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Dynamics of rice seed-borne bacteria from acquisition to seedling colonization

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Abstract

Background Rice cultivation relies on planting grains harboring beneficial microbiota. However, the origination, distribution, and transmission dynamics of grain-borne bacteria remain unclear.

Results Using rice grain as a model system, this study investigates the primary sources, major niches in seeds, and the dynamics of community acquisition, maintenance, and transmission between generations of grain-borne bacteria. Quantitative PCR and 16S rRNA gene sequencing demonstrate rice grains acquiring bacteria primarily from the external environment during panicle heading and flowering. These bacteria concentrate between the caryopsis and glumes, establishing sizable communities in developing seeds. The dominant taxa included *Pantoea*, *Pseudomonas*, and *Sphingomonas*. Throughout seed development and storage, community structure remains consistent while abundance fluctuates within one order of magnitude. Upon germination under axenic conditions, seed bacteria successfully colonize shoots and roots of offspring seedlings. However, bacteria transmitted solely through internal routes fail to form comparably large communities. Analysis of taxonomic composition uncovers dramatic reshaping from seeds to seedlings, potentially reflecting functional adaptation.

Conclusions We clarify seed-borne bacterial origination, acquisition timing, seed colonization, intergenerational transmission, and seedling diversification. Our findings provide novel insights into rice seed bacterial dynamics critical for microbiome management.

Keywords Seed-borne bacteria, Rice grain, Quantitative abundance, External route, Panicle booting and flowering, Functional adaptation

Background

Seeds serve as carriers, facilitating the transfer of microorganisms from parent plants to their offspring [1–6]. As pioneer colonizers, seed bacteria likely impact microbiome assembly and function in subsequent generations [7]. However, the distribution, origination, and transmission dynamics of rice seed-associated bacteria are not well-studied, especially their quantitative profiles.

The seeds of some gramineous plants, such as rice and wheat, are covered by a pericarp that is closely connected and inseparable from the seed coat. This unique fruit structure of gramineous plant seeds is termed the caryopsis [8, 9]. The caryopsis is covered by two glumes, the lemma and palea, which are connected by rachilla. Below the glumes are two smaller glumes, the 1st and 2nd sterile lemmas. These accessory structures, along with the caryopsis, make up the rice grain [10, 11]. In agricultural production, rice crops are cultivated by planting rice grains instead of caryopses, making these accessory structures

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a potential bacterial reservoir for the next generation of plants [12].

Bacteria have been identified to reside in various compartments of seeds, such as the seed coat, embryo, endosperm, and perisperm [13, 14]. 16S ribosomal RNA gene sequencing of rice seed compartments has shown that the outer surface of husks possesses higher richness and diversity of bacterial communities than the caryopsis [15]. Studies isolating fungi, bacteria, and yeasts from the lemma and palea of wheat and barley reveal a higher abundance of filamentous fungi in these structures compared to the caryopsis [16]. Bacterial seed-to-seedling transmission has been studied by allowing seedlings to grow in axenic culture, where seedlings may harbor microbial communities originating solely from seeds. A recent study, which involved planting hulled and unhulled rice seeds under axenic conditions, revealed that the seed coat acts as a microbial niche, limiting the taxonomic composition and diversity of bacterial communities in seeds and seedlings [5]. Currently, our understanding of bacterial abundance, origination, and transmission associated with seed accessory structures remains limited.

