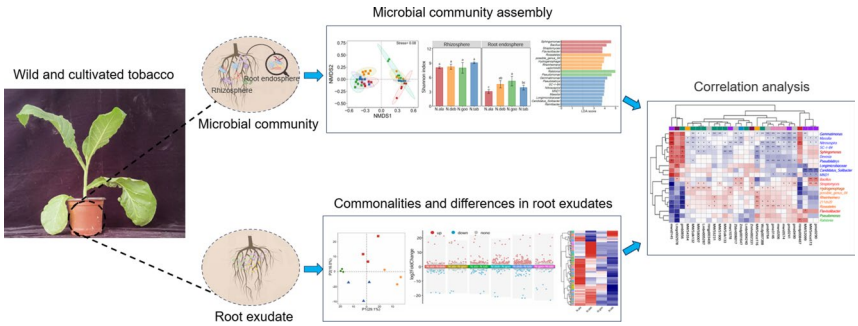
Graphical Abstract



Background

There are abundant and various microbes within and around the roots of plants, including bacteria, fungi, protozoa and viruses, etc. [1]. These microbial members are crucial in affecting plant growth and productivity [2–4]. It has been demonstrated that plants of different species, soil physicochemical properties and environmental factors shaped different microbial communities [5–7]. Plant species and genotypes can shape core microorganisms in roots, regulate the interactions between microorganisms, and change the associations of specific microorganisms with plants, thus regulating the assembly of microbial community in plant roots [8, 9]. Some studies have found that the composition and structure of the root-associated microbial communities differed between wild and cultivated species in barley [10], lettuce [11], sunflower [12] and common bean [13]. Moreover, the wild species of plants had stronger viability under biotic and abiotic stresses, which might be partly attributed to their associations with rhizosphere microbial community [14–16]. Therefore, uncovering the differences of microbial communities in plant species is necessary for deeply understanding the co-evolutionary theories of plant and microbiome interactions during plant domestication.

In nature, the continuous release of root exudates into the soil is an important measure for plants to cope with and adapt to complex environments. Root exudates not only promote plant adaptation to soil environments, but also provide nutrients for the early colonization of soil microorganisms, which play an active role in shaping root-associated microbiomes [17]. For example, Rudrappa et al. [18] previously found that *Arabidopsis thaliana* roots secreted malic acid to selectively recruit *Bacillus spp.* and thereby improved crop disease resistance. Neal et al. [19] found that benzoxazines secreted by maize roots not only induced maize disease resistance, but also recruited *Pseudomonas putida* to colonize the maize rhizosphere, thus influencing host growth and development. Several studies have proved that root exudates were different between different plant types, as well as between wild and cultivated species [20–23]. However, there is still a lack of comprehensive knowledge about how different species of plants regulate root exudates to form a characteristic rhizosphere microbiome.

Tobacco is an important model plant with abundant root exudates. The nutrients and energy substances secreted by the roots of tobacco were effective in maintaining tobacco normal growth, improving the soil environment, and resisting pests and diseases [24]. There exist a wide variety of tobacco species, and significant differences were observed in root exudates among different tobacco species. For example, more esters and fatty acids were detected in root exudates of the tobacco species Gexin 3 which was resistant to black shank disease, but more hydrocarbons and phenolic acids were detected in the susceptible species Xiaohuangjin 1025 [25]. By comparing the content of organic acids secreted from different tobacco species, Yang et al. [26] found that the high-K tobacco species ND202 could secret some specific exudates including 2,4-hexadienoic acid, nonadecanoic acid, 2,3-butanediol and 3-methyl-2-butanol when compared with two common species K326 and NC89. Different root exudates released by plants could recruit differently key microorganisms and affect the composition of root-associated microbiome, thereby improving the capacity of plants to adapt to the environment [1]. Therefore, it is important to study the differences of root exudates and microorganisms among different tobacco species and their potential correlations.

In this study, three wild tobaccos (*Nicotiana alata*, *Nicotiana debneyi* and *Nicotiana goodspeedii*) and one cultivated tobacco (*Nicotiana tabacum* Hongda) planting with the same conditions were used as study models. Amplicon sequencing and metabolic profiling were

performed to study the microbial communities and root exudates of different species of tobacco, respectively. Specifically, we aimed to (1) reveal the assembly patterns of rhizosphere and root endosphere microbiomes among different tobacco species; (2) compare the differences in root secretion metabolisms among different tobacco species; and (3) establish the relationships between root exudates and key microorganisms.

Methods

Sample collection and processing

Three wild tobacco species including *Nicotiana alata* (N.ala), *Nicotiana debneyi* (N.deb) and *Nicotiana goodspeedii* (N.goo), and one cultivated tobacco species of *Nicotiana tabacum* Hongda (N.tab) were used for this study. The seeds of them were purchased from the China Tobacco Germplasm Resource Platform. Tobacco seeds were soaked with 10% in sodium hypochlorite for 12 min and rinsed thrice using sterile water. The sterilized seeds were sown into pots (10 cm × 10 cm) containing potting soil mix (horticultural grade peat:vermiculite in a 9:1 vol:vol mixture), then covered by plastic film. After 21 days, the seedlings were transplanted into new pots. Thirty plants were prepared for each tobacco species. The seedlings were irrigated with water once a week and fertilized with water-soluble fertilizer once a week. The water-soluble fertilizer mainly includes 20% of total nitrogen, 20% of water-soluble phosphorus, 20% of water-soluble potassium, 0.05% of EDTA-Cu, 0.1% of EDTA-Fe, 0.1% of EDTA-Mn, 0.1% EDTA-Zn and 0.15% boron. The plant growth chamber was set at 60% relative humidity, 16 h light (28°C) /8 h darkness (25°C), and 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation. When tobacco grew at the six-leaf stage, two plants for each tobacco species were randomly collected and then mixed into a pooled sample. A total of five pooled samples were taken for each species.

In sampling, tobacco plants were pulled from the soil and the loose soil attached to the roots was shaken away. Rhizosphere samples were obtained by collecting soil close to the roots using a sterilized brush. The remaining roots were placed in sterilized phosphate buffered saline and sonicated for 1 min. Subsequently, roots were soaked in 75% ethanol for five minutes and washed thrice using sterilized water. Finally, the treated roots were snap-frozen in liquid nitrogen and then ground. The ground roots were considered as the endosphere samples. In total, 20 rhizosphere samples and 20 root endosphere samples were obtained from four tobacco species.

