The ISME Journal (2020) 14:1015–1029
https://doi.org/10.1038/s41396-020-0587-5

ARTICLE

Mycelial network-mediated rhizobial dispersal enhances legume nodulation

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Received: 25 August 2019 / Revised: 7 January 2020 / Accepted: 14 January 2020 / Published online: 23 January 2020

Abstract

The access of rhizobia to legume host is a prerequisite for nodulation. Rhizobia are poorly motile in soil, while filamentous fungi are known to grow extensively across soil pores. Since root exudates-driven bacterial chemotaxis cannot explain rhizobial long-distance dispersal, mycelia could constitute ideal dispersal networks to help rhizobial enrichment in the legume rhizosphere from bulk soil. Thus, we hypothesized that mycelia networks act as vectors that enable contact between rhizobia and legume and influence subsequent nodulation. By developing a soil microcosm system, we found that a facultatively biotrophic fungus, *Phomopsis liquidambaris*, helps rhizobial migration from bulk soil to the peanut (*Arachis hypogaea*) rhizosphere and, hence, triggers peanut–rhizobium nodulation but not seen in the absence of mycelia. Assays of dispersal modes suggested that cell proliferation and motility mediated rhizobial dispersal along mycelia, and fungal exudates might contribute to this process. Furthermore, transcriptomic analysis indicated that genes associated with the cell division, chemosensory system, flagellum biosynthesis, and motility were regulated by *Ph. liquidambaris*, thus accounting for the detected rhizobial dispersal along hyphae. Our results indicate that rhizobia use mycelia as dispersal networks that migrate to legume rhizosphere and trigger nodulation. This work highlights the importance of mycelial network-based bacterial dispersal in legume–rhizobium symbiosis.

Introduction

The spatial distribution of bacteria significantly influences their ecosystem services, for instance the interaction with host plants. Soil nitrogen-fixing bacteria (rhizobia) can establish beneficial interactions with legume plants and fix atmospheric dinitrogen (N_2) by forming a specialized organ, the nodule [1–3]. The access of rhizobia to the legume determines nodule initiation [4, 5]. Yet, rhizobia are poorly motile in soil, and nodulating rhizobia, except for seed-borne rhizobia, are derived from soil environment. Especially, root exudates mediate a short-distance recruitment (a few millimeters) of rhizobia, rhizobia, which are separated from legume hosts by a long distance that cannot reach their targets [6]. Moreover, it is impossible for rhizobia to cross the air-filled gaps between soil aggregates via flagellum-mediated active movement without the facilitation of flowing water or other vectors [5, 7]. Thus, it is still unknown how rhizobia migrate to legume rhizosphere from bulk soil.

Beneficial fungi could interact with rhizobia-infected legumes and produce additional effects, such as nutrient acquisition and plant fitness, in a relationship known as tripartite mutualism [8–11]. Simultaneous colonization of legumes with fungal and rhizobial symbionts is likely common in nature, as fungi and bacteria live together and their synchronous root infection is inevitable [12, 13]. Studies have reported that beneficial fungi enhance legume–rhizobium interactions via nutrient complementarity

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Supplementary information The online version of this article (https://doi.org/10.1038/s41396-020-0587-5) contains supplementary material, which is available to authorized users.

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or plant fitness improvement [9, 14]. Nothing is known about the direct interactions between microbial symbionts before symbiotic establishment or the concomitant effects on subsequent rhizobial associations with legume hosts. Fungal mycelia, representing most of the soil microbial biomass, are demonstrated to grow extensively in soil pores and form a mycelial network, which is known as the "fungal highway" [15]. Such mycelia are also known as an ideal channel for bacterial dispersal in structurally and chemically heterogeneous soil ecosystems. Indeed, it is found that specific bacteria can act as "hitchhikers" traveling along mycelial networks to new niches, where they play important ecological functions, such as bioremediation, foraging, biocontrol, and nutrient cycling [15–18].

The filamentous ascomycetes *Phomopsis* spp. are widely distributed in the natural environment, and contribute to ecosystem functions, such as litter decomposition and nutrient cycling [19, 20]. Our previous studies showed that a facultatively biotrophic fungus, *Phomopsis liquidambaris*, could establish a symbiotic association with peanut (*Arachis hypogaea*) and enhance nodulation and N_2-fixation under controlled and field-realistic conditions [8–11, 21, 22]. Several mechanisms have been elucidated regarding *Ph. liquidambaris*-mediated nodulation enhancement, including plant hormone modifications [11], leaf CO_2 fixation increasement [9], and soil microflora improvement [22]. Although no direct interactions between two root symbionts and concomitant effects on nodulation are known, *Ph. liquidambaris* inoculation increased bradyrhizobial diversity in peanut rhizosphere [22], suggesting the possible role of *Ph. liquidambaris* in rhizobial rhizosphere enrichment. Recent studies demonstrated that mycelia are able to redistribute water and nutrients and release water and carbon (C)-rich compounds to the mycosphere to create a hospitable microhabitat for bacteria [23–25]. In addition to its endophytic lifestyle, *Ph. liquidambaris* is able to degrade soil recalcitrant organic matter and survive in harsh environments [26–30]. Because of the strong saprophytic ability, *Ph. liquidambaris* could release substantial nutrients to the mycosphere, which may mediate its interaction with rhizobia [31]. Thus, an interaction between *Ph. liquidambaris* and rhizobia before their symbiosis with legume hosts may exist and participate in *Ph. liquidambaris*-mediated nodulation enhancement. Here, we hypothesize that mycelial networks can be used by rhizobia as a means to reach the rhizosphere from bulk soil, increase the contact frequency with legumes.

In the present study, using *Ph. liquidambaris*–peanut–rhizobia tripartite mutualism as a model, we first developed a soil microcosm, where rhizobium was inoculated at some distance away from the plant to study if mycelia of *Ph. liquidambaris* could transfer rhizobia from bulk soil to the rhizosphere and the concomitant effects on legume–rhizobia interactions. Next, we performed a series of plate experiments to study the dispersal modes of rhizobia along mycelial networks. Last, we performed transcriptome sequencing to reveal the potential genetic mechanisms of rhizobial dispersal on *Ph. liquidambaris* networks. The obtained results indicate that mycelial networks are important means for rhizobial migration from bulk soil to rhizosphere, and thereby initiate legume–rhizobium interaction.

Materials and methods

Microorganisms and growth media

Experiments were performed with *Ph. liquidambaris* strain B3 and peanut-nodulating rhizobium, *Bradyrhizobium yuanmingense* (hereafter *Bradyrhizobium*) [8, 22]. To mimic soil conditions, soil extraction agar (SEA, 1.5% agar) was used to activate *Ph. liquidambaris* [32]. A fungal plug was transferred to SEA and placed in an incubator at 28 °C for 7 days. The *Bradyrhizobium* was activated as in [8].

Soil microcosm design

Peanut (line Gan-Hua) seeds were obtained from the Ecological Experimental Station of Red Soil, China Academy of Science. After surface disinfection, seeds were germinated on 0.5 PDA (1%, w/v) at 28 °C until the radicle reached ~2 cm. The seedlings without microbial contamination were used. The soil was collected from the surface (0–10 cm) of a peanut paddock at Botany Garden of Nanjing Normal University (32° 6.318′ N, 118° 54.88′ E), China, and mixed thoroughly after passing through a 2 mm sieve. The main physicochemical properties and gravimetrical moisture have been described [9]. Each microcosm (12 cm length, 5 cm width, 12 cm height) contained 1.5 kg gramma-sterilized soil, and soil was kept at its original gravimetrical moisture (16%, w/w) with sterile water. Each microcosm contained two compartments, microbial and root, which were separated by a sterile 30 μm mesh [33]. The microcosm experiments contained the following treatments: (1) control setup of the microcosm without microbial inoculation or peanut cultivation; (2) microbial compartment with soil suspension inoculation and root compartment with peanut cultivation; (3) microbial compartment with *Ph. liquidambaris* and soil suspension inoculation and root compartment with peanut cultivation; (4) microbial compartment with *Bradyrhizobial* inoculation and root compartment with peanut cultivation; (5) microbial compartment with *Ph. liquidambaris* and *Bradyrhizobial* inoculation and root compartment with peanut cultivation; (6) microbial compartment with *Bradyrhizobial* inoculation

and root compartment without peanut cultivation; (7) microbial compartment with *Ph. liquidambaris* and *Bradyrhizobial* inoculation and root compartment without peanut cultivation; and (8) microbial compartment without microbial inoculation and root compartment with peanut cultivation and *Bradyrhizobial* inoculation.

