Environmental Response to Root Secondary Metabolite Accumulation in *Paeonia lactiflora*: Insights from Rhizosphere Metabolism and Root-Associated Microbial Communities

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**ABSTRACT** *Paeonia lactiflora* is a commercial crop with horticultural and medicinal value. Although interactions between plants and microbes are increasingly evident and considered to be drivers of ecosystem service, the regulatory relationship between microbial communities and the growth and root metabolites of *P. lactiflora* is less well known. Here, soil metabolomics indicated that carbohydrates and organic acids were enriched in the rhizosphere (RS) with higher diversity. Moreover, the variation of root-associated microbiotas between the bulk soil (BS) and the RS of *P. lactiflora* was investigated via 16S rRNA and internally transcribed spacer (ITS) amplicon sequencing. The RS displayed a low-diversity community dominated by copiotrophs, whereas the BS showed an oligotroph-dominated, high-diversity community. Hierarchical partitioning showed that cation exchange capacity (CEC) was the main factor affecting microbial community diversity. The null model and the dispersion niche continuum index (DNCI) suggested that stochastic processes (dispersal limitation) dominated the community assembly of both the RS and BS. The bacterial-fungal interkingdom networks illustrated that the RS possessed more complex and stable co-occurrence patterns. Meanwhile, positive link numbers and positive cohesion results revealed more cooperative relationships among microbes in the RS. Additionally, random forest model prediction and two partial least-squares path model (PLS-PM) analyses showed that the *P. lactiflora* root secondary metabolites were comprehensively impacted by soil water content (SWC), mean annual precipitation (MAP), pH (abiotic), and *Alternaria* (biotic). Collectively, this study provides a theoretical basis for screening the microbiome associated with the active components of *P. lactiflora*.

**IMPORTANCE** Determining the taxonomic and functional components of the rhizosphere microbiome, as well as how they differ from those of the bulk soil microbiome, is critical for manipulating them to improve plant growth performance and increase agricultural yields. Soil metabolic profiles can help enhance the understanding of rhizosphere exudates. Here, we explored the regulatory relationship across environmental variables (root-associated microbial communities and soil metabolism) in the accumulation of secondary metabolites of *P. lactiflora*. Overall, this work improves our knowledge of how the rhizosphere affects soil and microbial communities. These observations improve the understanding of plant-microbiome interactions and introduce new horizons for synthetic community investigations as well as the creation of microbiome technologies for agricultural sustainability.

**KEYWORDS** plant-microbe associations, bacterial-fungal interactions, dispersal limitation, microbial interdomain networks, rhizosphere metabolome, *Paeonia lactiflora*
Determining the composition and distribution of the rhizosphere (RS) microbes, as well as how they change from those of the bulk soil (BS) microbes, is critical for manipulating them to improve plant growth performance and increase agricultural yields (1, 2). The rhizosphere is an essential interface that allows plants and their soil environment to exchange resources (3). It is commonly acknowledged that plants can control rhizosphere microbial diversity and community by altering the composition of their root exudates (4). In other cases, the plant influences its microbiome by altering soil pH, decreasing competition for beneficial microorganisms, and providing an energy source, typically in the form of carbon-rich rhizodeposition (5). Plants utilize rhizobacterial assemblages in bulk soil pools to gain particular functional features required for plant fitness (2), and thus, the rhizosphere is a subset of the bulk soil microbe (6). The widespread consensus is that the diversity and composition of rhizosphere bacteria differ from those of bulk soil microbiota, which is linked to major changes in physicochemical parameters that drive niche differentiation (7). Aside from environmental variations across niches, the overall discrepancy in bulk soil and the rhizosphere is a significant driver of microbial distribution differences (8). The richness and composition of the rhizosphere microbes are influenced by plant species and soil properties (9). In addition, high-throughput sequencing of culture-independent marker genes (typically, 16S rRNA in bacteria and internal transcribed spacer [ITS] in fungi) has lately significantly expanded the repertory of soil microorganisms, and various studies have explored root-associated soil microbes, such as those for crops (10), Salvia miltiorrhiza (11), licorice (12), and citrus (13). Therefore, the composition and diversity of bacteria and fungi in *P. lactiflora* in bulk soil and the rhizosphere can be explored by amplicon sequencing.

Researching the microbiome on the soil-plant continuum is essential for investigating how these environmental factors respond to plant development and secondary metabolite (SM) synthesis (10). The effects of the microbiome on plant secondary metabolism of ginseng (14), licorice (15), etc., have been reported. Herbaceous peony (*Paeonia lactiflora* Pall.), a common garden plant in temperate climates (16) coming in a variety of flower types and colors, offers significant commercial benefits in the form of cut flowers, landscape architecture, and potted plants (17). Furthermore, the root of *P. lactiflora* is commonly used as a traditional medicine named Baishao, with analgesic and anti-inflammatory activities, to treat rheumatoid arthritis, hepatitis, and spasms (18). The root secondary metabolites of *P. lactiflora* are mainly represented by paeoniflorin (19). At present, the main research on *P. lactiflora* is focused on horticultural traits and pharmacological effects. However, research on the microbiome throughout the soil-plant continuum and how the microbiome interacts with *P. lactiflora* root secondary metabolites is limited. By considering the differences in soil metabolites, the different processes of microbial change in bulk soil and the rhizosphere can be improved (20). The metabolic profiles in root-associated soils contain a wide range of compounds that attract certain microbial species to develop intricate interactions with plants (21). Thus, chemical compounds in root-associated soil could influence soil microbes. Connecting chemicals to unique microbes can provide an understanding of the maintenance of microbial communities in root-associated soils (22). Overall, soil metabolites can disclose intricate molecular connections and metabolic functions in the soil microbiome community, as well as providing a mechanism of evaluating soil function (23).