DNA extraction and Illumina sequencing

0.5 g rhizosphere and endosphere samples were weighed and microbial DNA was extracted using the Mag-Bind® Soil DNA Kit (OMEGA Biotek Inc., Doraville, GA, USA). DNA concentration was assessed using a NanoDrop® 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and DNA quality was assessed using a 1.0% agarose gel. Bacteria were amplified using universal primers 799F and 1193R (Table S1) to amplify the gene sequences of the V5–V7 region of bacterial 16S rRNA, and fungi were amplified using primers ITS1F and ITS2R (Table S1) to amplify the gene sequences of ITS1 region. The quality of PCR products was controlled using Qubit 4.0 and Agilent 2100 bioanalyzers. Finally, sequencing was performed on the MiSeq platform (Illumina, San Diego, CA, USA) and 250 bp paired-end reads were generated.

Analysis of sequencing data

Amplicon sequencing data were processed using QIIME 2 [27]. The DADA2 [28] module was used for quality control, denoising, and chimera filtering. Valid sequences were clustered at 97% sequence similarity to generate unique amplicon sequence variants (Amplicon Sequence Variants, ASVs). The bacterial and fungal ASVs were then identified for species annotation in comparison with the SILVA (v138) prokaryotic database [29] and the UNITE (v2021.5.10) eukaryotic database [30], respectively. A "filter table" was then prepared using QIIME2 by removing mitochondrial, chloroplast and *Chloroflexi* phylum features, and retaining only feature sequences annotated to the phylum level and below.

To eliminate the effect of sequencing depth, the sequences of each sample was normalized to the minimum read number. The richness and diversity indices (Shannon index, Chao1 index, Simpson index and ACE index) were calculated to reflected the alpha diversity of microbial communities. Beta diversity of the community was calculated based on Bray–Curtis distance matrix and visualized using non-metric multidimensional scaling (NMDS). The Adonis function was used for permutation multivariate analysis of variance (PERMANOVA) statistical tests to assess the relative contributions of different factors to microbial community assembly. Linear discriminant analysis Effect Size (LEfSe) was used to find biomarkers with statistical differences between groups. And the filtering thresholds for the nonparametric factors Kruskal–Wallis rank-sum test was set to 0.05, and the LDA_score filtering threshold was set to 2. These analyses were performed on R software (v4.2.0). Spearman correlation analysis was performed on ASVs, and data with correlation coefficient $r > 0.9$ and $P < 0.05$ were selected, and then network visualization was performed on Gephi (v0.9.7) software. The node topology was classified according to the values of intra-module connections (*Zi*) and inter-module connections (*Pi*). The *Zi* and *Pi*

classification thresholds for microbial taxa were 2.5 and 0.62, respectively [31]. Phylogenetic studies of bacterial communities were performed by reconstructing unobserved states (PICRUSt2) to predict the potential functional characteristics of bacterial communities through 16S rRNA gene data [32].

Metabolites extraction and sequencing

100 g fresh rhizosphere soil was weighed, and the metabolites were extracted with 500 mL deionized water. The extracted metabolites were refrigerated at −80 °C overnight and then 60 mL was taken for vacuum freeze drying. The freeze-dried metabolites were added to 2 mL of 70% methanol extract, shaken for 15 min, and then sonicated in ice water for 15 min. Then the metabolites were centrifuged at 4℃ and 13,000 r/min for 3 min, and the supernatant was removed and filtered with a 0.22 μm microporous membrane and waited for detection [33]. Data were collected using ultra performance liquid chromatography (UPLC) (ExionLC™ AD) and tandem mass spectrometry (MS/MS) (QTRAP®6500+).

Analysis of metabolic data

The metabolites were quantitatively and qualitatively analyzed based on a local metabolite database. Chromatographic integrations and corrections were performed using MultiQuant software. Partial Least Squares Discrimination Analysis (PLS-DA) was performed using the ropls package in R software (v4.2.0). Based on the Variable Importance in Projection (VIP) obtained from the PLS-DA model, the differential metabolites were initially screened. Then the metabolites were analyzed for differences among different groups. The correlation between differential metabolites and key genera was analyzed and visualized on R software (v4.2.0).

Results

Both species and plant compartments affected microbiome assembly in the rhizosphere and root endosphere of tobacco

In total, 1,944,320 bacterial 16S rRNA and 2,650,890 fungal ITS high-quality reads were obtained from 40 samples. These reads were sorted into 11,690 bacterial ASVs and 9,385 fungal ASVs. NMDS and PERMANOVA analyses showed that the plant compartment had a greater effect on both bacterial (46.6%, $P=0.001$) and fungal (26.7%, $P=0.001$) communities than tobacco species (15.7% for bacterial community and 20.1% for fungal community, $P=0.001$ for both) (Fig. 1A and Table S2). Species had a greater effect on fungal community than that on bacterial community. For rhizosphere and root endosphere, NMDS analysis and PERMANOVA analyses showed that tobacco species caused significant differences in both bacterial and fungal communities ($P=0.001$) (Figure S1A). Wild tobacco species (N.ala, N.deb and N.goo) were well separated from cultivated tobacco (N.tab), and the three wild tobacco species were also clearly separated from each other in both the rhizosphere and root endosphere (Figure S1A and Table S3). The alpha diversity of rhizosphere microorganisms was