The preparation of soil suspension, microbial inoculation, collection of soil samples and determination of 16S rRNA [34], rhizobial symbiotic gene *nodC* [35], and housekeeping gene *recA* [36] abundance are described in the Supplementary Appendix. The primers for the target genes are listed in Supplementary Table S1.

Mycelia-based *Bradyrhizobial* dispersal assays

To monitor the attachment and dispersal of *Bradyrhizobium* on *Ph. liquidambaris* networks, a petri plate with a physical barrier was established. A sterile polystyrene ring (Ø 3.5 cm) was placed in the middle of the plate before SEA medium was poured. A fungal plug (Ø 5 mm) was inoculated in the middle of the ring and *Bradyrhizobium* was inoculated on the inner side of the ring with a bacterial inoculating loop. The plates with only *Bradyrhizobial* inoculation were also included as controls. The plates were then sealed with parafilm and placed at 28 °C for 7 days. The interactions of *Ph. liquidambaris* and *Bradyrhizobium* were observed by scanning electron microscope (JEOL, Japan). The experiments were carried out with three individual replicates, and representative scanning electron micrographs are shown.

Stage-dependent *Ph. liquidambaris–Bradyrhizobial* interaction assays

To study the interaction between *Ph. liquidambaris* and *Bradyrhizobium* at different stages simultaneously, five groups of a fungal plug (Ø 5 mm) and a 5-μl *Bradyrhizobial* suspension were inoculated on both sides of a square petri dish (13 × 13 cm) with SEA medium in diagonal rows, generating a reverse V-shape of increasingly far inoculation sites [37]. The *Bradyrhizobial* inoculation sites from near to far away from *Ph. liquidambaris* plugs were named S1–S5. Plates with only *Bradyrhizobial* inoculation were also created as controls (S0). The plates were then sealed with parafilm and placed at 28 °C for 7 and 14 days, respectively.

For *Bradyrhizobial* growth assays, the medium of each site from S0 to S5 was collected at 7 and 14 days after inoculation (dai). The medium was then mixed well with sterile water and spread on the surface of yeast mannitol agar plates (Ø 9 cm) supplemented with 30 mg l^{-1} natamycin after serial dilutions. Natamycin was used to inhibit the growth of *Ph. liquidambaris*. The plates were then sealed with parafilm and placed at 28 °C for 7 days. The number of *Bradyrhizobial* colonies on plates with 5–30 single colonies was counted. The experiment was carried out with three individual replicates.

For *Bradyrhizobial* motility assays, the medium of different sites was collected at 7 and 14 dai. Images and videos were directly taken with a Zeiss Axio Imager A1 microscope (Zeiss, Jena, Germany) [38]. The experiment was carried out with three individual replicates.

RNA-seq

For RNA-seq, *Bradyrhizobia* at S0 and S2 were sampled by scraping the medium surface with a sterile razor blade at 14 days after co-inoculation. Harvested cells were immediately frozen with liquid nitrogen and stored at −80 °C. The methods of RNA extraction, sequencing, and annotation are described in the Supplementary Appendix. The experiment was performed with three individual replicates. The RNA-seq reads are deposited in the Sequence Read Archive service of the GenBank database under the accession number SUB6177629.

RNA extraction and qRT-PCR analysis

Bradyrhizobium at each site from S0 to S5 after 14 days cocultivation with *Ph. liquidambaris* and *Bradyrhizobium* after fungal exudates (0 and 5 mg ml^{-1}) incubation for 24 h were collected for RNA extraction. The RNA extraction and qRT-PCR procedures are described in the Supplementary Appendix. The *Bradyrhizobial* 16S rRNA gene [39] was used as reference gene to normalize the expression of selected genes using the $2^{-\Delta\Delta Ct}$ method [40]. The experiment was performed with three individual replicates and three technical replicates, and sterile water was used as a negative control. The selected genes and primers are listed in Supplementary Table S1.

Statistical analyses

All experiments were performed in at least three individual replicates. The data are expressed as mean with standard error (SE). Statistical analyses were performed with SPSS v. 18.0 (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance via Tukey's honestly significant difference or Student's *t* test.

Results

Soil microcosm: *Ph. liquidambaris* networks facilitate *Bradyrhizobial* dispersal in soil conditions

To determine whether *Ph. liquidambaris* can serve as a bridging network for rhizobial migration to peanut

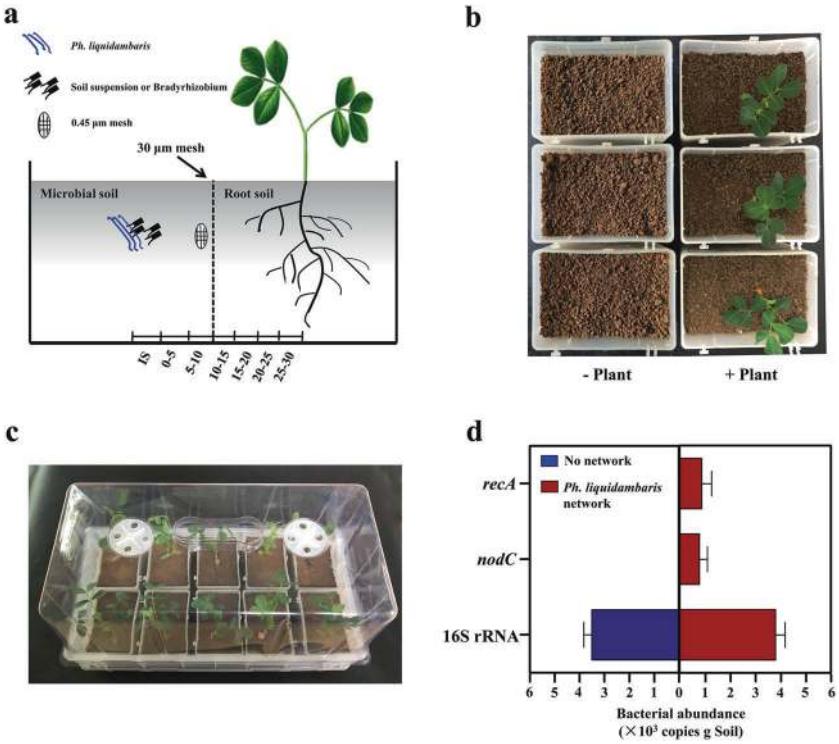


Fig. 1 *Ph. liquidambaris* transfers soil rhizobia to the rhizosphere of peanut. a, **b** A soil microcosm system containing microbial and root compartments was established to determine whether *Ph. liquidambaris* networks transfer rhizobia to the rhizosphere of peanut. The microbial and root compartments were separated by a sterile 30 μm mesh. Roots were confined to the root soil, but fungi and rhizobia in the microbial soil were able to cross the mesh and enter root soil. The treatments with peanut cultivation contained individual seedling planted in root compartment. Sterile 0.45 μm mesh in soil microcosm was used to observe *Ph. liquidambaris–Bradyrhizobium* interaction. **c** Microcosms were placed in transparent boxes with closed lids to minimize water evaporation and avoid microbial contamination. **d** 16S rRNA, *nodC*, and *recA* copies in the rhizosphere soil of peanut. At 15 dai, rhizosphere soil of peanut was collected to determine the copy numbers of 16S rRNA, *nodC*, and *recA* when the microbial compartment was inoculated with soil suspension with or without *Ph. liquidambaris*. Data and error bars are the mean ± SE ($n = 4$). CFUs colony-forming units; dai days after inoculation; IS inoculation site.

rhizosphere, a soil microcosm system was set up (Fig. 1a–c). Soil suspensions with or without *Ph. liquidambaris* were inoculated into the microbial compartment, which was separated from the peanut plant. In the presence of *Ph. liquidambaris*, rhizobial marker genes *nodC* and *recA* and *Ph. liquidambaris*-specific *ITS* were detected in peanut rhizosphere at 15 dai, which were not seen in the absence of *Ph. liquidambaris* (Fig. 1d; Fig. S1).

Furthermore, for determining *Ph. liquidambaris*-mediated rhizobial dispersal rates, a rhizobial species, *B. yuanmingense*, with or without *Ph. liquidambaris* was inoculated into the microbial compartment. *Bradyrhizobium* was quantified in soils at different distances from the inoculation site by colony-forming units (CFUs) and the abundances of *nodC* and *recA*, respectively. In the absence of mycelia, no *Bradyrhizobium* was detected beyond 15 mm away from the inoculation site at 9 dai (Fig. 2a, b; Fig. S2). By contrast, in the presence of mycelia, *Bradyrhizobium* reached 10–15 and 20–25 mm at 3 and 6 dai, respectively (Fig. 2a). At 9 dai, *Bradyrhizobial* colonies were detected in rhizosphere soil (Fig. 2a, b; Fig. S2). These results indicate that *Ph. liquidambaris* helped rhizobial migration to rhizosphere from bulk soil.