Here, this study collected two soil compartments (bulk soil [BS] and rhizosphere [RS]) from four primary areas of *P. lactiflora* production and performed amplicon sequencing, physicochemical property determination, and soil metabolism determination. Consequently, we presumed that (i) copiotrophic microbes are more common in the RS than in the BS (1), (ii) the diversity of the microbes in the BS is higher than that in the RS (10), (iii) the complexity of the bacterial-fungal interkingdom network in the rhizosphere is higher than that in the bulk soil, and therefore, the bacterial-fungal interkingdom network in the rhizosphere is more robust than that in the bulk soil (24), and (iv) the soil properties and microbial community of *P. lactiflora* would be partially
correlated with root secondary metabolites (15). Specifically, our aims are to (i) detect the class and abundance differences of metabolites in *P. lactiflora* between the BS and RS, (ii) explore the compositional structure and dynamic ecological processes of bacterial and fungal communities between the BS and RS, and (iii) investigate the regulatory relationship between the microbiome and the root secondary metabolome of *P. lactiflora*.

**RESULTS AND DISCUSSION**

**Variations of soil metabolomics between the BS and RS.** Through soil metabolomics, we can detect rhizosphere sediments secreted by plant roots, such as border cells, exudates, and mucilage, which influence the variation and activity of microorganisms in plant roots (25). To construct soil metabolic profiles of the bulk soil (BS) and the rhizosphere (RS), we performed untargeted metabolomic analysis of soil samples from 24 sampling sites in the four major *P. lactiflora* production areas in China (Bozhou in Anhui [AH], Zhongjiang in Sichuan [SC], Heze in Shandong [SD], and Pan’an in Zhejiang [ZJ]). Among all soil samples, 85 metabolites including carbohydrates (13, 15.29%), lipids (42, 49.41%), organic acids (5, 5.88%), and others (25, 29.41%) were discovered and classified (Fig. 1A and see Table S1 in the supplemental material). Notably, the relative abundance of organic acids (BS, 45.99%; RS, 43.11%) and carbohydrates (BS, 11.32%; RS, 88.68%) was higher in the RS than in the BS, while the relative abundance of lipids (BS, 56.89%; RS, 43.11%) was higher in the BS.

In general, plants have traditionally used rhizosphere secretions (including sugars, organic acids, amino acids, fatty acids, and secondary metabolites) to attract microorganisms from bulk soil to the rhizosphere for their own benefit, thereby shaping the rhizosphere microbial community (26). According to two groups of researchers (5, 27), the majority of the root exudates of various plants included organic acids and sugars, emphasizing the critical ecological function that these two classes of metabolites play in the recruitment or extinction of certain microbes in root-associated soils. The organic acids enriched in the rhizosphere in this study were mainly pipecolic acid and citrazinic acid (Table S1). Organic acids can increase the availability of phosphorus in soils, serve as metal chelators in the rhizosphere, act as electron donors in microbial anaerobic metabolism, and modulate enzyme activities (5). Previous studies have shown that under hypotonic stress, after cell decay, or during seed germination, pipecolic acid is released in the rhizosphere as an osmoprotectant for soil bacterial osmotically stressed cells (28).

Moreover, in root-associated soils, sugars are necessary for sustaining bacterial populations (20). Here, the D-threitol, D-fructose, D(+)-talose, D-allose, D-glucitol, sucrose, etc., are the predominant carbohydrates enriched in the rhizosphere (Table S1). Carbohydrates and carbohydrate derivatives in general can be processed further by live cells to supply energy, and a variety of intermediates are engaged in the production of essential elements for life, including amino acids, fatty acids, and nucleic acids (29). Previous studies have shown that adding sugars from pine root exudates to soils affects the abundance and activity of various dominating taxa, like *Proteobacteria*, *Actinobacteria*, and *Firmicutes* (30). Some sugars in root exudates regulate rhizosphere bacteria’s chemotactic reactions, biofilm formation, and gene expression in addition to acting as carbon sources for microbes (31).

However, with the rise of new methodological approaches such as exometabolomics, microbial genomics, etc., root exudates were found to be highly complex mixtures containing metabolites from various chemical classes (32). Remarkably, some primary metabolites were detected by gas chromatography-mass spectrometry (GC-MS) in this study, among which lipids were the largest number detected, which is contrary to prior research (20). The relative abundance of benzoic acid, 2,4,7,9-tetra-methyldec-5-ene-4,7-diol, methyl 2,6-dihydroxybenzoate, succinic acid, quinolinic acid, inositol, etc., was significantly upregulated in the rhizosphere. Previous studies have demonstrated complex subterranean interactions between plant roots and soil...
microbes through lipids (33). Clearly, plant lipids actively shape the microbiome that inhabits the rhizosphere and subsequently colonizes its root tissue. Furthermore, rhizosphere deposition and plant-microbe signaling are key subterranean processes in which lipids as chemical language are highly involved (34). Among known root exudates, benzoic acid is not rapidly and efficiently degraded by soil microorganisms, and its accumulation eventually alters soil microbial composition (35).