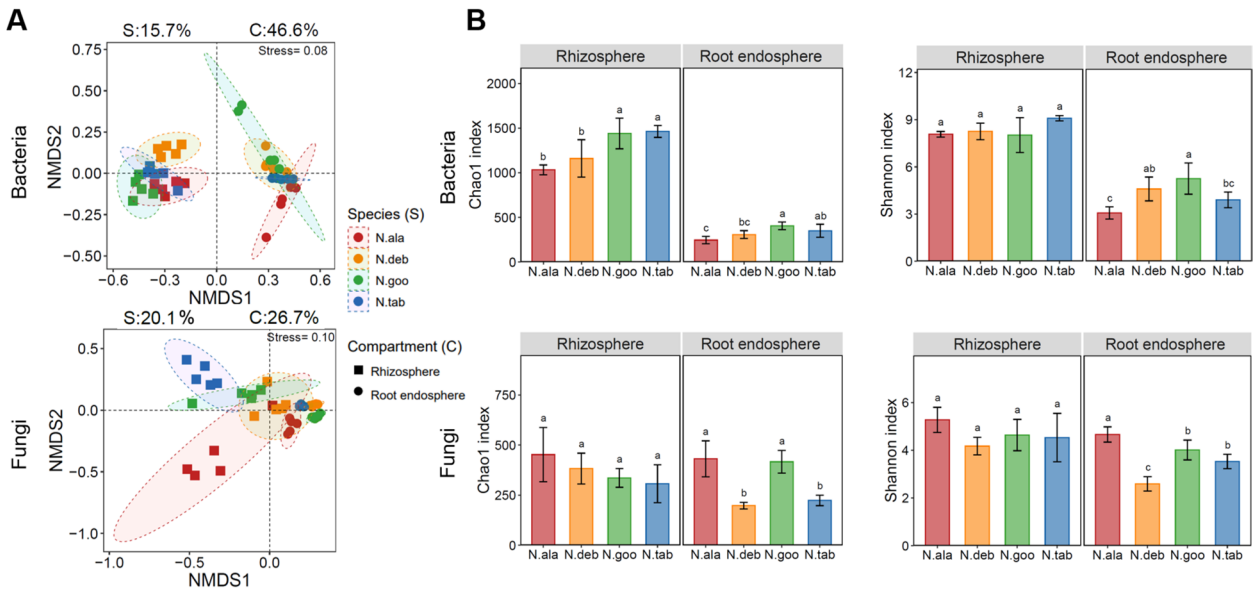


Fig. 1 The influence of tobacco species and plant compartments on the diversity of rhizosphere and root endosphere microbiomes. **A** Non-metric multidimensional scaling ordination (NMDS) analysis of microbial communities. "S" denoted the effect of species; "C" denoted the effect of plant compartments. **B** Alpha diversity analysis of microbial communities. Different letters indicate significant differences in the TukeyHSD test

not significantly affected by species. In the root endosphere, the bacterial community in N.goo showed the highest alpha diversity, and followed by N.deb, N.tab and N.ala, respectively, where the bacterial diversity in N.goo and N.ala were significantly different (ANOVA; $P < 0.05$). But the fungal community showed a different pattern. The fungal community in N.ala showed the highest alpha diversity, and followed by N.goo, N.tab and N.deb, respectively, where the fungal diversity in N.ala was significantly different with N.deb and N.tab (ANOVA; $P < 0.05$), and N.goo and N.deb were significantly different (ANOVA; $P < 0.05$) (Fig. 1B and Figure S1B).

Based on the distribution of total ASVs, we found that the number of ASVs was significantly higher in the rhizosphere bacteria than that in the root endosphere (ANOVA; $P < 0.01$), with little difference for fungi. In the rhizosphere and root endosphere, the four tobacco species all possessed a large number of specific bacterial or fungal ASVs (Figure S2A, B). Taxonomic classification demonstrated that both bacteria and fungi phyla were mildly regulated by species and plant compartments (Figure S3A, B). For the bacterial community, the rhizosphere was mainly composed of *Proteobacteria* (55.07–65.71%), *Bacteroidota* (4.60–14.14%), *Actinobacteria* (2.91–6.28%), *Gemmatimonadota* (2.93–7.38%), *Acidobacteriota* (3.27–4.74%) and *Patescibacteria* (2.59–6.32%), and the root endosphere was mainly composed of *Proteobacteria* (85.99–87.43%) and *Firmicutes* (9.46–11.71%) (Figure S3A). For the fungal community, the rhizosphere was mainly composed of *Ascomycota* (24.99–57.87%) and *Mortierellomycota* (0.57–6.87%), and the root endosphere was mainly composed of *Ascomycota* (11.17–36.26%) and *Basidiomycota* (1.56–7.18%) (Figure S3B). Both bacterial and fungal genera were influenced by species and plant compartments (Figure S3C, D). For the bacterial community, the rhizosphere was mainly composed of *Pseudomonas* (1.06–7.54%), *Ralstonia* (1.99–18.82%), *Sphingomonas* (1.81–7.20%), *Pseudomonas* (1.06–7.54%) and *Candidatus_Kaiserbacteria* (1.90–5.93%), and the root endosphere was mainly composed of *Pseudomonas* (1.03–35.65%) and *Bacillus* (8.60–11.43%) (Figure S3C). For the fungal community, the rhizosphere was mainly composed of *Fusarium* (3.54–16.36%), *Acremonium* (0.37–31.39%), *Lecanicillium* (0.01–10.38%), *Cercophora* (0.78–12.82%) and *Humicola* (0.36–8.93%), and the root endosphere was mainly composed of *Fusarium* (1.24–10.44%), *Simplicillium* (0.15–9.42%) and *Clitopilus* (0.40–6.77%) (Figure S3D).

LDA plots based on LEfSe analysis showed the differential microorganisms in different groups at the family and genus levels (Fig. 2). The number of differential bacteria in the four species were significantly more than that of fungi. For the bacterial families, there were 4, 4, 2 and 10 differential families for N.ala, N.deb, N.goo and N.tab in the rhizosphere, and 1, 4, 11 and 4 differential families for N.ala, N.deb, N.goo and N.tab in the root endosphere (Fig. 2A). For the fungal families, the differential families in the rhizosphere were only detected in N.ala (5) and