There are three proposed routes for the source and transmission of seed bacteriome [4]: 1) the external route (horizontal transmission), where bacteria acquired from the environment colonize on and/or within seeds; 2) the internal route (vertical transmission), where endophytic bacteria enter seeds via the xylem or nonvascular tissue; 3) the floral route, where both environmentally-acquired and endophytic bacteria colonize on and/or within seeds via the flower. These routes, however, have largely yet to be experimentally validated. Seed-to-seed transmission of bacterial communities across generations has been reported in *Setaria viridis*, *Crotalaria pumila*, rice, radish and tomato [5, 17–20]. These studies compared community dissimilarity between the harvested/sown parent seeds and the progeny seeds, suggesting vertical transmission of seed-borne microbes across two or three generations. For example, previous studies on the longitudinal transmission of the bacterial community from seed to seed in rice sequenced the microbiota composition of developing and developed rice seeds in two consecutive years [5, 6]. The authors found that the bacterial compositions of progeny seeds were similar to those of parent seeds, identifying the parental seeds and stem endosphere as major sources of progeny seed microbial communities. However, microbiota overlap alone does not prove transmission [4]. To confirm which route primarily contributes to the establishment of seed-borne bacteria, methods to differentiate these routes are needed. Tracking studies using fluorescent-labeled bacteria offer one of the most precise methods for determining transmission routes [21, 22]. For example, a constitutively eGFP-expressing bacterial pathogen, *Clavibacter michiganensis* subsp. *michiganensis* GCMM-22, was used to demonstrate that bacteria could access tomato seeds through two routes: an internal route via the xylem and an externally route via tomato fruit lesions [21]. Moreover, further quantification of microbiota population sizes may enhance our understanding of how these vertically transmitted bacteria assemble and contribute to the plant microbiota.

In comparison to vegetative organs, seeds generally harbor bacterial communities with less diversity [2, 23]. Proteobacteria is the dominant phylum found in a variety of plant seeds, with common genera including *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Micrococcus*, *Pantoea*, *Enterobacter*, *Stenotrophomonas*, *Xanthomonas*, *Cellulomonas,* and *Acinetobacter* [24, 25]. The genera *Pantoea*, *Methylobacterium*, *Sphingomonas*, and *Pseudomonas* were identified as the main members of rice seeds and can stably exist in both developing and mature seeds [5, 6].

Many bacteria residing in seeds are challenging to cultivate and may be inactive or dormant [4, 25], making detection methods at the nucleic acid level, that are independent of culture, more effective in evaluating the structure and population size of the microbiota [26, 27]. However, bacterial 16S rRNA gene sequencing has a limitation: it co-amplifies plant organellar DNAs, especially in plant tissues that contain limited numbers of bacteria. Recently, several improved methods have been developed to specifically amplify bacteria-specific 16S rRNA gene sequences from plant whole genomic DNAs [28–30], enhancing the use of 16S rRNA gene sequencing in deciphering seed bacterial communities.

In this study, we found that rice seeds harbor abundant bacteria within their accessory structures, while caryopses themselves are almost bacteria-free. Rice seeds primarily acquire bacteria through external routes during panicle heading and flowering, and transfer these bacteria to different organs of the seedlings upon germination. In contrast, bacteria originating solely from internal routes are unable to establish a diverse bacterial community in the corresponding seedlings. These novel insights into seed-borne bacteria will promote the development of next-generation breeding strategies from the perspective of engineering the seed microbiome.

Results

Abundant seed-associated bacteria concentrated between caryopsis and glumes in rice grain

Rice cultivation involves planting the whole grain rather than just the caryopsis. Consequently, we investigated the bacterial community within the entire rice grain

(referred to as 'internal grain') and compared it to the community within the isolated caryopsis (referred to as 'internal caryopsis'). We quantified bacterial abundance in rice seeds from different cultivars and planting areas using quantitative PCR (q-PCR). Q-PCR revealed that internal grains harbored an average bacterial abundance of 1.88×10^4, while the internal caryopsis harbored undetectable levels of bacteria (Fig. 1A). This trend of higher bacterial abundance in the internal grain compared to the internal caryopsis was consistent across different locations and cultivars (Fig. 1B).

To further illustrate the distribution of bacteria in rice seeds, we quantified bacterial abundance in different grain compartments using Nipponbare seeds harvested six weeks later. Aligning with the above results, internal grain contained an average of 9.86×10^3 bacteria, while most caryopses had undetectable levels (Fig. 1C). Removing the caryopsis from the internal grain yielded the remaining seed accessory structures, which contained a bacterial abundance of 6.61×10^3, comparable to the level in the internal grain (Fig. 1C). Further separation and measurement within the accessory structures revealed an average abundance of 4.68×10^2 bacteria (Fig. 1C). These results indicated that most bacteria reside in the region between the caryopsis and glumes (Fig. 1D). Culture-dependent methods confirmed the presence of culturable bacteria in this niche (Fig. S1).