In addition, the abundance of soil metabolism in the RS was different from that in the BS. The diversity of the soil metabolites in the RS was significantly higher than that in BS (Fig. 1B to F; Supplemental Results, Table S1, and Fig. S1A and B). Notably, BS and RS soil metabolic classes were not particularly different, probably because the BS is also influenced by certain plants (samples were collected at 20 cm from the roots in a P. lactiflora field). This study’s results differed slightly from prior research on peppers (bulk soil was gathered 1 m away from the peppers) (36).
The rhizosphere affects distribution and diversity of microbes in the BS and RS. Bulk soil (BS) can be regarded as a “microbial seed bank” from which rhizosphere (RS) microorganisms are recruited, and hence, the diversity of rhizosphere microorganisms is reduced (1). To understand the differences in microbial composition between the BS and RS, statistical analyses were performed on the annotated phyla and genera. A taxonomic dendrogram (Fig. 2A) revealed the most numerous types of amplicon sequence variant (ASV) classifications of bacteria at the phylum level: *Proteobacteria* (36.74%), *Acidobacteria* (17.14%), and *Bacteroidetes* (7.14%). Among the bacterial phyla in the RS, *Bacteroidetes* (BS, 35.27%; RS, 64.73%), *Firmicutes* (BS, 21.26%; RS, 78.74%), and *Proteobacteria* (BS, 29.79%; RS, 70.21%) were enriched in the RS. Normally, these phyla are widely suited to C-rich environments (typically found in the rhizosphere) for high metabolic activity, fast growth, and reproduction and thus are sometimes referred to as copiotrophs (3, 21). Conversely, *Chloroflexi* (BS, 82%; RS, 18%), *Acidobacteria* (BS, 64.98%; RS, 35.02%), and *Actinobacteria* (BS, 72.24%; RS, 27.76%) were enriched in the BS. Except for *Actinobacteria*, most of the other phyla (*Acidobacteria*, *Nitrospirae*, *Gemmatimonadota*, and *Chloroflexi*) enriched in the BS were defined as oligotrophs (1). Numerous studies demonstrate that oligotrophs develop more slowly under resource-poor conditions while copiotrophs grow more quickly (37). While oligotrophs effectively exploit resources at the expense of growth rate, copiotrophs grow more quickly and depend on the availability of resources (38). It is worth noting that some phyla are not absolutely copiotrophs or oligotrophs. Both copiotrophs and oligotrophs were present in the *Bacteroidetes* and *Proteobacteria* phyla as reported in previous studies (39). Therefore, further exploration is needed according to a more rigorous physiological ability and at a more refined taxon level. The relative abundance of bacterial genera showed that the abundance of genera *Pseudomonas* (BS, 10.48%; RS, 89.52%), *Sphingomonas* (BS, 39.65%; RS, 60.35%), and *Massilia* (BS, 14.01%; RS, 85.99%) in the RS was higher than that in the BS (Fig. 2B). For fungi (Fig. 2C), the most abundant phyla were *Ascomycota* (79.22%), *Basidiomycota* (10.93%), and Mortierellomycota (9.85%), which is found to be a common pattern in soils globally (40). Only the abundance of Basidiomycota was higher in the RS (BS, 33%; RS, 67%). The subkingdom Dikarya, sometimes known as the “higher fungi,” is made up of the two major divisions Basidiomycota and Ascomycota, which together make up the kingdom Fungi (41). The phylum Glomeromycota is entirely comprised of photoautotroph symbiotic fungi, such as arbuscular mycorrhizal fungi (AMF) (42). However, the relative abundance of Glomeromycota in the BS is higher than that in the RS, but the relative abundance of this phylum was low for the whole. The relative abundance of bacterial genera showed that the abundance of genera *Pseudomonas* (BS, 10.48%; RS, 89.52%), *Sphingomonas* (BS, 39.65%; RS, 60.35%), and *Massilia* (BS, 14.01%; RS, 85.99%) in the RS was higher than that in the BS (Fig. 2B). The relative abundance of fungal genera showed that the abundance of genera *Penicillium* (BS, 24.24%; RS, 75.76%), *Hymenula* (BS, 32.01%; RS, 67.99%), and *Chaetomium* (BS, 36.40%; RS, 63.60%) in the RS was higher than that in the BS (Fig. 2D). Indicator analysis (Table S3) showed that bacterial indicators were mainly enriched in the phylum *Proteobacteria*, while those in fungi were mainly in the phyla Ascomycota (BS) and Basidiomycota (RS).

Community diversity is another statistic frequently used in soil microbiome investigations, where greater diversity is typically regarded as advantageous for the soils as a whole (43). The alpha diversity index was calculated to quantify the species richness of microbial communities, and the results showed that the Chao1 index was significantly higher in the BS than in the RS, and the bacterial community was higher than the fungal community (Fig. 3A). This result is consistent with the research results of microbiome analysis such as those for corn (10) and licorice (15). In contrast, the diversity of soil metabolism was greater in the RS than in the BS (Fig. 1C and D); therefore, subsequent studies can be combined with new technologies such as microbial genomics and epimetabolomics to understand the metabolic niches in the rhizosphere.
FIG 2 Taxonomic dendrograms of the detected microbial communities display the ASV distribution. Different colors represent different phylum levels. Each point represents the corresponding classification (Continued on next page)
microbiome more clearly. The results of latitudinal diversity were shown in Fig. S2A. The key environmental factors influencing microbial diversity were determined using Spearman correlation (Fig. S2E).

Principal-coordinate analysis (PCoA) is used to describe the differences between samples, and Shi et al. (44) developed an adjusted principal-coordinate analysis (aPCoA) based on PCoA, by adjusting for covariates to exclude their effects. aPCoA ordinations and permutational multivariate analysis of variance (PERMANOVA) (Fig. 3C) revealed that origin site (bacteria, $R^2 = 27.5\%, P = 0.001$; fungi, $R^2 = 29.9\%, P = 0.001$) explained the majority of the variation in bacterial and fungal communities, followed by compartment niche (bacteria, $R^2 = 6.4\%, P = 0.001$; fungi, $R^2 = 5.6\%, P = 0.001$). Moreover, microbial community dissimilarity was significantly lower in the RS than in the BS (Fig. 2D), indicating a homogenizing effect of plants on community structure (6). The selection effects of the rhizosphere on particular species are primarily responsible for the variations in the bacterial and fungal community between the RS and BS (45). These results provide potential evidence for inferring rhizosphere effects (46). The beta diversity pattern includes two independent components: turnover (species replacement) and nestedness (species loss) (47). The diversity decomposition analyses revealed that species replacement processes dominated microbe compositional dissimilarities (contributing 79.17% and 76.21% for bacterial and fungal diversity, respectively), whereas richness difference processes attributed only 20.83% and 23.79% on average (Table S4). For both bacteria and fungi, species replacement processes were significantly higher in the BS than in the RS. The richness difference processes of bacteria in the BS were significantly lower than in the RS, while the fungi in the BS were significantly higher than in the RS (Fig. S3). The spatial turnover of microbes was typically consistent with the distance-decay trend at the spatial scale (Fig. S2E).