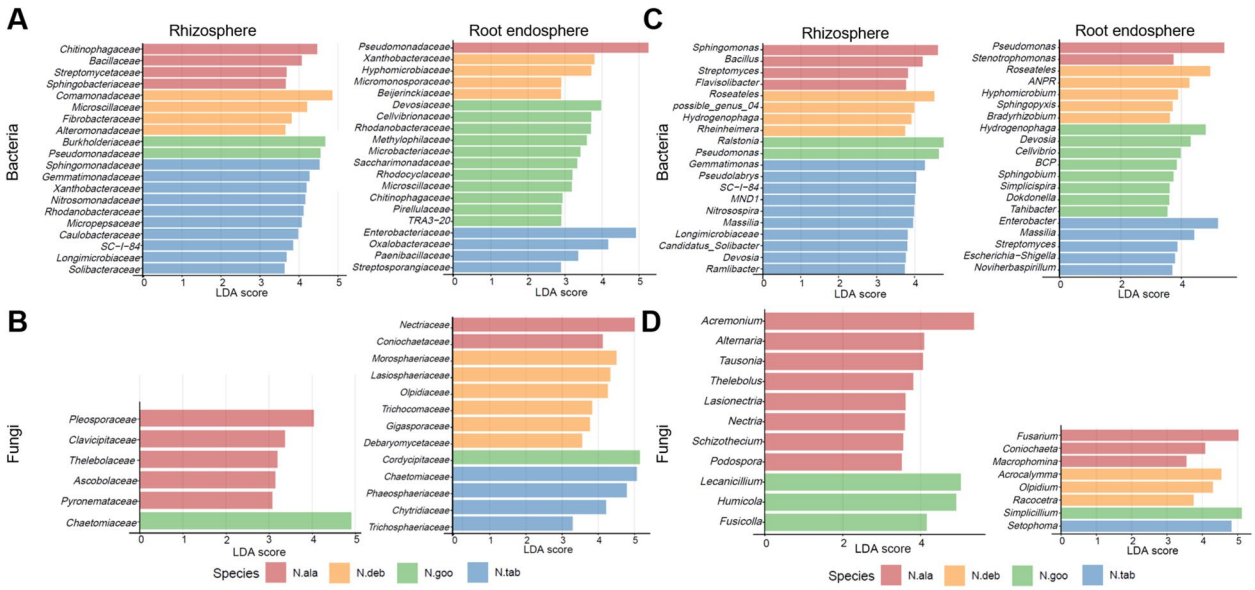


Fig. 2 Differences in microbial communities in the rhizosphere and root endosphere of different tobacco species. **A** Differential bacteria at the family level of different tobacco species (top 20). **B** Differential fungi at the family level of different tobacco species. **C** Differential bacteria at the genus level of different tobacco species (top 20). **D** Differential fungi at the genus level of different tobacco species. *ANPR Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium*; *BCP Burkholderia–Caballeronia–Paraburkholderia*

N.goo (1), and the differential families in the root endosphere were detected in all the four species (2 in N.ala, 6 in N.deb, 1 in N.goo and 4 in N.tab) (Fig. 2B). For the bacterial genera, there were 4, 4, 2 and 10 differential genera for N.ala, N.deb, N.goo and N.tab in the rhizosphere, and 2, 5, 8 and 5 differential genera for N.ala, N.deb, N.goo and N.tab in the root endosphere (Fig. 2C). For the fungal genera, the differential genera in the rhizosphere were only detected in N.ala (8) and N.goo (3), and the differential genera in the root endosphere were detected in all the four species (3 in N.ala, 3 in N.deb, 1 in N.goo and 1 in N.tab) (Fig. 2D).

Species affected microbial co-occurrence networks

Co-occurrence network analysis showed that the interactions between microorganisms were stronger in wild species than in cultivated tobacco, as evidenced by a greater number of nodes and edges. Except for N.deb (124 nodes and 948 edges), the networks of the other two wild species N.ala (145 nodes and 1,396 edges) and N.goo (153 nodes and 3,194 edges) were significantly more complex than that of N.tab (100 nodes and 999 edges). Nodes belonging to fungal taxa were higher than those to bacterial taxa in the three wild tobacco species, while the opposite pattern was observed in the cultivated tobacco N.tab. Negative correlation stabilizes fluctuations in communities with disturbances and promotes network stability. Compared with other three species, microbes in N.goo had a stronger negative within-boundary interaction (53.6%) than other three species (N.ala: 39.4%; N.deb: 41.0%; N.tab: 48.8%) (Fig. 3A). The degrees of bacterial and fungal nodes in N.goo were the highest, followed by N.ala, N.deb and N.tab, respectively (Fig. 3B), indicating more complex networks in wild tobacco species. Similar results were observed in individual plant compartments (Figure S3).

Based on *Zi* and *Pi*, all the nodes were classified as peripherals, connectors, module hubs, and network hubs. The results showed that 20 connectors (10 bacteria and 10 fungi) and 2 module hubs (2 fungi) were found in the N.ala network, 5 connectors (3 bacteria and 2 fungi) were found in the N.deb network, 7 connectors (3 bacteria and 4 fungi:) were found in the N.goo network, and 13 connectors (9 bacteria and 4 fungi) were found in the N.tab network (Fig. 3C).

The functional profiles of the microbiomes in different tobacco species

In order to investigate the effect of species on the functioning of communities, PICRUSt2 was used to predict the metagenome of bacterial communities. NMDS analysis and PERMANOVA analysis based on KO level

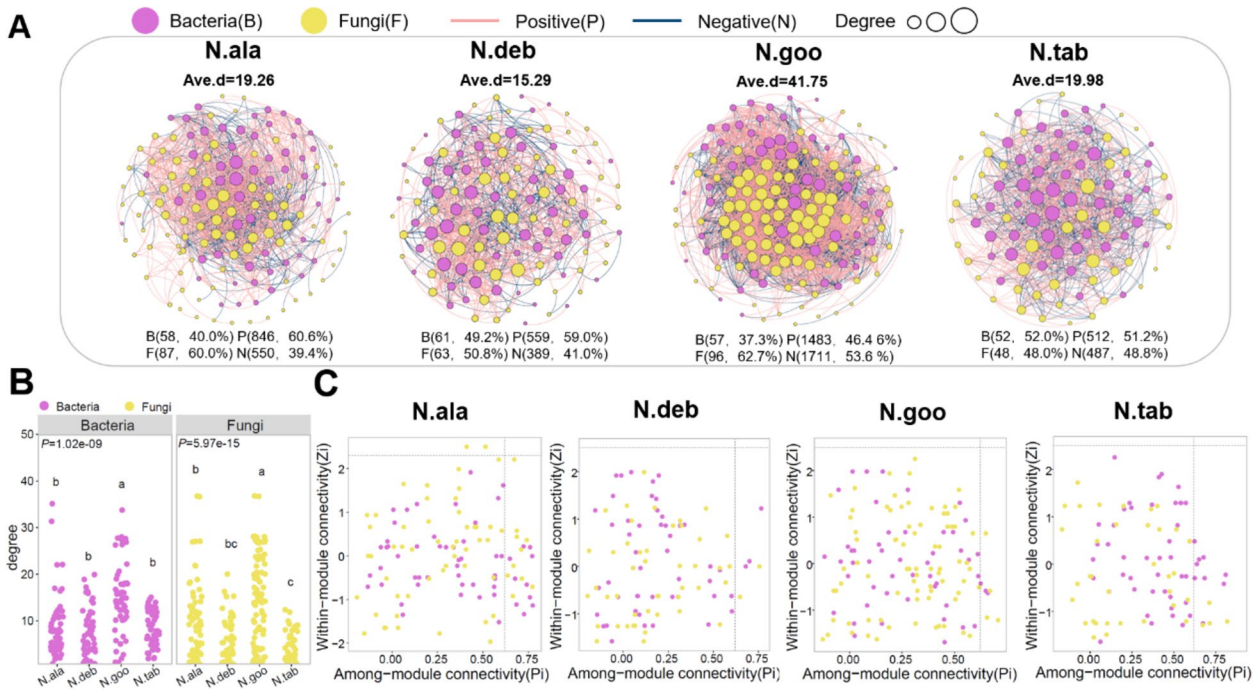


Fig. 3 Symbiotic networks between bacteria and fungi. **A** Symbiotic network analysis of different tobacco species showing different network patterns among microbial kingdoms. **B** Node degree of different tobacco species. Different letters indicated significant differences determined by ANOVA test. **C** Topological roles of these nodes were defined from scatter plots of intra-module connectivity (*Zi*) and inter-module connectivity (*Pi*). *Zi* > 2.5, *Pi* > 0.62 for network hubs, *Zi* > 2.5, *Pi* < 0.62 for module hubs, *Zi* < 2.5, *Pi* > 0.62 for connectors, *Zi* < 2.5, *Pi* < 0.62 for Peripherals nodes