We then tested the effects of the host plant on mycelia-based *Bradyrhizobial* dispersal in soil environment. In the absence of peanut, *Ph. liquidambaris*-mediated *Bradyrhizobial* dispersal could not be detected beyond 20 mm at 9 dai, which was less than that with peanut cultivation (Fig. 2a, b; Fig. S2), suggesting that host plants acted as attractants that direct mycelia-based *Bradyrhizobial* dispersal.

Soil microcosm: *Ph. liquidambaris*-based *Bradyrhizobial* dispersal triggers nodulation

To directly observe *Ph. liquidambaris–Bradyrhizobium* interaction in soil environment and on root surface, meshes (0.45 μm) and peanut roots were sampled for scanning electron microscopy at 9 dai. The meshes were used as the soil itself is not suitable for sample preparation procedure of scanning electron microscopy. No fungal network was observed in soil pores without *Ph. liquidambaris*

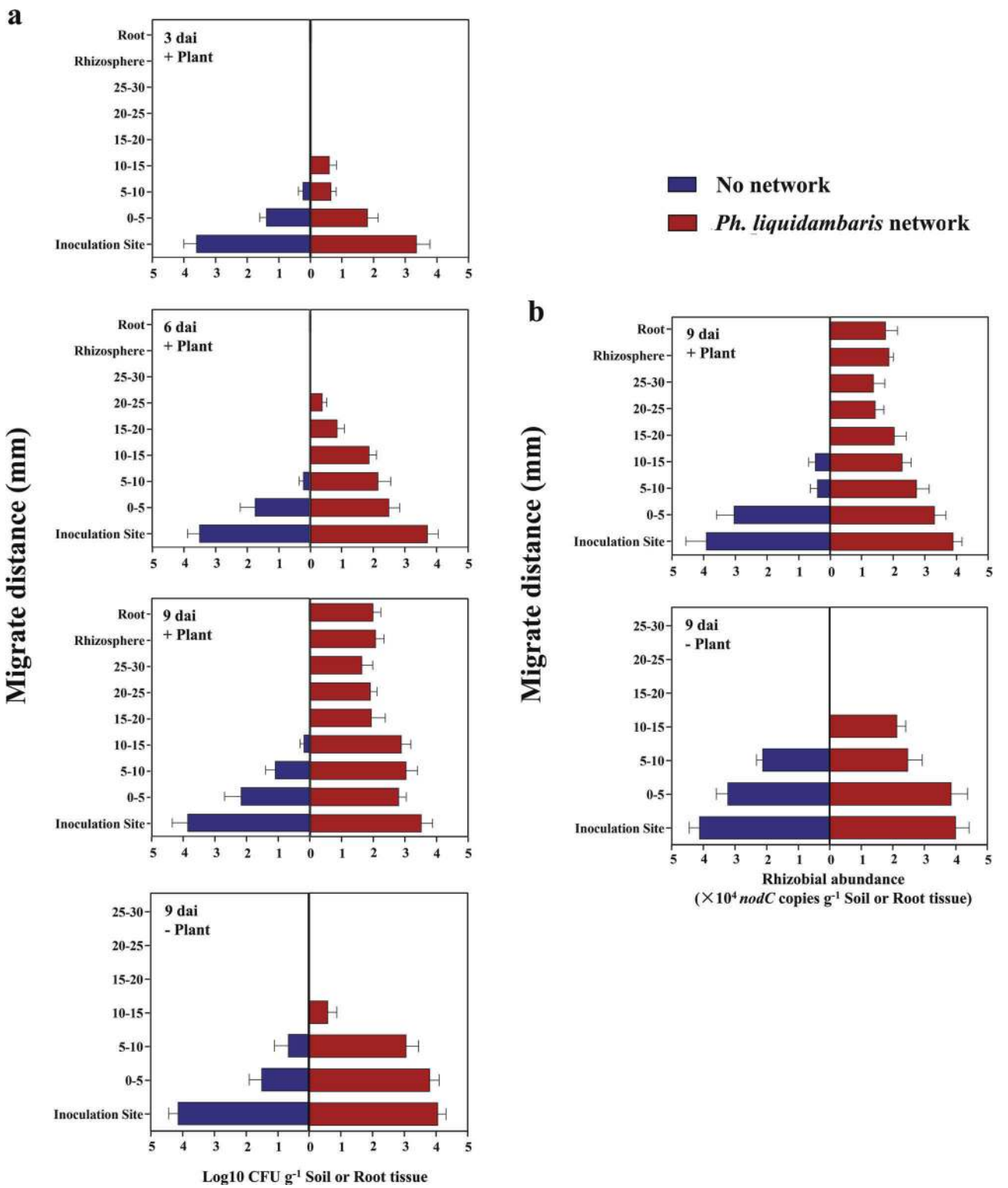


Fig. 2 *Ph. liquidambaris* facilitates *Bradyrhizobial* dispersal in soil conditions. a *Bradyrhizobial* number was obtained by CFU determination from the soil at various collection distances at 3, 6, and 9 dai when the microbial compartment was inoculated with *Bradyrhizobium* with or without *Ph. liquidambaris* and the root compartment was planted with or without peanut. **b** *Bradyrhizobial* number was obtained by qPCR of *nodC* from the soil at various collection distances at 3, 6, and 9 dai when the microbial compartment was inoculated with *Bradyrhizobium* with or without *Ph. liquidambaris* and the root compartment was planted with or without peanut. Data and error bars are the mean ± SE ($n = 4$). CFU colony-forming units; dai days after inoculation.