16S rRNA gene sequencing was utilized to analyze the bacterial community structure within the internal grain. Grains harvested six weeks later were used for this analysis. At the phylum level, Proteobacteria predominated the community (99.6%, Fig. 1E). At the genus level, the dominant members included *Pantoea* (66.4%) and *Pseudomonas* (30%), followed by *Sphingomonas* (2%) and *Aureimonas* (0.6%) (Fig. 1F). These findings align with previous characterizations of seed-associated bacteria [6, 31]. The observed Ace index at the operational taxonomic units (OTU) level was under 30 (Fig. 1G), significantly lower than reported for rice leaves and roots [29, 30, 32], indicating relatively low species richness in the rice seed microbiome.

Collectively, our findings demonstrate an abundance of bacteria carried by rice grains, emphasizing the crucial role of the entire grain, including both the caryopsis and seed accessory structures, in housing the seed-borne bacterial community. Notably, the caryopsis itself contains minimal bacteria, underscoring the importance of the structures surrounding the caryopsis in nurturing this vital bacterial community.

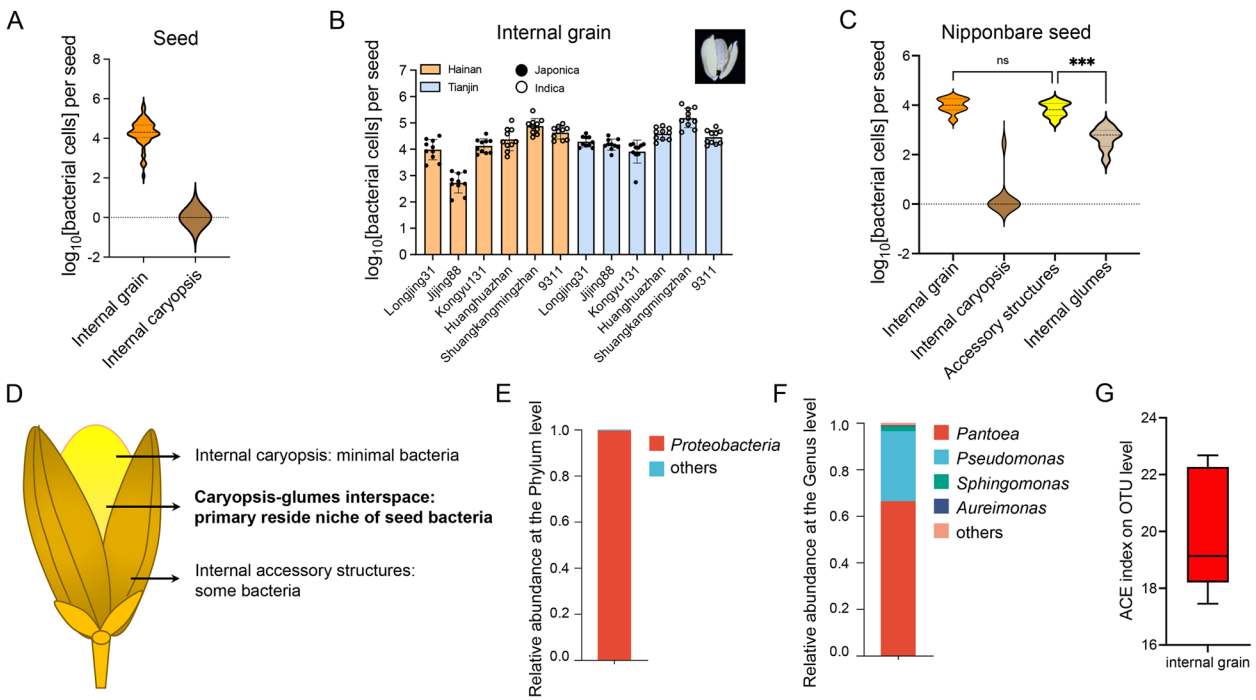

Fig. 1 Composition and distribution of bacteria in rice grain. **A-D** qPCR showing bacterial abundance in different niches of rice grains. Means and SDs in (**A**) were calculated from 120 seed samples harvested from 6 cultivars planted in 2 areas, and in (**B**-**D**) were calculated from 10 seed samples each. ns, not significant; ***, $p < 0.001$. **E** Schematic diagram showing bacterial distribution in different niches of rice grains. **F-G** Relative abundance of bacterial taxa in internal grain at the phylum (**F**) or genus (**G**) levels. Taxa with > 0.1% relative abundance are shown. **H** Bacterial community richness shown by the ACE analysis

Seed-borne bacterial community remains stable during development