showed that both plant compartments (29.7%) and species (20.1%) had significant effects ($P < 0.01$) on bacterial community function (Fig. 4A). NMDS analysis of each compartment further showed that species elicited significant differences ($P < 0.001$) in bacterial community functioning, with well segregation among different species (Figure S5). Importantly, in the root endosphere, the N.tab group had a significantly higher functional diversity than the N.goo group ($P < 0.05$) (Fig. 4B).

In addition, some C, N and P cycling-related genes had different patterns in different plant compartments or different species (Fig. 4C). Functional genes involved in Denitrification (e.g., *norB*, *nirK*, *nrfh*, *nirS* and *nosZ*), N fixation (e.g., *nifH*, *nifD* and *nifK*) and Nitrification (e.g., *amoC*, *amoA* and *amoB*) were much more abundant in the rhizosphere microbiome. While genes involved in C degradation (e.g., *vanA*, *amyA* and *xylB*), C fixation (e.g., *cbbL*) and P transport (e.g., *pstC*, *pstA*, *pstB* and *pstS*) were more abundant in the root endosphere microbiome. The effects of species were more pronounced in the root endosphere than in the rhizosphere. In the rhizosphere, *nirD* and *nirB* in the pathway of N reduction were enriched in the three wild species and depleted in the N.tab group. In the root endosphere, *pstC*, *pstA*, *pstB* and

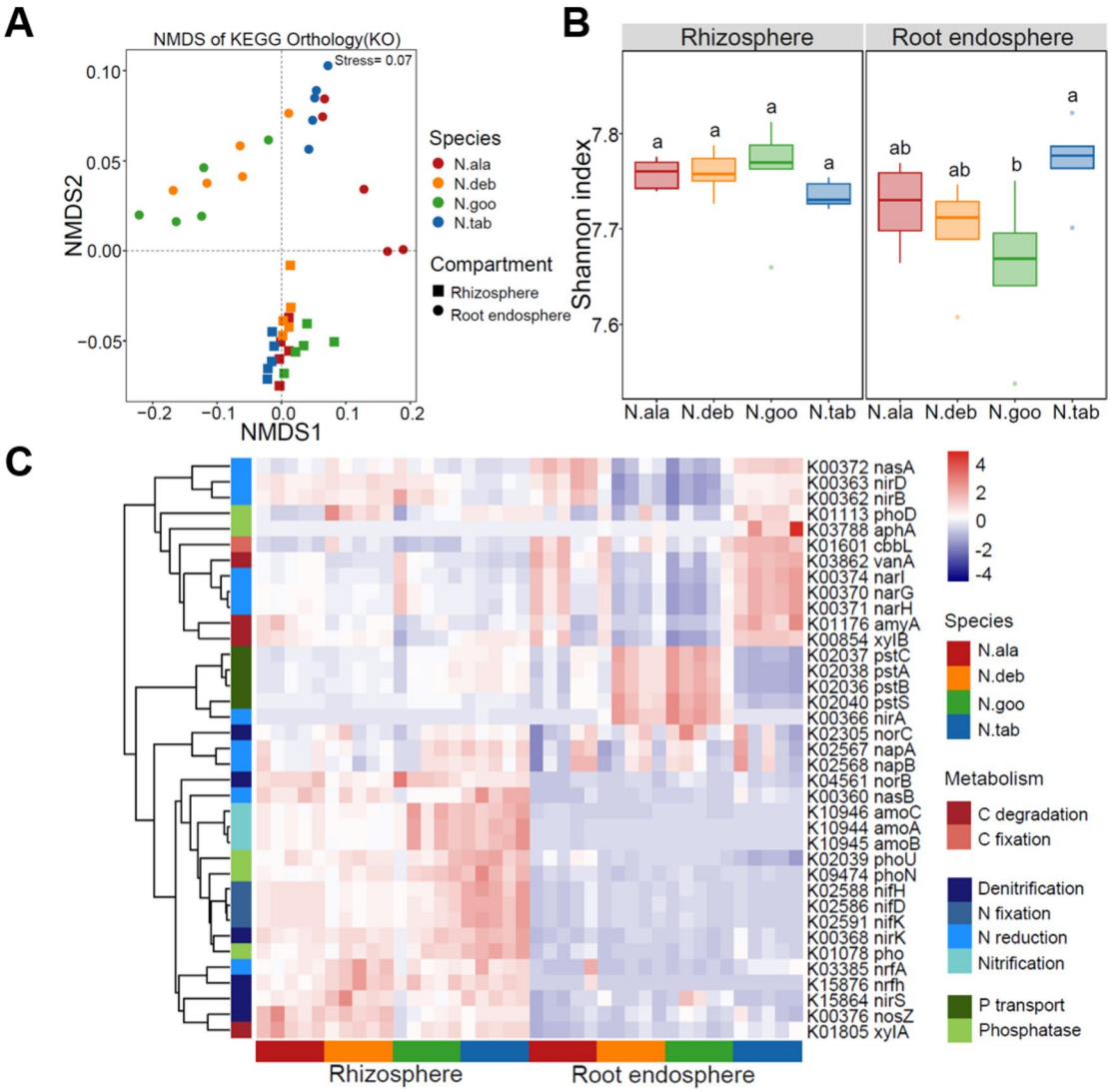


Fig. 4 PICRUSt predicted the functional distribution of microbial communities between different tobacco species at the KO level. **A** The KO-based NMDS showed significantly different microbial function across different species and plant compartments. **B** Functional diversity of rhizosphere and root endosphere microbiomes in different tobacco species. Different letters indicated significant differences as determined by the Tukey HSD test. **C** Heat map showing the relative abundance of functional genes (based on KO) involved in C, N and P cycling, which varied among different species and plant compartments

pstS in the pathway of P transport were enriched in the wild species N.deb and N.goo, but the phosphatase gene *aphA* was enriched only in the N.tab group (Fig. 4C).