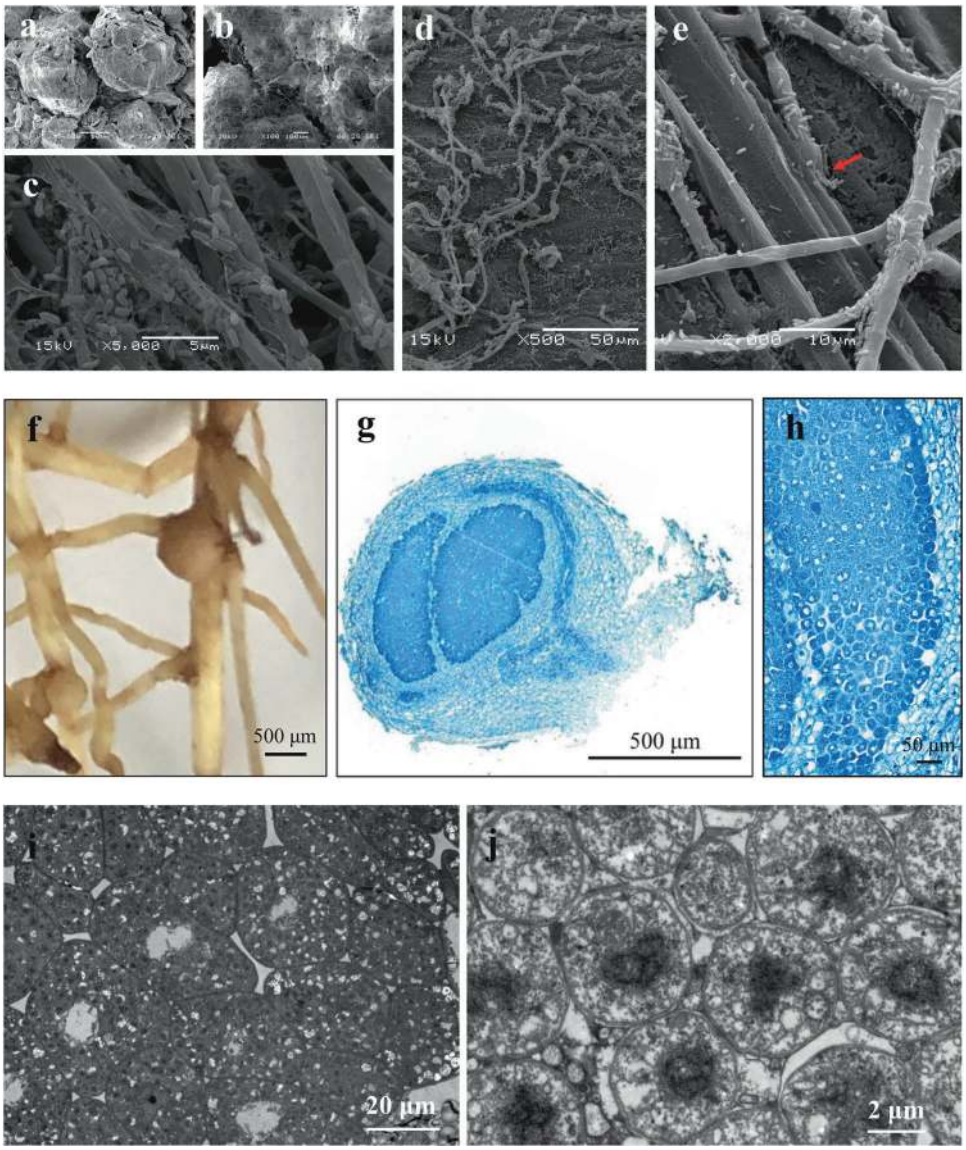


Fig. 3 *Ph. liquidambaris* facilitates *Bradyrhizobial* dispersal, promotes *Bradyrhizobial* root infection, and triggers peanut–*Bradyrhizobium* interaction. a SEM image showing no fungal network in soil pores of microcosm without *Ph. liquidambaris* inoculation. **b** SEM image showing fungal networks formed by *Ph. liquidambaris* in soil pores of microcosm with *Ph. liquidambaris* inoculation. **c** SEM image showing the attachment of *Bradyrhizobium* on *Ph. liquidambaris* hyphae. *Bradyrhizobium* was attached on the hyphal surface and formed fiber-like biofilms in soil. At 9 dai, the meshes (0.45 μm) were sampled for scanning electron microscopy to observe *Ph. liquidambaris–Bradyrhizobium* interaction in soil environment. **d** SEM image showing *Ph. liquidambaris–Bradyrhizobium* interaction on the root surface. **e** SEM image showing the root invasion of hyphae-attached *Bradyrhizobium* through crack sites (red arrow). At 9 dai, peanut roots were sampled for scanning electron microscopy to observe *Ph. liquidambaris–Bradyrhizobium* interaction on root surface. **f** Nodules on the lateral root of peanut in the presence of *Ph. liquidambaris* and *Bradyrhizobium*. No visible nodule was observed on the root in the absence of *Ph. liquidambaris*. **g** Toluidine blue-stained transverse section of nodules. **h** Higher-magnification images from **g**. The infected cells were well organized in the N_2-fixation zone. **i**, **j** TEM images showing the symbiosomes and bacteroides. The bacteroides were well organized in symbiosomes. At 45 dai, root nodules were collected and prepared for transmission electron microscopy to observe nodule ultrastructure. The experiments were carried out with three individual replicates, and representative micrographs are shown. SEM scanning electron microscope; TEM transmission electron microscope; dai days after inoculation.

inoculation (Fig. 3a; Fig. S3a, b). By contrast, *Ph. liquidambaris* extended vastly in soil pores, generating fungal networks to link soil aggregates after inoculation (Fig. 3b; Fig. S3c). Meanwhile, *Ph. liquidambaris* hyphae were able to reisolate from soil microcosm with *Ph. liquidambaris* and bradyrhizobial co-inoculation rather than the microcosms of control and bradyrhizobial inoculation (Fig. S4).

A mass of *bradyrhizobium* formed dense surface-attached biofilms on the hyphal surface (Fig. 3c). At the root surface, a tripartite interaction, including

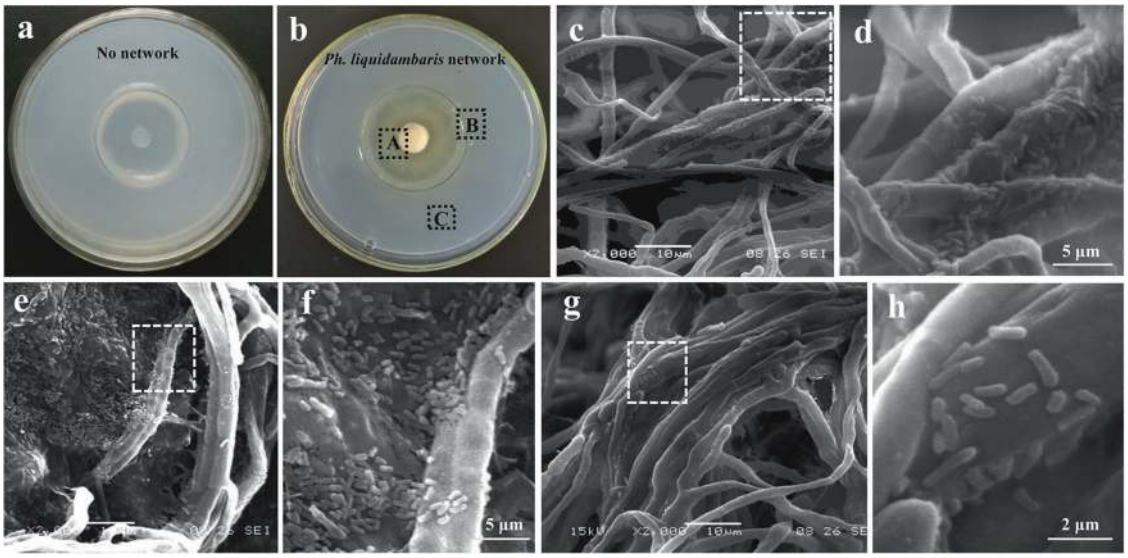


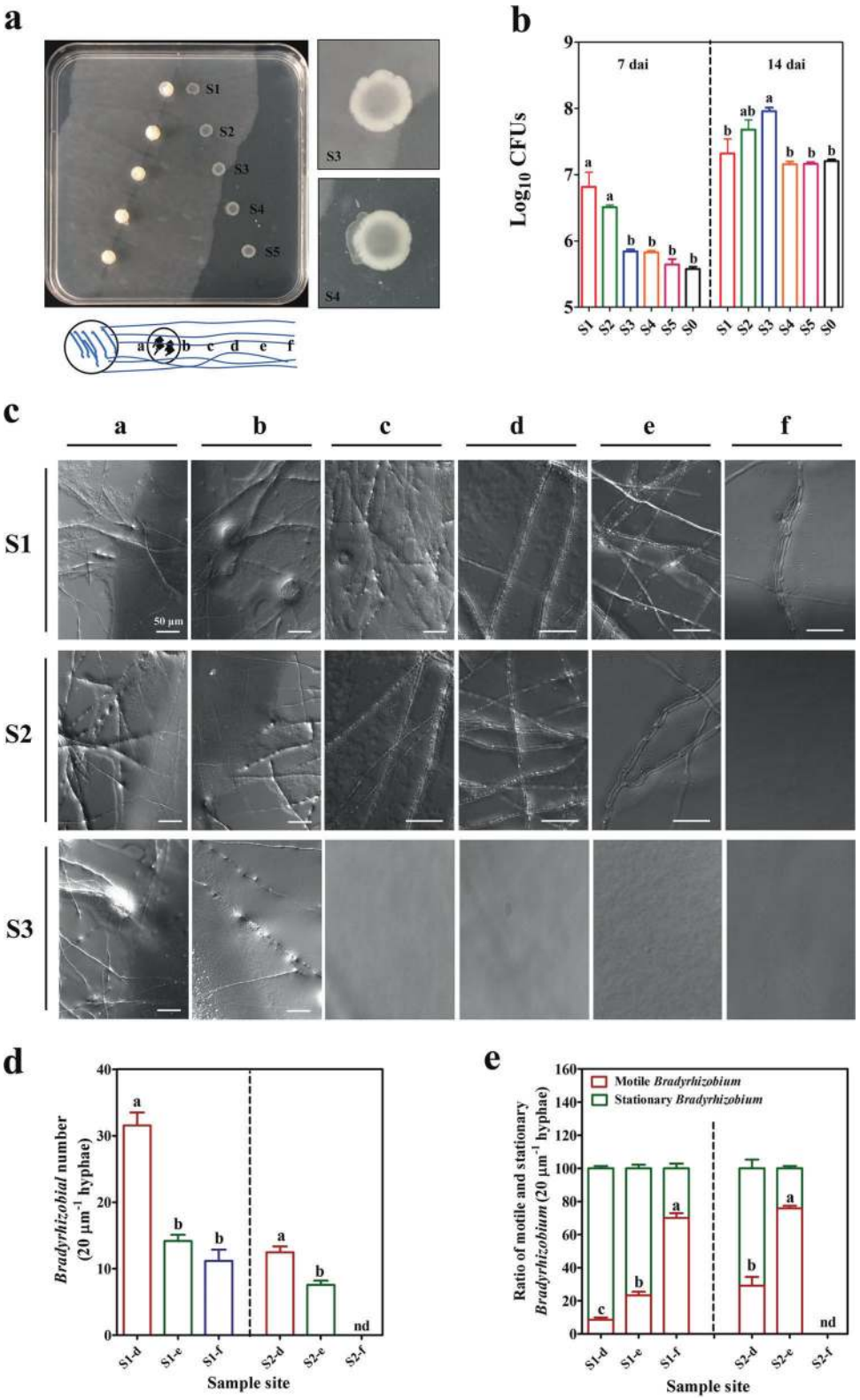
Fig. 4 *Ph. liquidambaris* networks help *Bradyrhizobial* dispersal. a Plate with a sterile polystyrene ring with *Bradyrhizobium* inoculation alone. **b** Plate with a sterile polystyrene ring with *Ph. liquidambaris* and *Bradyrhizobium* co-inoculation. A–C sites refer to sampling sites for scanning electron microscopy. **c**, **d** SEM images showing *Bradyrhizobium* attached to and dispersed from mycelial networks of *Ph. liquidambaris* at A site. **e**, **f** SEM images showing *Bradyrhizobium* attached to and dispersal from mycelial networks at B site. **g**, **h** SEM images showing *Bradyrhizobium* attached to and dispersal from mycelial networks at C site. **d**, **f**, **h** Higher-magnification images from **c**, **e**, and **g**. The experiments were carried out with three individual replicates, and representative micrographs are shown. SEM scanning electron microscope.