Bacteria and fungi in the same region have large differences in cell structure and ecological niche but are also closely related. Therefore, it is necessary to comprehensively understand the relationship between bacteria and fungi in the BS and RS (Fig. 3B, Supplemental Results, Fig. S2B to D, and Table S5).

**Community assembly was governed by stochastic processes primarily belonging to dispersal limitation.** To gain insight into the microbiome, it is essential to understand the ecological mechanisms that shape the communities (48). Previous studies (49, 50) have indicated that the mechanisms of community assembly can be classified into stochastic processes based on the neutral hypothesis (dispersal limitation, etc.) and deterministic processes based on the niche theory (environmental filtration and biotic interactions). The results of beta nearest taxon index ($\beta$NTI) and $R_{\text{Bray}}$ (Raup-Crick) demonstrated that stochastic processes dominate the assembly of microbial communities in the BS and RS, especially dispersal limitation (Fig. 4A and B). Meanwhile, the deterministic process focused on heterogeneous selection (Fig. 4A). Heterogeneous selection is selection under heterogeneous abiotic and biotic environmental circumstances, resulting in more-dissimilar community structures (51). For bacteria, the RS had a larger relative contribution of stochastic processes mostly related to dispersal limitation (~97.8%) than the BS (~41.3%). Likewise in fungi, dispersal limitation occupied the highest proportion in the stochastic processes of the BS (69.2%) and RS (62.0%). Random fluctuations in the relative abundance of species are principally represented by stochastic processes, which include random birth, death, and dispersion occurrences (52). Previous studies have found that dispersal limitation plays an important role in driving beta diversity at small and medium sample sizes (53). It is important to note that the dispersion limit cannot be regarded as the only indicator of a stochastic process because dispersion can be either deterministic or stochastic (51).
For results of other analytical methods (modified stochastic ratio [MST] [Fig. 4C] and dispersal niche continuum index [DNCI] [Table S6]) to quantify community assembly, please see the Supplemental Results for details.

The Sloan neutral community model (NCM) elucidated the possible role of stochastic events in the formation of bacterial and fungal communities in the BS and RS (Fig. 4D and Fig. S4). The relative contribution of stochastic processes explained 23% and 56% of the community variance for the total bacterial and fungal communities, respectively. This indicated that the overall fungal community was more impacted by stochastic processes than was the total bacterial community. In contrast, the entire fungal community’s migration rate (0.001) was lower than that of the total bacterium community (0.008), indicating that species dispersion was more limited in the total fungal community (Fig. 4D). For two soil compartments (Fig. S4), the migration rate of the RS (bacteria, 0.009693; fungi, 0.0022) was higher than that of the BS (bacteria, 0.009684; fungi, 0.0016). The microbial community immigration rate in the RS was higher than that in the BS, indicating that the dispersing capacity of most microbial taxa in the RS was greater than that in the BS. Furthermore, according to the neutral model, stochastic processes should be taken into account even in a highly selective environment like the rhizosphere since dispersion restriction is important in the construction of microbial communities (Fig. 4D). The $R^2$ of fungi in the BS was higher than that in the RS, suggesting that the aggregation of fungal communities in the BS was more driven by stochastic processes than was that in the RS. In general, because of the selected role of root excretions (54), deterministic processes account for a greater percentage of rhizosphere microbe assembly than that in bulk soil, which agreed with the results for fungi.
To investigate the relative role of deterministic and stochastic mechanisms in microbial community assembly, community-level habitat niche breadths (Bcom) were estimated (Fig. 4E). Both in the BS and in the RS, bacteria were found to have a considerably higher Bcom value than fungi ($P < 0.001$), and the niche breadth of the BS was larger than that of the RS but not significantly so. According to a prior study, species with larger niche breadths may exhibit more metabolic adaptability and be less subject to deterministic processes (55). However, the $R^2$ (NCM) of bacteria in the RS was lower than that in the BS, which may be related to sampling scale, historical factors, random drift, and other factors (53). Based on the size-plasticity theory (also known as the “body size effect”), ecological determinism would rise with organism size since smaller organisms (such as bacteria) are less environmentally filtered than bigger organisms (56). Conversely, the $R^2$ of the neutral model was higher for fungi than for bacteria, suggesting a greater contribution of stochastic processes to fungi. In addition, the niche breadth index was used to distinguish generalists and specialists based on whether it was more or less than simulated chance, respectively (Fig. 4F). In the BS, generalists have the largest proportion of bacteria (63%), while specialists have the largest proportion of fungi (54%). In the RS, specialists have the largest proportion of both bacteria (67%) and fungi (58%). Furthermore, deterministic processes have a greater impact on environment specialists with restricted ecological niche widths than on generalists with broad ecological niche widths (57). Species were divided according to the simulated null distribution, with the
highest proportion of specialists in the RS, which also appeared to be related to rhizosphere effects.

Distance-decay relationships (DDRs) are known to be affected by both deterministic and stochastic processes (58). The distance-decay pattern, which predicts decreased community resemblance with rising geographic distance, has been intensively studied in the biogeography of microbiota (59). The similarity of bacterial and fungal communities in the BS and RS decreased with increasing geographical distance, indicating that significant DDR patterns were present in both bacteria and fungi (Fig. S2C). The distance-decay pattern was established using four ecological processes: mutation, drift, dispersion, and selection. The slope of the distance-decay curve is increased by selection and drift, but this connection is weakened by dispersal (60). For bacteria, the DDR slope of the RS is steeper than that of the BS, which implies that historical factors (such as geographic isolation) have a stronger impact on the rhizosphere (45). In contrast, the DDR slope of the RS in fungus was lower than that of the BS, which might be attributed to the function of environmental choice in fungal community spatial variation (61).