Differences in root exudates of different tobacco species

The differences in root exudate composition among the four species were analyzed by PLS-DA model (Fig. 5A). The results showed that the species exerted a remarkable influence on root exudate composition and the sum of the contributions of the first and second principal components was 45.1% (Fig. 5A). A total of 884 metabolites were detected, including lipids (23.27–31.75%), phenolic acids (14.27–21.23%), quinones (3.73–10.42%), alkaloids (5.77–7.64%), terpenoids (4.83–7.65%), amino acids and derivatives (2.13–3.63%), nucleotides and derivatives (2.28–4.96%), organic acids (2.02–3.27%), lignans and coumarins (0.31–0.57%), flavonoids (0.2–0.38%), and several unclassified metabolites (24.18–26.72%) (Figure S6). The differential metabolites of any two species were compared using a volcano plot, which showed all the three wild species enriched a number of metabolites when compared with the cultivated N.tab (N.ala vs N.tab: 161, N.deb vs N.tab: 314, N.goo vs N.tab: 98) (Fig. 5B, Table S4).

A total of 189 differential metabolites were screened (VIP ≥ 1 and $P < 0.05$), and analyzed by hierarchical cluster analysis (Fig. 5C). The differential metabolites among different species showed the N.ala group had the most differential metabolites, while N.tab had the fewest differential metabolites. Using VIP ≥ 1, $P < 0.001$ as the screening criteria, 29 highly significantly differential metabolites were screened out, and eight differential metabolites belonging to different classifications were selected to compare their abundance in different species (Fig. 5D). The results showed that 2-hydroxyisobutyric acid, 4-methylbenzoic acid, fraxetin (7,8-Dihydroxy-6-methoxycoumarin), β-ureidoisobutyric acid, and O-anisic acid (2-methoxybenzoic acid) differed significantly between wild and cultivated species. LysoPE 18:1(2n isomer), nicotinic acid, 5'-deoxyadenosine and 3,3'-di-(3-methylbutanoyl) sucrose were significantly different among the four species (Fig. 5D). These results

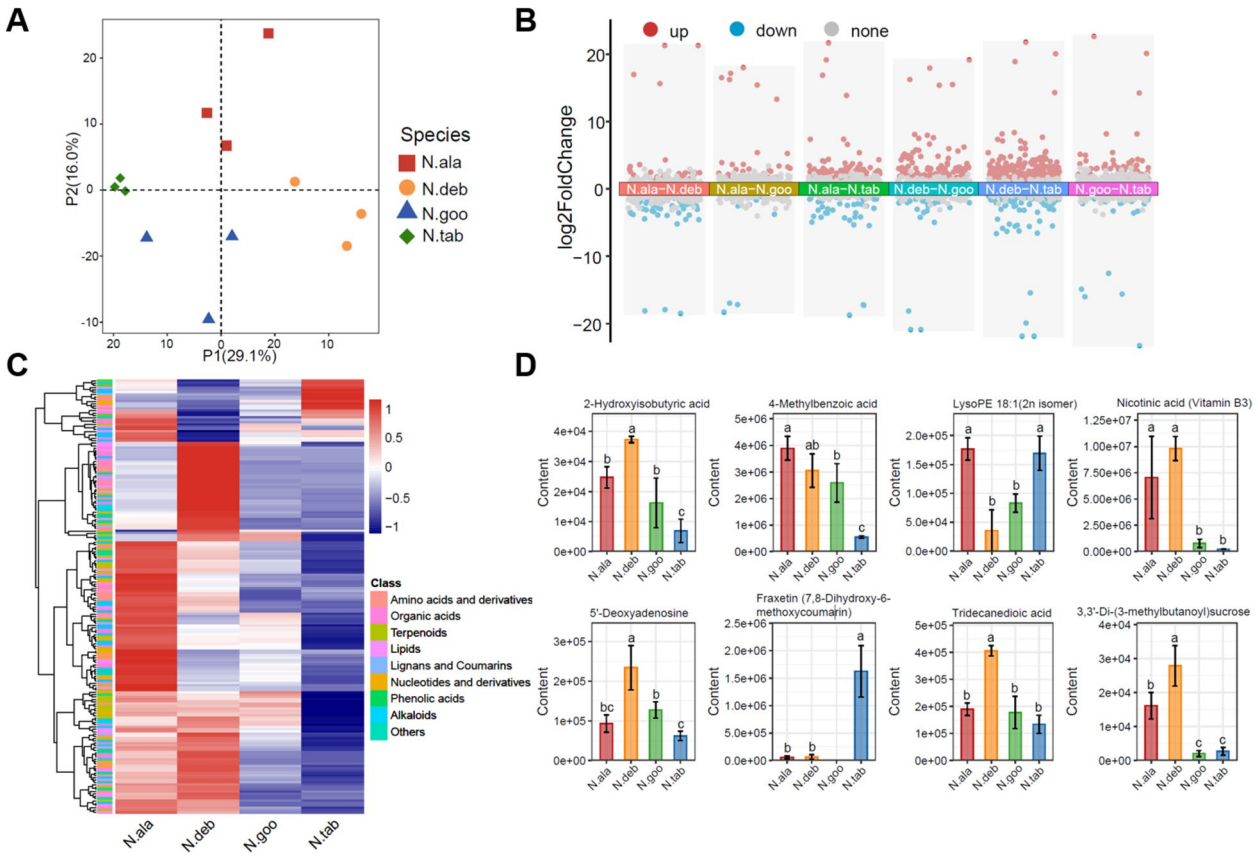


Fig. 5 Differential analysis of tobacco root exudates of different species. **A** PLS-DA showing the difference of metabolites among four species of tobacco. **B** Volcano plot showing differential metabolites between any two species. **C** Heat map showing the relative abundance of differential metabolites among the four species of tobacco. **D** The contents of some differential metabolites in wild and cultivated tobaccos. Different letters indicated significant differences as determined by the TukeyHSD test

suggested that tobacco species affected the composition and accumulation of metabolites in the rhizosphere soil.