Bradyrhizobium, *Ph. liquidambaris*, and plant, was observed (Fig. 3d, e). Dense biofilms were also formed by *Bradyrhizobium* on hyphae at the root surface (Fig. 3e). Some hyphae began to infect roots via cracks on the root surfaces, which might facilitate the root invasion of attached *Bradyrhizobium* (Fig. 3e). When *Bradyrhizobium* was directly inoculated in root compartment, we observed that *Bradyrhizobium* attached to the cracks on the root surfaces (Fig. S5b). No *Ph. liquidambaris* hypha or *Bradyrhizobium* was found on root surface of *Bradyrhizobial* inoculation in microbial compartment (Fig. S5a).

At 45 dai, we harvested peanut roots and analyzed peanut–*Bradyrhizobium* nodulation. No visible nodule was formed on the root when *Bradyrhizobium* was inoculated in the microbial compartment alone. With both inoculation of *Ph. liquidambaris* and *Bradyrhizobium* in the microbial compartment, 2 ± 0.5 nodules per plant were formed on lateral roots (Fig. 3f; Supplementary Table S2). When *Bradyrhizobium* was directly inoculated in peanut rhizosphere or the root surface at the root compartment, 3 ± 0.75 nodules were formed per plant, mainly on the crown root (Fig. S6a; Supplementary Table S2). By histological and transmission electron microscope observations, these nodules showed a normal organization pattern, a central N_2-fixation area and vascular bundles surrounding the nodule cortex (Fig. 3g; Fig. S6b). Plant cells that were infected by *Bradyrhizobium* were assembled tightly in the N_2-fixation area (Fig. 3h; Fig. S6c). Moreover, the ultrastructure of symbiosomes and bacteroides was normal, and each symbiosome contained 48.70 ± 12.47 bacteroides (Fig. 3i, j; Supplementary Table S2), indicating that these nodules could fix N_2. Similarly, effective nodules with 50.30 ± 10.55 bacteroides per symbiosome were observed when *Bradyrhizobium* was delivered directly into root compartment (Fig. S6d, e; Supplementary Table S2). These results suggest that *Ph. liquidambaris*-based *Bradyrhizobial* dispersal triggered peanut–*Bradyrhizobium* nodulation.

Plate interaction: *Ph. liquidambaris* networks help *Bradyrhizobium* dispersal

To address the direct interactions between *Ph. liquidambaris* and *Bradyrhizobium*, three different plate experiments were performed. First, the nested plate system showed that without *Ph. liquidambaris* inoculation, *Bradyrhizobium* was not able to cross the ring (Fig. 4a). By contrast, in the presence of *Ph. liquidambaris*, *Bradyrhizobium* crossed the ring and reached the outer compartment, i.e., site C (Fig. 4b). Scanning electron microscopy further detail the process: *Bradyrhizobium* was attached on the hyphal surface at A site, indicating that *Bradyrhizobium* migrated along established *Ph. liquidambaris* networks toward fungal plugs (Fig. 4c, d). Then, when the growing hyphae reached the *Bradyrhizobial* inoculation site, *Bradyrhizobium* migrated to the surface of hyphae from the growth medium and crossed the ring (Fig. 4e–h). Moreover,



we found that *Ph. liquidambaris* was able to help other three peanut nodule-occupying rhizobia [21], cross the ring (Fig. S7).

The dispersal of *Bradyrhizobium* on mycelial networks detected in above-mentioned experiment could be due to the passive dispersal when *Bradyrhizobium* is pushed

◀ **Fig. 5 Analysis of *Bradyrhizobial* dispersal on mycelial networks of *Ph. liquidambaris* at 14 dai. a** *Ph. liquidambaris* and *Bradyrhizobium* were inoculated on a square plate in a diagonal row. At 14 dai, *Ph. liquidambaris* began to interact with *Bradyrhizobium* at S3. At S4, *Bradyrhizobium* was attracted by *Ph. liquidambaris* before physical contact. The sketch refers to sampling sites of square plate above for microscopic observation. The big circle represents fungal plug and the small circle represents *Bradyrhizobial* colony. a–f in the sketch represent the sampling sites. a, b in the sketch represent the sampling sites at left and right of *Bradyrhizobial* colony. **b** Effects of *Ph. liquidambaris* on *Bradyrhizobial* growth at S0–S5 at 7 and 14 dai. *Bradyrhizobial* number was determined by CFU. Data and error bars are the mean ± SE ($n = 3$) and different letters indicate significant differences among different sites at the same dai (one-way analysis of variance with Tukey's test, $P < 0.05$). **c** Different stages of *Ph. liquidambaris–Bradyrhizobial* interaction at 14 dai showing *Bradyrhizobial* dispersal along mycelial networks. Samples were collected from **a** and directly observed with a Zeiss Axio Imager A1 microscope. Bars, 50 µm. **d** Average number of *Bradyrhizobium* on hyphae of different sampling site of **c**. **e** Average ratio of motile and stationary *Bradyrhizobium* on hyphae of different sampling site of **c**. The number of total, motile, and stationary *Bradyrhizobium* on 20 µm hyphae was counted from images and time-lapse videos, respectively. The experiments were performed with three individual replicates and each replicate contained 15 images or time-lapse videos over 60 s. Data and error bars are the mean ± SE ($n = 3$) and different letters indicate significant differences among different sites (one-way analysis of variance with Tukey's test, $P < 0.05$). dai days after inoculation.

horizontally across surface by the growing hyphae as filamentous fungi extend by apical growth [38, 41]. Thus, by developing synthetic glass fiber networks simulating mycelial networks, we further determined whether *Bradyrhizobium* used active motility or passive dispersal along mycelia [38]. At 7 and 14 dai, *Bradyrhizobium* migrated out from the inoculation spot and followed the topology of the glass fiber networks (Fig. S8a, b). Although we could not rule out the possibility that capillary forces contribute to this process [42], these results suggested the involvement of bacterial active motility during *Bradyrhizobial* dispersal along mycelial networks. Third, existing fungal networks of *Ph. liquidambaris* was created to determine whether *Bradyrhizobium* could disperse on established fungal networks. At 7 dai, *Bradyrhizobium* rapidly dispersed across the existing fungal networks (Fig. S8c), with a 5.71-fold increase in dispersal distance, which further confirmed the involvement of bacterial active motility during *Bradyrhizobial* dispersal along mycelial networks.