Deterministic processes include the selection of abiotic environmental factors (environmental filtering) and the mutual antagonism and synergy between species. Furthermore, soil physicochemical characteristics such as pH, wetness, and soil characteristics can influence root-associated microbial communities (62). The results of the Mantel test, canonical correspondence analysis (CCA), and hierarchical partitioning analysis indicated that the cation exchange capacity (CEC) significantly affected the microbial community diversity of the BS and RS (Supplemental Results, Tables S5 and S7, and Fig. S5 and S6). Notably, the results of the hierarchical partitioning indicated that environmental variables had a quite low interpretation rate (bacteria, 6.3%; fungi, 8.1%) and a large level of the unexplained component (bacteria, 93.7%; fungi, 91.9%). Correspondingly, it is speculated that the deterministic process is dominated by species-to-species interactions.

The complexity and stability of the interkingdom network of the RS were higher than those of the network of the BS. Cooccurrence patterns are frequent and essential to comprehending the composition of microbes (14). Interkingdom network analysis was performed to assess the impact of bacterial-fungal interkingdom interactions between the BS and RS (Fig. 5A and B and Fig. S7A and B). To determine the difference in the complexity of the microbial network between the BS and RS, the changes in the topological parameters (Table S8) of the microbial network were calculated. The cooccurrence network of the BS consisted of 229 nodes (bacteria, 149; fungi, 80) and 3,149 edges (positive, 1,663; negative, 1,486), whereas those of the RS consisted of 382 nodes (bacteria, 284; fungi, 98) and 3,176 edges (positive, 2,073; negative, 1,103). This was consistent with the complexity of the bipartite network in the BS and RS. Specifically, the BS had higher network connectivity (i.e., average degree) than the RS. The above results showed that the complexity of the RS bacterial-fungal interkingdom network was stronger than that of the BS network, which was similar to the results for licorice (15). However, in the microbial network results of different developmental stages of maize, the network complexity of the BS and RS at different stages is different (10). The samples in this study were collected at the mature stage of P. lactiflora, and the next step in the study of network complexity may require sampling at different periods. The intricate linkages seen in the RS networks might be attributed to more frequent nutrient exchanges and more stable network topologies that can withstand external disturbance (15). The links for bacteria and fungi themselves were mostly positive. In contrast, the association between bacteria and fungi was mainly negative in the BS, while positive links were higher than negative correlations in the RS (Fig. 5C). In this study, the positive correlation between microbes was stronger in the RS than in the BS, indicating that the rhizosphere’s microbes may engage in substantial mutualistic interactions (63).

Based on natural connectivity analysis, the highest robustness was observed in the RS network. Meanwhile, species extinction (resilience to node loss) of the interkingdom
network was simulated to compute the robustness of the network. The RS network was more stable ($P < 0.001$) than the BS network under either random node loss or targeted removal of module hubs (Fig. 5D). The network vulnerability (the highest reduction in network efficiency when a single node was removed from the network) was likewise lower in the RS (0.01072515) than in the BS (0.01960996), indicating that RS networks are more resistant. According to the natural connectivity analysis, the BS network had more natural connectedness than the RS network when fewer nodes were randomly deleted, and the RS network had higher natural connectivity when more nodes were removed (Fig. 5E). Studies of network stability with warming control show that network stability rises with network complexity, particularly relative modularity, which is congruent with macroecological data. Simultaneously, our findings confirm MacArthur’s claim that ecological complexity contributes to stability (24). Additionally, the level of network complexity was assessed using a newly constructed metric known as cohesion. Positive cohesion can define the extent of cooperative conduct in the community, whereas negative cohesion can show the level of competitive activity in the community (24). Positive cohesion of the RS network was significantly higher than
that of the BS network, while negative cohesion of the RS network was marginally but not significantly higher than that of the BS network (Fig. 5F). Since rhizosphere microbes are driven to homogeneous selection by root exudates, more cooperative (positive) linkages in the rhizosphere might exist (54).

Keystone nodes that contribute significantly to forming the network structure were discovered in all interkingdom networks based on within-module connectivity (Z) and among-module connectivity (P), along with 10 module hubs, 64 connectors, and 7 hubs (Fig. S7C). In the BS, network hubs contained only 6 fungi, and connectors contained 38 bacteria and 17 fungi. In the RS, network hubs had 1 fungus, connectors included 7 bacteria and 2 fungi, and module hubs had only 10 fungi (Table S8).

**Integrated modulations of *P. lactiflora* root secondary metabolites.** Secondary metabolites (SMs) of *P. lactiflora* (Table S9) are the material basis for its clinical efficacy and an important component of *P. lactiflora* defense against pathogenic attack and environmental stress (64). Abiotic and biotic environments that are constantly changing have an impact on the appropriate synthesis and accumulation of SMs, which are tightly regulated in both space and time (65). The *P. lactiflora* root secondary metabolites were influenced by both abiotic (location, soil, and climate) and biotic (diversity and composition of microbes) variables. Soil water content (SWC), mean annual precipitation (MAP), and pH influenced the secondary metabolites of *P. lactiflora* roots (Fig. 6A, Supplemental Results, Table S10, and Fig. S8).

Plant microbiomes have been proven in many studies (66) to impact host plant productivity of essential medicinal components such as alkaloids, steroids, terpenoids, etc. For example, studies have shown that plant-microbiome interactions can increase the biomass yield of *Salvia miltiorrhiza* and affect tanshinone production. The unique seed-associated microbiome (*Pantoea, Pseudomonas, Sphingomonas,* and *Dothideomycetes*) of *S. miltiorrhiza* contains a gene pool associated with the synthesis of tetraterpene backbones and other compounds, thereby providing additional metabolic capacity to the host plant (11). In addition, several studies have reported the beneficial effects of arbuscular mycorrhizal symbiosis on plant growth and secondary metabolite accumulation in licorice roots (12). The microbiome related to the secondary metabolism of *P. lactiflora* was screened, which provided a theoretical basis for the subsequent isolation and culture of bacteria and fungi. The random forest regression model analysis was performed to identify microbial genera (bacteria-BS, *Rhodoplanes* and *Pseudoxanthomonas*; bacteria-RS, *Erwinia* and *Sphingobium*; fungi-BS, *Apiotrichum* and *Solicoccozyma*; fungi-RS, *Alternaria* and *Vishniacozyma*) affecting paeoniflorin (Supplemental Results and Fig. S9).