Relationship between tobacco root exudates and microorganisms

Correlation analysis was used to investigate the relationship between differential microorganisms and differential metabolites among different species. The results indicated that the correlation between differential microorganisms and differential metabolites in the rhizosphere (Fig. 6) was much greater than that in the root endosphere (Figure S7), and fungi showed fewer correlations with metabolites compared to bacteria. Among them, the metabolite O-anisic acid (2-methoxybenzoic acid) (mws0145) had the most positive correlations with the differential bacteria in the rhizosphere, showing a significantly positive correlation with 6 bacteria and a significantly negative correlation with 1 bacteria. For example, *Gemmatimonas* and *Nitrosospira* that were enriched in N.tab showed significantly positive correlations with O-anisic acid (2-methoxybenzoic acid) ($P<0.01$), and *Roseateles* that was enriched in N.deb showed a significantly negative correlation with O-anisic acid (2-methoxybenzoic acid) ($P<0.01$). While metabolite β-ureidoisobutyric acid (pme3146) had the most negative correlations with the differential bacteria in the rhizosphere, showing a significantly negative correlation with 7 bacteria and a positive significantly correlation with 2 bacteria (Table S5). For example, *Gemmatimonas, Nitrosospira, SC-I-84* and *Pseudolabrys* that were enriched in N.tab showed significantly negative correlations with β-Ureidoisobutyric acid ($P<0.01$), and *Roseateles* that was enriched in N.deb showed a significantly positive correlation with β-ureidoisobutyric acid ($P<0.01$). The rhizosphere bacteria *Streptomyces*, *Hydrogenophaga* and *Roseateles* had the most positive correlations with

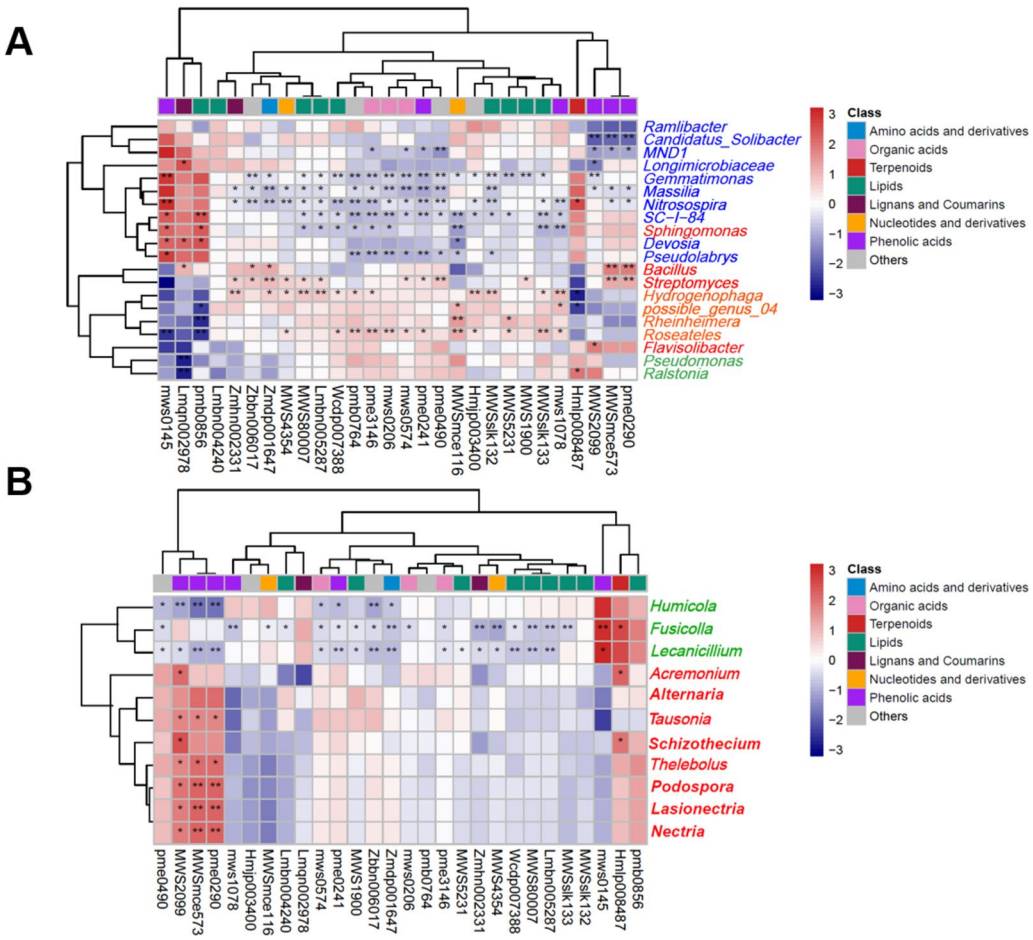


Fig. 6 Correlation analysis between differential metabolites and microorganisms in the rhizosphere. **A** Correlations between differential metabolites and rhizosphere bacteria. **B** Correlations between differential metabolites and rhizosphere fungi. The colors of the row names in the heatmap represent differential microbial genera in different tobacco species, red: N.ala, orange: N.deb, green: N.goo, blue: N.tab. *ANPR Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium*; *BCP Burkholderia–Caballeronia–Paraburkholderia*

metabolites and positively correlated with 12 metabolites. The rhizosphere bacterium *Nitrosospira* had the most negative correlations with metabolites and negatively correlated with 19 metabolites (Fig. 6A).

Discussion

Plant root-associated microbial communities are key factors affecting plant growth [34, 35]. Changes in the structure of plant root-associated microbial communities were driven by a combination of host and environmental factors [31]. Revealing the effects of plant species on the assembly and function of the root-associated microbiomes, as well as that on the composition and content of root exudates, is important for enhancing our understanding of plant–microbiome interactions. In this study, the results indicated that the structure and function of root-associated microbial communities and components of root exudates were partly influenced by tobacco species. This finding is consistent with studies on wheat [36], rice [37], and licorice [38], demonstrating that plant species were crucial in shaping the plant microbiomes.