After confirming the bacterial community carried by mature rice seeds during storage, we further investigated the abundance and composition of the seed-associated bacterial community throughout seed development. Grains were examined at 0, 7, 15, 24, and 40 days after pollination (DAP) representing flowering to physiological maturity. Q-PCR revealed consistent bacterial abundance within internal grains across developmental stages, reaching a peak of 7.34×10^5 cells per seed at physiological maturity (Fig. 2A). Conversely, internal caryopses exhibited negligible bacteria throughout development (Fig. 2A).

16S rRNA gene sequencing analysis revealed remarkable similarity in taxonomic compositions across stages. Proteobacteria dominated (> 90%), followed by Firmicutes and Actinobacteria (Fig. 2B). Community size standardized to 1.00 at 0 DAP showed coefficients of 1.22, 3.38, 3.02, and 7.48 at 7, 15, 24, and 40 DAP, respectively (Fig. 2C). The dominant genera, including *Pantoea*, *Pseudomonas,* and *Sphingomonas*, remained prevalent throughout seed development, collectively comprising over two-thirds of the bacterial community. The remaining third was made up of genera such as *Agrobacterium*, *Xanthomonas*, *Methylobacterium*, and *Aureimonas*. Notably, the abundance of *Pantoea* increased dramatically, exceeding 50% by the physiological maturity stage (Fig. 2C). Venn analysis showed that 78% of OTUs were shared among all stages, with no unique OTUs (Fig. 2D), indicating minimal developmental impact on bacterial taxa. The bacterial taxa composition and the quantities throughout development are similar to storage-stage seeds (Fig. 1F-G). Ace index at OTU level remained low throughout (Fig. 2E), consistent with the storage-stage seeds (Fig. 1H).

These findings demonstrate that the seed-borne bacterial community maintains composition and abundance throughout development and storage, with minor fluctuations in population size across stages.