Weighted gene coexpression network analysis (WGCNA) was used to compress the data of two soil compartments and found the brown (BS) and pink (RS) modules, which were highly linked to the root secondary metabolites, in order to perform dimensionality reduction analysis on the complex bacterial and fungal abundance data (Fig. S10A and B). Overall, the random forest model and cooccurrence network analysis identified the hub ASVs related to paeoniflorin (Fig. S10C and D). In order to accurately identify the taxa of the key ASVs, NCBI BLAST searching was performed, and the highest-alignment sequences were selected to construct maximum likelihood trees (ML trees) with the key ASVs in the BS and RS (Fig. 6B and Table S11). The key bacteria identified in the BS were *Sphingomonas agri* (BASV000058), *Ramilbacter monticola* (BASV000145), *Sphingomonas sediminicola* (BASV000202), and *Brevitalea aridisoli* (BASV000331), and the fungus was *Alternaria alternata* (FASV000012). In the RS, the key bacteria detected were *Sphingobium limeticum* (BASV000196) and *Variovorax urelyticus* (BASV000243), and the fungi were *Cephalosporium gramineum* (FASV00003), *Mortierella globalpina* (FASV000047), and *Linnemannia amoeboidea* (FASV000579). WGCNA screening found that *Alternaria tenuissima* and *A. alternata* were significantly correlated with paeoniflorin content. *A. tenuissima* is widespread, occurring on a wide range of different plant hosts in numerous countries under various environmental conditions (67). Interestingly, *A. tenuissima*, a fungal endophyte, has been shown to influence growth and selenium absorption in its bioaccumulation host *Astragalus bisulcatus* (68). The ethyl acetate (EA) crude
FIG 6 Interactions among environment, soil metabolites, root secondary metabolites, and microbiotas. (A) Correlations of the microbial community and soil metabolite structures (Bray-Curtis distance) with geographical variables (blue), climatic characteristics (green), (Continued on next page)
extract of endogenous A. alternata obtained from Ziziphus spina-christi included alka-
loids, tannins, flavonoids, glycosides, phenolics, and terpenoids, according to preliminary
phytochemical screening. This demonstrated the potential of Alternaria as an important
candidate microbial resource for promoting the growth and secondary metabolite accu-
mulation of P. lactiflora. Additionally, these can provide the material basis of biological
bacterial fertilizer for P. lactiflora cultivation. Altogether, further mycobacterial investiga-
tions are required to confirm that the bacteria and fungi discovered in this work that are
connected to the production of paoniflorin are indeed candidate microorganisms for
enhancing the secondary metabolites of P. lactiflora. The study of isolation of P. lactiflora
endophytes showed that Alternaria tenuissima, Aspergillus flavus, and Penicillium com-
mune can all produce paoniflorin, and A. flavus can produce paoniflorin in an amount
of 342.4 μg/L (69). Furthermore, in this study, BLAST search was used to identify similar
sequences of the above three fungi (Fig. S11). In addition, the random forest results indi-
cated that only the genus Alternaria was identified in the BS and RS, and it was positively
correlated with the content of paoniflorin.

Two partial least-squares path models (PLS-PMs) were created independently for
the bulk soil and rhizosphere to clearly illustrate the regulatory linkages among these
factors (Fig. 6A and B). Using the multiple regression analyses outlined in the previous
results, a representative for each component in the PLS-PMs was cautiously chosen.
Only the variables that were significant were kept in the equations. The goodness-of-fit
index (GoF) was used to judge the overall fitting degree of the model, and the results
showed that the fitting degrees of the BS (0.7171) and RS (0.6656) were both good. In
the BS, geography (path coefficient = 0.787) and soil (−0.715) were seen in a substan-
tial positive and negative major impact on root secondary metabolites ($R^2 = 0.994$).
Soil metabolites ($R^2 = 0.231$) were directly and positively affected by geography (1.902) and
climate (1.8777). Fungal composition ($R^2 = 0.443$) was affected only by soil (0.696),
while bacterial composition ($R^2 = 0.951$) was influenced by geography (−0.710) and
soil (−0.281). The PLS-PM of the RS was more complicated than that of the BS. Root
secondary metabolites in the RS model were mostly explained by all factors ($R^2 = 0.971$) and were significantly affected by the soil (0.279), key bacteria (0.158), climate
(−0.611), and geography (−0.179). The direct effects on the soil metabolites ($R^2 = 0.569$) were climate (−0.542) and soil (0.505). Geography had significant direct influ-
ences on bacterial diversity (−0.723) and fungal diversity (−0.780). The soil had the great-
est impact on the bacterial composition (−0.559) and fungal composition (0.887).
Furthermore, according to the source model of plant microbiome (SMPM) (Fig. 7), bac-
terial communities in the RS were obtained from the BS (34.7%) and unknown sources
(65.3%). The fungal community of the RS contained 40.1% from the BS and 59.9% from
unknown sources.

**Conclusion.** In conclusion, this study provides a systematic understanding of the
differences and diversity of the bacterial and fungal communities and soil metabolome
between the BS and RS at four origins of P. lactiflora. The following conclusions were
obtained. (i) The abundance of soil metabolism in the RS was different from that in the
BS, in which both carbohydrates and organic acids were enriched in the RS.