Stage-dependent *Ph. liquidambaris–Bradyrhizobial* interaction assays

Ph. liquidambaris and *Bradyrhizobium* were inoculated on a square petri dish in a diagonal row, which enabled the simultaneous sampling of fungi and bacteria at different interactive stages (Fig. 5a). We first quantified the *Bradyrhizobial* CFUs at 7 and 14 dai. These two time points were chosen because *Ph. liquidambaris* and *Bradyrhizobium* began to interact at S2 and S3 at 7 and 14 dai, respectively (Fig. 5a; Fig. S9). *Ph. liquidambaris* networks increased *Bradyrhizobial* growth at S1 and S2 at 7 dai, and this positive effect at S1 was decreased at 14 dai, while the cell number of *Bradyrhizobium* at S3 was increased when *Ph. liquidambaris* reached the *Bradyrhizobial* colony at 14 dai. By contrast, no significant difference in *Bradyrhizobial* growth was found at S4 or S5 in relative to S0 (Fig. 5b).

We then captured the interactions of *Ph. liquidambaris* and *Bradyrhizobium* at macroscopic and microscopic scales at 14 dai. At this time, the *Bradyrhizobium* at the colony edge of S4 was stimulated toward the fungal plug (Fig. 5a), suggesting that *Bradyrhizobium* was attracted by *Ph. liquidambaris* before physical contact. To directly observe the *Bradyrhizobial* dispersal along mycelial networks, we used time-lapsed microscopy of *Bradyrhizobium* and *Ph. liquidambaris* at S1–S3. The interaction between *Ph. liquidambaris* and *Bradyrhizobium* at S3 was expected to represent the initial stage of interaction establishment, as fungal colony had just come into physical contact with the *Bradyrhizobium* [43]. At S3, *Bradyrhizobium* at the edge of the colony began to swim along hyphae toward *Ph. liquidambaris* (Fig. 5c; Movie S1). At S1 and S2, *Ph. liquidambaris* networks had already established interactions with *Bradyrhizobium* and facilitated their long-distance dispersal (Fig. 5a, c). When analyzing bacterial motility along the direction of mycelial growth, we found that pioneer *Bradyrhizobium* on the growing hyphae was still motile (Movies S2 and S3), but most *Bradyrhizobium* on the established mycelial networks had converted to stationary state (Movie S4). The *Bradyrhizobial* dispersal mode was supported by the quantitative data of motile and stationary *Bradyrhizobial* number on *Ph. liquidambaris* surface at S1 and S2. Along the direction of mycelial growth, the *Bradyrhizobial* number and ratio of stationary *Bradyrhizobium* decreased gradually (Fig. 5d, e), while the ratio of motile *Bradyrhizobium* was gradually increased (Fig. 5e).

Bradyrhizobium was attracted by *Ph. liquidambaris* at the "no-physical-contact" stage and *Bradyrhizobial* number was increased at the early interactive stage, indicating that hyphal exudates might be involved in this process. We then collected hyphal exudates of *Ph. liquidambaris* and tested their effects on *Bradyrhizobial* growth, biofilm formation, and chemotactic behaviors. The addition of hyphal exudates at 5 and 10 mg ml^{-1} showed higher *Bradyrhizobial* optical densities (ODs) than those in the control (Fig. 6a). Meanwhile, *Bradyrhizobial* biofilm formation and chemotaxis were significantly enhanced in the presence of hyphal exudates at 1, 5, and 10 mg ml^{-1} (Fig. 6b, c).

In brief, these results indicated that *Bradyrhizobial* motility, growth, and proliferation were involved in their

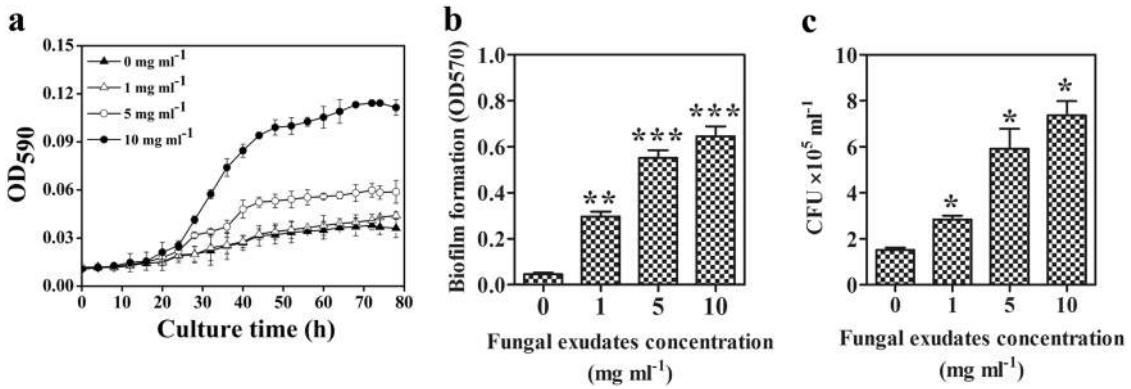


Fig. 6 Effects of *Ph. liquidambaris* exudates on *Bradyrhizobial* growth, biofilm formation, and chemotaxis. a *Ph. liquidambaris* exudates increased *Bradyrhizobial* growth. The OD_{590} values were recorded every 4 h for 78 h. **b** *Ph. liquidambaris* exudates increased *Bradyrhizobial* biofilm formation. **c** Chemotactic response of *Bradyrhizobium* toward *Ph. liquidambaris* exudates. The chemotactic response of *Bradyrhizobium* toward *Ph. liquidambaris* exudates was evaluated by capillary assay. Data and error bars are the mean ± SE ($n = 3$) and asterisks indicate significant differences between control and fungal exudates treatments (Student's *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). CFU colony-forming units.

dispersal along mycelial networks while fungal exudates might contribute to this process.

Transcriptomic analysis of *Bradyrhizobium* to *Ph. liquidambaris*

To reveal the underlying mechanisms driving *Bradyrhizobial* dispersal on *Ph. liquidambaris* networks, *Bradyrhizobium* at S0 (B) and S2 (E + B) was sampled for RNA-seq at 14 dai. *Bradyrhizobium* at S2 was chosen, as bacteria at this stage showed high reproduction and motility in the presence of mycelial networks (Fig. 5b, c). Transcriptome analysis showed that *Bradyrhizobium* underwent a global gene expression shift in the presence of mycelia, with 831 differentially expressed genes (expression change of greater than twofold, $P < 0.05$), including 368 genes with increased expression levels and 463 genes with decreased expression levels (Supplementary Table S3). Gene Ontology (GO) enrichment analysis of differentially expressed genes was first performed. Most of differentially expressed genes with predicted function annotations were assigned to metabolism, environmental information processing, and cellular processes (Fig. 7a). Then, we performed KEGG pathway enrichment analysis for the differentially expressed genes. KEGG pathway analysis showed that amino acid and derivatives metabolism, carbohydrate metabolism, energy metabolism membrane transport, signal transduction, and cell motility were enriched (Fig. 7b). Within the significantly upregulated genes, 55 genes were associated with metabolism, including amino acids and derivatives (24), carbohydrates (23), cofactors and vitamins (8), nucleotides (6), and lipids (5). In total, 29 genes with significantly decreased expression were associated with metabolism, mainly that of carbohydrates (12), amino acids and derivatives (9), cofactors and vitamins (5), and lipids (3) (Fig. 7b; Supplementary Table S4). Consistently, several genes related to transport of nutrients in *Bradyrhizobium* were regulated, for instance glucose/mannose transport system substrate-binding protein, multiple sugar transport system ATP-binding protein, branched-chain amino acid transport system ATP-binding system, and phospholipid/cholesterol/gamma-HCH transport system permease protein (Supplementary Table S3).