This study examined the effects of tobacco species on bacterial and fungal communities in the rhizosphere and root endosphere. It was found that the assembly of the rhizosphere and root endosphere microbiomes was affected by both plant compartments and species (Fig. 1A). Several studies have showed that plant compartments are key factors affecting plant-associated microbiome assembly [39–41]. Similarly, we found that the rhizosphere and root endosphere formed different microbial communities (Fig. 1A), which further confirmed that plant compartments were the main selection force in determining the composition of plant-associated microbiomes [42]. For different plant species, it was found that tobacco species had more influence on fungal community than bacterial community, which was consistent with the effects of rice species on the composition of bacterial and fungal communities [43]. The composition of fungi community changed greatly between wild and cultivated rice, while the bacteria community was relatively conserved [43], and this conclusion may also be applicable in tobacco. We also found that the tobacco species showed a more significant effect on the alpha diversity and functional diversity of the root endosphere microbiome than on the rhizosphere microbiome (Figs. 1B, 5B). And genes associated to the C, N, and P cycles had different patterns in different plant compartments or in different species (Fig. 5C). This may be attributed to differences in the microorganisms contained within the seeds before planting and the vertical transmission of these microorganisms [44–46]. Moreover, when soil microorganisms entered the plant roots, there was a process of selective screening of specific microbial taxa and functions, and only microorganisms that could adapt to the specific root environment and interact with the host could successfully colonize and reproduce [47, 48]. This study provided new evidence that plant species influenced both the structure and function of the host root-associated microbiome.

Each plant has a specific rhizosphere microbiome [49]. Even rhizosphere microbial composition is different among different genotypes of the same plant species [50]. For example, *indica* and *japonica* rice, among which, *indica* rice species attracted more nitrogen cycling-related bacteria in the roots [51]. The reason for the difference was not only related to the filtration effect of plant hosts, but also might be affected by the type and content of plant root exudates [52]. Some evidence has indicated that *Bacillus* [53], *Streptomyces* [54] *Pseudomonas* [55], *Sphingomonas* [56], *MND1* [57, 58] and *Massilia* [59] could colonize various plant compartments in plants and have significant effects on plant growth. In our study, different tobacco species also recruited different microbial members. For example, *Bacillus*, *Streptomyces*, *Pseudomonas* and *Sphingomonas* were enriched in wild tobacco rhizosphere, whereas *MND1* and *Massilia* were enriched in cultivated tobacco rhizosphere (Fig. 2). Colonization of these specific microorganisms was involved in the assembly of root-associated microbial community. These findings can deepen the understanding of the mechanism of plant–soil–microbial interaction and provide theoretical reference for breeding and improvement strategies.

There were also frequent interactions between different microorganisms within the microbial community, including symbiosis, parasitism, predation, competition, etc. These interactions not only affected the stability of the community, but also had profound effects on the environment and host health [60]. Negative regulation among microorganisms, known as ecological competition, could enhance the stability of microbial communities by inhibiting cooperative instability [61]. Plants might benefit from microbial competition, thereby increasing resistance to external pressures [62]. In this study, the wild species N.goo had the strongest negative interactions, which might enhance the stability of microbial networks. Besides, fungal ASVs were most represented in the network among the three wild tobacco species, whereas bacterial ASVs were most represented in the network of cultivated tobacco N.tab (Fig. 3A). This phenomenon was similar to the study on wheats which suggested that fungi preferred the ecological environment created by wild plants and their associated root exudates [36].

Previous studies have demonstrated that root exudates could influence the plant health by regulating the structure of soil microbial community [63, 64]. In this

study, different root exudates were detected in different tobacco species, and the most differential root exudates were found between the cultivated tobacco and three wild tobacco species (Fig. 5), suggesting that tobacco species affected the and composition accumulation of root exudates. Various organic substances contained in these root exudates could regulate the flow of nutrients and energy required for the colonization of root-associated microorganisms, thus affecting the dynamic changes of root-associated microbial communities [22, 65]. A recent study [66] has found that strong correlations were observed between microorganisms and metabolites, and bacteria dominated the symbiotic network. Our results were consistent with this study and showed that bacteria had more correlations with metabolites than fungi, and microorganisms in the rhizosphere had more correlations with metabolites than that in the root endosphere (Fig. 6, Figure S7), indicating that root exudes had a greater impact on rhizosphere bacteria. In addition, most of the microorganisms enriched in cultivated tobacco were negatively correlated with differential metabolites, whereas most of the microorganisms enriched in wild species were positively correlated with differential metabolites (Fig. 6). This may be related to the high abundance and diversity of root exudates in wild tobacco (Fig. 5C). These results demonstrated that different species of tobacco might recruit specific microorganisms by producing different root exudates, which was similar to the study on *Arabidopsis thaliana* [67]. The rhizosphere bacteria *Streptomyces*, *Hydrogenophaga*, *Roseateles* and *Nitrosospira* were significantly correlated with most of the differential metabolites (Fig. 6A), and these bacteria have been shown to stimulate plant growth potential and enhance plant stress resistance [54, 68–70]. This further proved that microorganisms and plants interacted with each other through corresponding secretions, thus affecting plant growth and health [71]. Collectively, these findings could provide new evidence for the influence of root exudates on microbial community assembly.

Conclusion

In this study, we studied the regulatory effects of different tobacco species on root-associated microbiomes and root exudates using amplicon sequencing and metabolome detection. It was found that tobacco species had significant effects on the diversity, composition, symbiotic network and functional of microbial communities in the rhizosphere and root endosphere. The composition and abundance of root exudates released by different tobacco species also showed significant differences. Moreover, there were certain correlations between root exudates and microbial communities, which might explain the differences in microbial communities among different species.

Abbreviations

N.ala	*Nicotiana alata*
N.deb	*Nicotiana debneyi*
N.goo	*Nicotiana goodspeedii*
N.tab	*Nicotiana tabacum* Hongda
ASV	Unique amplicon sequence variant
NMDS	Non-metric multidimensional scaling
PERMANOVA	Permutational multivariate analysis of variance
LEfSe	Linear discriminant analysis effect size
ANOVA	One-way analysis of variance
UPLC	Ultra performance liquid chromatography
MS/MS	Tandem mass spectrometry
PLS-DA	Partial least squares discrimination analysis
VIP	Variable importance in projection
S	Species
C	Compartments
ANPR	*Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium*
BCP	*Burkholderia–Caballeronia–Paraburkholderia*

Supplementary Information

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Supplementary material 1.

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Author contributions

M.L. Gu, J.M. Tao and P.J. Cao designed the study. M.L. Gu wrote the manuscript. M.L. Gu, J.M. Tao and S.Z. Yu performed the experiments. M.L. Gu, J.J. Jin, H. Su and Z.X. Yang conducted the statistical and bioinformatics analysis. J.M. Tao and P. Lu contributed to conceptualization and funding acquisition. J.M. Tao, H.H. Shang, J.F. Zhang, and P.J. Cao were involved in the revision of the manuscript. All authors reviewed the manuscript.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Genome Sequence Archive in National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences (https://bigd.big.ac.cn/gsa), under accession number CRA016784 (16S) and CRA016783 (ITS).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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