**FIG 6** Legend (Continued)
edaphic properties (yellow), root secondary metabolites (orange), soil metabolites (pink), and microbial communities (purple) in the bulk
soil and rhizosphere determined using the Mantel test. The color of the line represents the significance of the differences (P values). The
size of the line represents the size of the correlation coefficients (Mantel’s r). The pairwise correlations of these variables are shown with
a color gradient representing the Spearman correlation coefficient. B/F_BS/RS, bacteria/fungi in the bulk soil/rhizosphere; M_BS/RS, soil
metabolites in the bulk soil/rhizosphere. Geographical variables include longitude, latitude, and altitude. Climatic variables include solar
radiation (Srad), wind speed (wind), mean annual temperature (MAT), and mean annual precipitation (MAP). Refer to Tables S13 in the
supplemental material for specific variables of soil and climate. Soil metabolites involving metaPCA1_BS and metaPCA1_RS represent
the first axes of principal-component analysis (PCA) of soil metabolites in the bulk soil and rhizosphere. Microbial communities
containing B_apcoa1_BS, B_apcoa1_RS, F_apcoa1_BS, and F_apcoa1_RS represent the first axes of adjusted principal-coordinate analysis
(aPCoA) of bacterial and fungal communities in the bulk soil and rhizosphere based on Bray-Curtis distances. (B) Identification of key
ASVs associated with paoniflorin content predicted by random forest model. Ridgeline plots show the relative abundance of key ASVs
in the bulk soil and rhizosphere. The key ASVs and the sequences obtained from the BLAST search results on NCBI are constructed
using the maximum likelihood method to construct a phylogenetic tree.
Copiotrophs were enriched in the rhizosphere, while oligotrophs were enriched in the BS. Similarly, both bacterial and fungal alpha diversity and beta diversity were significantly lower in the RS than in the BS. Additionally, cation exchange capacity (CEC) significantly affected the microbial community diversity of the BS and RS. (iii) Stochastic processes (dispersal limitation) dominated the community assembly of both the RS and BS. (iv) The bacterial-fungal interkingdom network of the RS was more complex and stable than that of the BS. (v) The microbes of high-quality P. lactiflora production were deduced by constructing biotic and abiotic model pathways that affect the growth and metabolism of P. lactiflora. Meanwhile, multiple abiotic (SWC, MAP, pH, etc.) and biotic (Alternaria, etc.) variables influenced the secondary metabolites of P. lactiflora roots. Overall, this study strengthens the understanding of the impact of rhizosphere effects on soil composition and diversity. These results can offer an important theoretical basis and technical support for the improvement of P. lactiflora quality and future synthetic community studies.

**MATERIALS AND METHODS**

**Sampling sites and analyses of environmental characteristics and soil metabolites.** Roots and two root-associated compartments of soil (bulk soil [BS] and rhizosphere [RS]) of *Paeonia lactiflora* were collected from 24 sampling sites in the four major *P. lactiflora* production areas (see Table S12 in the supplemental material) in October 2020. The four chosen origins were Bozhou in Anhui (AH), Zhongjiang in Sichuan (SC), Heze in Shandong (SD), and Pan’an in Zhejiang (ZJ). The sites were collected in a Z pattern, with six (5 by 5 m² per plot) serving as biological duplicates for each planted area (approximately 0.5 ha). The agricultural *P. lactiflora* areas had grown for 4 years with comparable treatment and management measures. Five topsoil cores (0- to 15-cm soil surface depth and ~20 cm away from the roots) and the accompanying intact and healthy roots were collected for each plot. These topsoils from the roots were passed through a sterile 2-mm sieve and were defined as bulk soil (BS). The roots and the soil that could not be shaken off them were put into a 50-mL centrifuge tube. Phosphate-balanced normal saline (PBS) was added to the centrifuge tube. Then, the centrifuge tubes were shaken on the platform (20 min, 180 rpm) (70). After removal of the roots, the remaining samples were centrifuged at 4,000 × g.
for 20 min at 4°C to collect the rhizosphere soil (RS; defined as that 1 mm of soil tightly attached to the roots) (71). For bulk soil (BS), rhizosphere (RS), and root samples, these samples were taken at random and blended as one biological replicate. Overall, 48 samples (four fields × six plots × two compartments) were collected. Before DNA extraction, all samples were collected and transported on dry ice and kept at −80°C. For the investigation of edaphic characteristics and root secondary metabolites, BS and root samples were employed, respectively. The BS and RS samples were used for the soil metabolites and microbial sequencing analyses.

Edaphic physicochemical characteristics (e.g., soil texture, soil water content, pH, and available phosphorus [Table S13]) and soil enzyme activities (e.g., soil catalase and soil urease [Table S13]) of the BS were measured according to previous protocols (72, 73). The meteorological data (Table S13) for all sampling locations, including the mean annual precipitation (MAP), mean annual temperature (MAT), solar radiation (Srad), and wind speed (wind), were taken from the WorldClim database version 2.0 (http://www.worldclim.org) (74).

Soil metabolic composition of the BS and RS was determined by GC-MS using previous methods (75). For the analysis of soil metabolomes of the BS and RS, principal-component analysis (PCA) was performed using the vegan package. Venn diagrams were drawn using the VennDiagram package (76) to identify unique and common soil metabolites. The Bray-Curtis distances of soil metabolites were counted by the vegan package. The EdgeR package (77) was used to calculate the differentially accumulated soil metabolites (DAM) between the BS and RS.

**Analyses of root secondary metabolome.** The major root secondary metabolites (Table S9) were determined and quantification of P. lactiflora was performed by liquid chromatography-mass spectrometry (LC-MS). We used 11 standards including albiflorin, benzoylxylopaeoniflorin, benzoylpaeoniflorin, cianidin-3,4'-dihydroxy-3',5'-dihydroxyanthocyanidin, cianidin-3,4'-dihydroxy-3',5'-dihydroxyanthocyanidin, dehydrobenzoylpaeoniflorin, ethyl gallate, gallic acid, odyxypaeoniflorin, paeoniflorin, paeonol, and paeonol 4'-O-glucoside. The primers for amplifying bacteria were 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTATCTAAT) in the V3-V4 hypervariable region of 16S rRNA (78), while the primers for amplifying fungi were ITS3_KYO2 (GGAAGTAAAAGTCGTAACAAGG) and TS4 (TCCTCCGCTTATTGATATGC) in the ITS2 region (79).