Giving that the *Bradyrhizobium* at S2 had a high cell number and motility compared with those at S0, we expected the genes associated with cell division, the chemosensory system, flagellar biosynthesis and assembly, and motility would be differently regulated by *Ph. liquidambaris* networks. An increase in nucleotide metabolism and protein synthesis of *Bradyrhizobium* in cocultivations was determined in the presence of *Ph. liquidambaris* (Fig. 7c; Supplementary Table S5). The expression of genes associated with purine and pyrimidine metabolism, bacterial peptidoglycan biosynthesis and large-subunit ribosomal protein components was increased in cocultivations (Fig. 7c; Supplementary Table S5). Among the differentially expressed genes involved in bacterial chemotaxis and motility, methyl-accepting chemotaxis proteins (MCPs) (GA0061099_1001674, GA0061099_1003438, GA0061099_1002562), and chemotaxis signaling proteins CheA (GA_0061099_10071, GA0061099_10021), CheR (GA0061099_1006673), CheY (GA0061099_1006671), and FliC (GA0061099_1001941) were significantly upregulated, while genes encoding flagellar M-ring protein FliF (GA0061099_1001939), flagellar P-ring protein precursor Flgl (GA0061099_1001946), flagellar biosynthetic protein FlhB (GA0061099_1001952), and ribose transport system substrate-binding protein rbsB (GA0661099_1010150) were downregulated in the presence of *Ph. liquidambaris* (Fig. 7d, e;

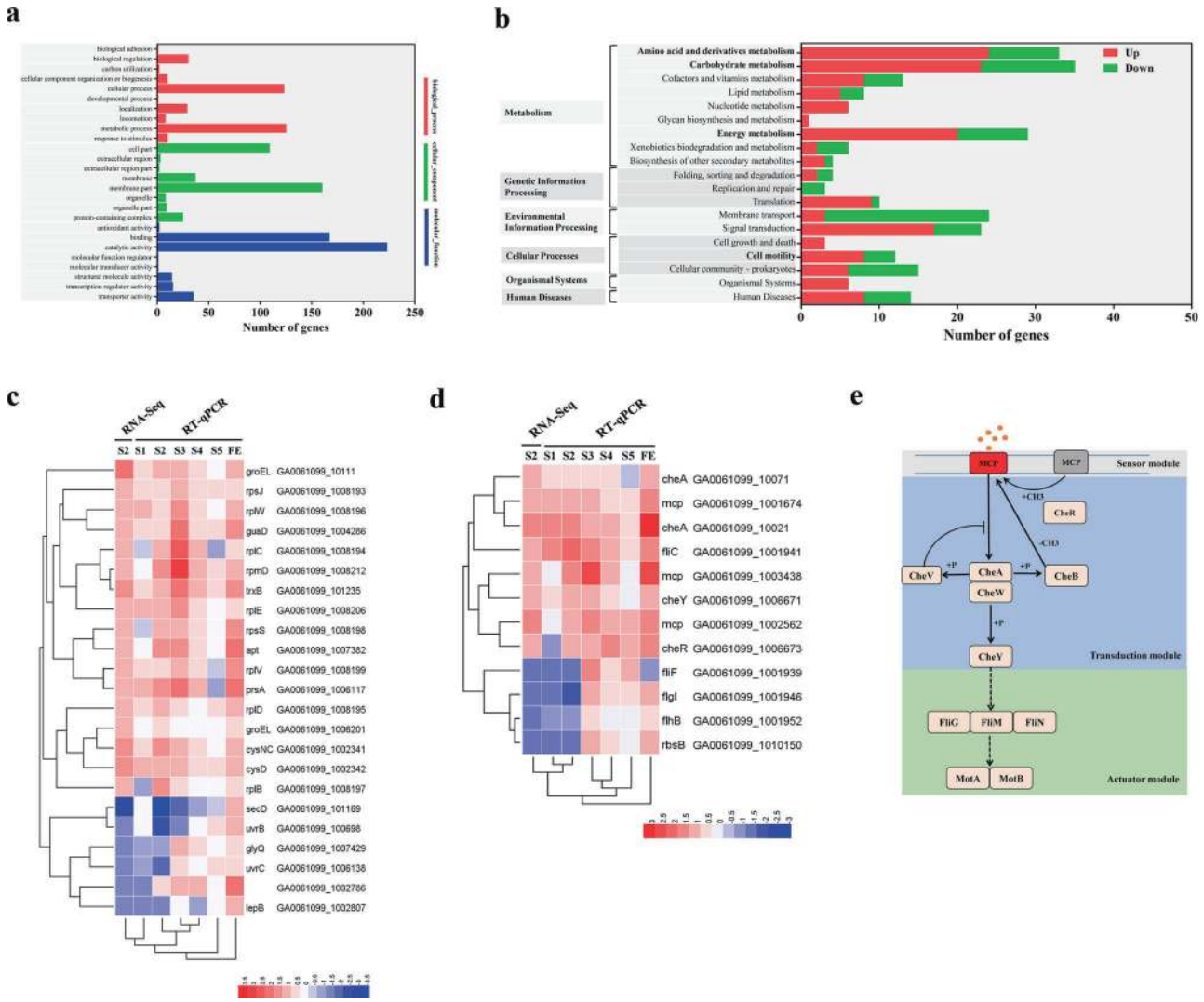


Fig. 7 RNA-seq reveal the potential genetic mechanisms of rhizobial dispersal on *Ph. liquidambaris* networks. a Number of differentially expressed genes of GO categories related to biological process, cellular component, and molecular function in *Bradyrhizobium* inoculation alone (B) vs *Bradyrhizobium* and *Ph. liquidambaris* co-inoculation (E + B). **b** KEGG analysis of differentially expressed genes in B vs E + B. **c** Expression of *Bradyrhizobial* differentially expressed genes associated with bacterial division at S0-S5 at 14 dai by RNA-seq and qRT-PCR. The responses of these differentially expressed genes to fungal exudates were also included. **d** Expression of *Bradyrhizobial* differentially expressed genes associated with bacterial chemotaxis, flagellar biosynthesis, and motility at S0–S5 at 14 dai by RNA-seq and qRT-PCR. The responses of these differentially expressed genes to fungal exudates were also included. **e** Bacterial swimming chemotaxis pathway, which includes sensor, transduction, and actuator modules. dai days after inoculation; FE fungal exudates.

Supplementary Table S5). In addition, genes associated with biofilm formation (GA0061099_1001800, GA0061099_1007256, GA0061099_1002199) were increased in cocultivations (Supplementary Table S3).

To understand the dynamic transcriptional regulation of *Bradyrhizobial* motility and proliferation at different stages of interactions, we employed qRT-PCR to analyze the relative expression of genes associated with motility and proliferation at S1–S5 with respect to S0. The transcriptional responses underlying *Bradyrhizobial* motility and proliferation to fungal exudates were also included. The expression of selected genes of *Bradyrhizobium* at S2 using qRT-PCR showed similar trends to RNA-seq (Fig. S10; Supplementary Table S5). Most of cell division-related genes are induced at S1–S4 (Fig. 7c). The *Bradyrhizobial* chemosensory system had already been primed in cocultivations before physical contact, as some of the tested *mcp* and *che* genes, especially *mcp* (GA0061099_1003438) and *cheR* (GA0061099_1006673), were upregulated at S4 (Fig. 7d). The tested flagellar biosynthesis- and motility-related genes showed a stage-dependent expression pattern. The flagellar biosynthesis gene *fliF*, *flgl* and motility-related gene *rbsB* were upregulated at S3–S4, but decreased at S1–S2. The *fliC* was induced at S1–S4 (Fig. 7d). Most of

the tested genes were significantly induced when *Bradyrhizobium* was incubated with *Ph. liquidambaris* exudates (5 mg ml^{-1}) in respect to 0 mg ml^{-1} exudates (Fig. 7c, d), suggesting that fungal exudates might play an important role in the *Ph. liquidambaris*-mediated *Bradyrhizobial* gene expression shift.

The *Bradyrhizobial* detoxification process was not enhanced when the bacteria were grown with *Ph. liquidambaris*. Genes encoding glutathione synthase (GA0061099_1002255), glutathione S-transferase (GA0061099_1003480), most of multidrug efflux pumps and the NodT family of efflux pumps were decreased in cocultivations (Supplementary Table S3).

Discussion

Mycelia are effective dispersal networks that contribute to the maintenance of bacterial ecological functions, such as bioremediation and predation [15–17]. However, little is known about the role of mycelia-based bacterial dispersal in legume–rhizobium symbiosis. Previous studies showed that *Ph. liquidambaris* enhanced nodulation of peanut and increased population of *Bradyrhizobium* sp. in rhizosphere under continuous cropping system that is rhizobium-deficient [9, 22], suggesting the possible role of *Ph. liquidambaris* in rhizobial rhizosphere enrichment.

Here, we developed a microcosm system to test *Bradyrhizobial* migration in soil in the presence or absence of *Ph. liquidambaris*, and revealed that *Ph. liquidambaris* helped *Bradyrhizobial* migration to peanut rhizosphere and, hence, triggered nodulation. In addition, *Ph. liquidambaris* might facilitate the root invasion of hyphae-attached *Bradyrhizobium* through crack sites. Mycelia-based *Bradyrhizobial* crack infection has potential to increase subsequent nodulation as crack entry is essential for peanut–*Bradyrhizobium* nodulation [44, 45]. To gain insight on *Ph. liquidambaris–Bradyrhizobium* interaction, we performed *Bradyrhizobial* dispersal modes assays and transcriptomic analysis. The results indicated that bacterial proliferation and motility mediated *Bradyrhizobial* dispersal along *Ph. liquidambaris*, while fungal exudates might contribute to this process.