**Statistical analyses.** All statistical analyses were carried out in the R environment (v4.1.3; http://www.r-project.org/). The ggplot2 package was used to illustrate the majority of the findings. Both ANOVA and posterior analysis calculations were performed using the EasyStat package (https://github.com/taowenmicro/EasyStat). Furthermore, the false-discovery rate approach was used to correct all of the P values (85). The classification tree of ASVs was drawn with Cytoscape (77). The Chao1 index, computed with the picante package, was used to examine the richness of species diversity (alpha diversity) (86). The equilibrium of the microbial communities was assessed using the bacterium/fungus ratio of the Chao1 index. Latitude patterns of alpha diversity of microbial communities were analyzed using the vegan package and linear regression. Correlations between alpha diversity and environmental variables, as well as bacterial and fungal alpha diversity, were analyzed using Spearman’s coefficient and linear regression. The beta diversity of microbes was calculated by computing the Bray-Curtis distance matrix and then reconciled using adjusted principal-coordinate analysis (aPCoA) (44), which excluded the effect of sampling location. Both analysis of molecular variance (AMOVA) and PERMANOVA were performed by the vegan package.
with 999 permutations. The relationship between bacterial and fungal community composition (Bray-Curtis dissimilarity) was explored using both the Mantel test and linear regression. Bacterial and fungal data were examined for association using the Procrustes analysis of the vegan package. The univariate linear regression analysis was used to study the distance-decay relationship (DDRs) between microbial distribution (Bray-Curtis distance) similarity and geographical location, where the slope of the DDR might change from compartment to compartment, representing various species’ turnover rates. Compositional differences between compartments were divided into replacement and richness difference components (Podani family, Sørensen dissimilarities) by the ade4 package (87, 88).

The assembly process of bacterial and fungal communities was calculated using a null model (89). According to jNTI and RCerm (Bray-Curtis), the ecological process was divided into five processes: stochastic processes (dispersal limitation, homogenizing dispersal, and undominated) and deterministic processes (heterogeneous selection and homogeneous selection) (10). To assess whether dispersal or niche processes dominated community construction, calculations were performed using the DNCI package (90). The modified stochastic ratio (MST), which was used for the relative importance of deterministic (MST < 0.5) and stochastic (MST > 0.5) processes, was computed by the NST package (91). The neutral community model (NCM), niche breadth calculations, and niche generalist and specialist divisions were performed according to our previous study (92).

The analyses of the bacterial-fungal interkingdom networks of the BS and RS were conducted by using the interdomain ecological network analysis pipeline (IDENAP) (93) on iNAP (https://onlinelibrary.wiley.com/doi/full/10.1002/imt2.13). The interdomain networks were constructed using the correlation coefficient $r$ or $>0.6$ as the threshold based on the SparCC method. In addition, the bipartite networks were also constructed based on the above-described method. According to previous methods (92), the nodes in the network were classified according to within-module connectivity (Z) and among-module connectivity (P): module hubs, peripherals, connectors, and network hubs. The relationship (the number of edges) between bacteria and bacteria, fungi and fungi, and bacteria and fungi was calculated according to previous studies (94). The complexity of the interkingdom network (e.g., total number of nodes, total number of links, and average connectivity [Table S3]) was calculated by the igraph package (95). Natural connectivity was used to compare the robustness values of the interkingdom network (96). Furthermore, the complexity after randomly removing 50% of nodes and after targeted removal of module hubs and vulnerability were also calculated according to the previous study (24). The cohesion was calculated based on the null model (97).

Using the linkET package, the Mantel test was used to investigate Spearman’s linkages between root secondary metabolites and environmental variables (microbes, soil metabolites, etc.). Multiple reaction monitoring (MRM) analysis was utilized to explore the influence of substantially correlated factors on microbes (Bray-Curtis distance) within every compartment by the ecodist package (98). Canonical correspondence analysis (CCA) was used to investigate the relative impact of significant environmental variables on microbial composition. The relative importance of explanatory variables for canonical analysis was obtained using the hierarchical partitioning of the rdacca.hp package, and the UpSetV package was used to visualize the results (99). For the selected important environmental variables, PCA and smooth regression analysis of additive models were performed using previous methods.

For the macroenvironmental data, the selection was based on the correlation and random forest model results with the secondary metabolites of *P. lactiflora* root. Random forest regression was performed through rPermute and A3 packages to obtain the significance of variables and the full model, respectively. The mean annual precipitation (MAP) map of sampling sites was drawn with ArcMap 10.7 (100). The univariate regression on secondary metabolites of *P. lactiflora* root and environmental variables was performed with the “lm” function of R. To obtain relevant genera of paeoniflorin-related fungi and bacteria in the BS and RS, the RandomForest software was employed, and fitted curves were utilized to explain the link between the abundance of the 10 leading genera and paeoniflorin concentration. Key modules significantly associated with root secondary metabolites were obtained using WGCNA (101). ASV and root secondary metabolites in the above key modules were analyzed by the random forest model through the linkET package. The cooccurrence network based on the key modules was constructed through the igraph package. BLAST searching on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) properly identified the important ASVs, and the maximum likelihood trees (ML trees) were generated by using sequences with the greatest recognition scores. Ridgeline plots show the relative abundance of key ASVs. Paeoniflorin-related fungi reported in the previous study (69) were screened from fungal sequences by local BLAST search, and maximum likelihood trees were constructed. Finally, the plspm tool was used to create partial least-squares path models to recapitulate the link among root secondary metabolites, soil metabolites, microbiome, and environment (102). The goodness-of-fit index (GOF) was generated to assess the model’s overall fit.

**Data availability.** The raw sequence data reported in this paper are available in the NCBI Sequence Read Archive under accession no. PRJNA855546.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1.** PDF file, 1.9 MB.

**SUPPLEMENTAL FILE 2.** XLSX file, 0.1 MB.
ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (U1812403-1, 82073960, and 82274045), the National Science & Technology Fundamental Resources Investigation Program of China (2018FY100701), and the Open Research Fund of State Key Laboratory of Southwestern Chinese Medicine Resources (NO.SCMR20210).

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