Dispersal mode analyses indicated that *Bradyrhizobial* cells on the growing and established hyphal networks shared different functions in new niche colonization. The ratio of motile and static *Bradyrhizobium* showed opposite trends along the direction of mycelial growth: the ratio of motile *Bradyrhizobium* was decreased and the ratio of static *Bradyrhizobium* was gradually increased. This suggested that *Bradyrhizobium* changed behavior along *Ph. liquidambaris* networks. The pioneer *Bradyrhizobium* on the growing hyphae was motile, which explored new niche with the growing hyphae. While a large number of *Bradyrhizobium* on the established hyphae was static and detached from hyphal surface for niche occupation. Thus, *Bradyrhizobial* cell proliferation and motility mediated its dispersal along mycelia.

Similar to the roles of root exudates in plant–microbe interactions, hyphae release a substantial amount of C-rich compounds, including sugars, organic acids, amino acids, and polyols to the mycosphere, where they mediate fungal–bacterial interactions [25, 31, 33, 46]. For example, arbuscular mycorrhizal fungus exudates stimulate the growth and phytate mineralization of phosphate-solubilizing bacteria [33, 46]. During *Bradyrhizobial* dispersal along mycelia, fungal exudates might contribute to this process with dual roles. Before physical contact, *Bradyrhizobium* was attracted by *Ph. liquidambaris*, suggesting that fungal exudates functioned as chemo-attractants providing information about a nearby nutrient-rich niches. At early interactive stage, *Bradyrhizobial* number was increased, indicating that *Ph. liquidambaris* provided nutrients for *Bradyrhizobial* reproduction. This conclusion was further supported by analyzing the responses of *Bradyrhizobial* growth and chemotactic behavior to *Ph. liquidambaris* exudates. As we did not quantify the *Ph. liquidambaris* exudates, we could not unambiguously identify the primary chemo-attractants and nutrients from *Ph. liquidambaris* that *Bradyrhizobium* utilized in the present study. Further works with a combination of transcriptomics and exometabolomics at different interactive stages would help to reveal the chemical exchange between interactive partners [47].

The transcriptomic analysis underpinned the results from soil microcosm experiment and dispersal mode assays. *Bradyrhizobial* genes associated with cell division were induced by *Ph. liquidambaris*, explaining the increase in cell number of *Bradyrhizobium* during its interaction with *Ph. liquidambaris*. *Bradyrhizobial* genes associated with carbohydrate, amino acid, and derivatives metabolism were induced by *Ph. liquidambaris*, suggesting that mycelia provide nutrients to support *Bradyrhizobial* proliferation, which is also reported in other fungal–bacterial interactions [46, 48]. In addition, most genes with functions assigned to production and conversion of energy were increased by mycelia, confirming that *Bradyrhizobium* utilized nutrients derived from fungi to raise their bioenergetic status [32, 49]. Biofilms are important for the fitness of bacteria [50] and have been reported to enable bacterial biomass accumulation during microbial interspecies interaction [51]. Correspondingly, genes associated with bacterial biofilm formation were induced when *Bradyrhizobium* was cocultivated with *Ph. liquidambaris* and biofilms were formed on the surface of mycelia by *Bradyrhizobium* and *Ph. liquidambaris* exudates stimulated *Bradyrhizobial* biofilm formation, suggesting that biofilm might contribute to *Bradyrhizobial* cell increase.

Bradyrhizobial genes associated with chemosensory system, flagellar biosynthesis, and motility were induced at the early interactive stage, highlighting the role of chemotaxis and flagellum-mediated motility in *Bradyrhizobial* dispersal along *Ph. liquidambaris* hyphae [32]. This combined with *Bradyrhizobial* dispersal along glass fiber networks and established mycelial networks suggested that *Bradyrhizobium* swim and/or swarm along mycelial networks. The transmembrane chemoreceptors MCPs were induced before physical contact, suggesting that *Bradyrhizobium* had already sensed *Ph. liquidambaris*. This notion was further confirmed by the activation of chemotactic signal transduction system and flagellar biosynthesis process, especially methyltransferase CheR, histidine kinase CheA, and FliC. CheR catalyzes the conversion of specific glutamyl residues to glutamyl methyl esters on the cytosolic side of MAPs and induces the autophosphorylation of CheA, which increases bacterial tumbling [51–53].

Unlike one study of [48], which reported that the detoxification process of *Burkholderia* was activated when cocultivated with *A. alternate* or *F. solani*, our results showed that genes associated with antioxidant system and drug efflux pumps of *Bradyrhizobium* were not enhanced in the presence of *Ph. liquidambaris*. One possible explanation for the differences is that *Ph. liquidambaris* did not produce any antimicrobial compounds during its interactions with *Bradyrhizobium*. In addition, we cannot rule out the involvement of other detoxification pathways. Compared with other soil bacteria, the rhizobial genome, especially that of *Bradyrhizobium*, is large and diverse, contributing to their lifestyle switches and environmental adaptations [54, 55]. Thus, the genomic traits of rhizobia probably confer their ability to degrade and utilize fungal-derived secondary metabolites, which are detrimental to other soil bacteria. In addition, detoxification is an energy-consuming process and might be shut off, which saves the resources for *Bradyrhizobial* growth and motility. Together, these results suggest that *Bradyrhizobial* chemosensory system, flagellum-mediated motility, and cell proliferation participated in their dispersal and growth on *Ph. liquidambaris* networks, which might be mediated by hyphal exudates.

The dispersal of rhizobia in soil environment is poor [56], and rhizobia are deficient and not uniform in degraded soil, for instance, acidic soil, saline soil, and arid soil [57]. Thus, the nodulation of legumes under these soil environment is normally deficient [57, 58]. To increase the symbiotic potential of legumes in rhizobially deficient environment, rhizobial inoculants have been introduced to legumes via on-seed (seed-applied) or in-furrow (soil-applied) methods [4, 59, 60]. On-seed inoculation can deliver rhizobia to the rhizosphere of the emerging root, but in-furrow inoculation has more advantages, including separating the rhizobia from seed-applied bactericides and insecticides, avoiding the damage to seeds, and increasing the rhizobial number in the soil of legumes, which germinate epigeally, such as peanut, soybean, and subterranean clover [59, 60]. However, in-furrow inoculation increases the average distance between rhizobia and legume hosts. Our result that mycelia transfer rhizobia to the rhizosphere and, hence, trigger peanut–rhizobium symbiosis provide evidence that this drawback could be effectively solved by in-furrow co-inoculation of rhizobia and beneficial fungi. Moreover, filamentous fungi are better adapted to harsh soil environment than bacteria [23, 25, 61]. Thus, co-application of rhizobia and beneficial fungi could improve the nodulation competitiveness of inoculated rhizobia over native soil rhizobia, as fungal–bacterial interactions increase bacterial adaptive plasticity and survival in the soil environment by providing microhabitats and nutrients [18, 24].

Conclusions

Our study indicates that mycelial networks transfer rhizobia to the rhizosphere from bulk soil and, hence, initiate legume–rhizobium interaction. Mycelia-based enrichment of rhizobia in the rhizosphere is probably common in the soil environment, as a relatively high proportion of saprotrophic fungi also have facultative biotrophic capacity and mycosphere is a hospitable microhabitat for rhizobia. A recent study of 201 species of wood-decay basidiomycetes and a microcosm system showed that 16.9% (34) of tested fungi could enter into facultative biotrophic relationships with plant roots [62, 63]. In addition to nutrients provision, the mycosphere is a weakly acidic environment, which favors the colonization of rhizobia, especially the *Bradyrhizobium* genus [64, 65]. Our study reveals long-distance rhizobial enrichment to the rhizosphere, which cannot be mediated by root exudates. Moreover, our study provides a theoretical basis for the practical use of beneficial fungi and rhizobia in combinations to improve the symbiotic efficiency of leguminous crops in degraded lands.

Acknowledgements We thank Yin-Ping Zhang and Chao-Chao Hu of Analytical & Testing Center, Nanjing Normal University, for their help with the microscopic imaging. This work was financially supported by the National Natural Science Foundation of China (NSFC no. 31870478; 31370507); the Priority Academic Program Development of Jiangsu Higher Education Institutions; and Doctor Breeding Project of Nanjing Normal University (1812000006317).

Author contributions WZ, XGL, and CCD designed the experiments, analyzed the data, and wrote and revised the manuscript. WZ, KS, MJT, FJX, and MZ performed experiments. All of authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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