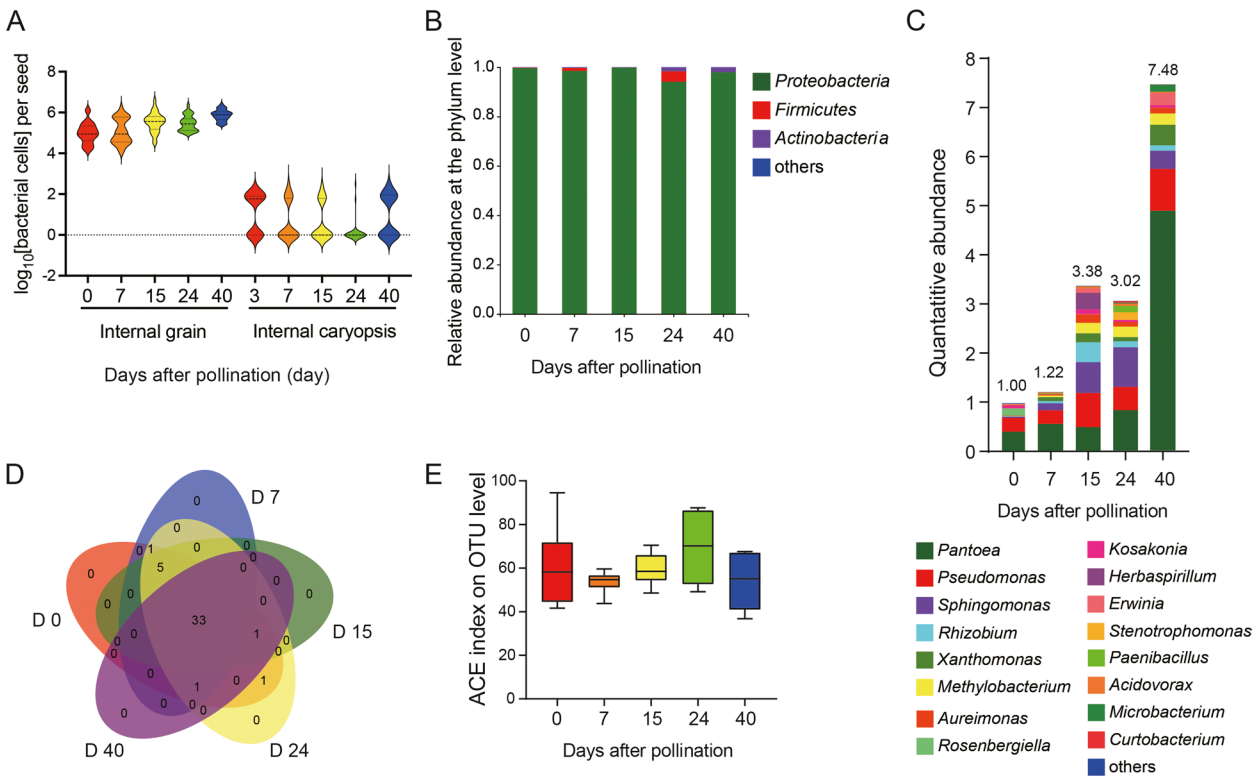

Fig. 2 Composition of seed-borne bacterial community during seed development. **A** qPCR showing bacterial abundance in different niches of developing rice seeds. Data were calculated from 30 seed samples. **B** Relative abundance of bacterial taxa in internal grain at the phylum level; taxa with > 0.1% relative abundance is shown. **C** Quantitative abundance of bacterial taxa in internal grains at the genus level; taxa with > 0.1% relative abundance is shown. Abundance coefficients (1.00, 1.35, 3.31, 3.16, 7.41) were calculated from qPCR results in Fig. 2A. **D** Venn diagrams at the OTU level. **E** Bacterial species richness shown by the ACE analysis

Seed bacteria predominantly originate from the external environment during panicle heading and flowering

To determine the primary sources of rice seed-borne bacteria, we established a control condition where seeds from greenhouse-grown plants were not inoculated with bacteria. In this scenario, rice seeds at the panicle heading and flowering stages had minimal to negative bacteria on their surface (Fig. 3A). Subsequent greenhouse cultivation revealed very low internal grain bacterial levels (~100 cells/seed) across development stages (Greenhouse, Fig. 3B). These results provide evidence: if internal route exists for rice seeds to acquire bacteria, it plays a minor role in shaping the abundant bacterial community typically found within mature seeds.

We also examined the bacterial abundance in seeds at the panicle heading and flowering stages harvested from paddy field-grown plants. SEM observation revealed high abundances of bacteria residing on their surface (Fig. 3C). Subsequent cultivation in the paddy field revealed high levels of bacterial colonization in the internal grains (Paddy field, Fig. 3B). These results strongly support the external environment as a major source for rice seed-borne microbiota.

To further rule out the internal route's role in the paddy field cultivation conditions, rice plants were transplanted from the paddy field to a greenhouse before the booting stage and grown until seed harvest. No bacteria were observed on the exterior surface of the seeds in the transplanted plants when they grew into the flowering stage (Fig. 3D). Seeds harvested from the transplanted plants also exhibited significantly lower bacterial levels than those grown in the paddy field (Fig. 3B), further supporting the link between abundant seed-borne microbiota and bacterial acquisition from the external environment. Notably, the consistent presence of abundant bacterial communities in seeds grown under natural field conditions suggests that specific environmental factors in the field play a crucial role in bacterial acquisition. However,

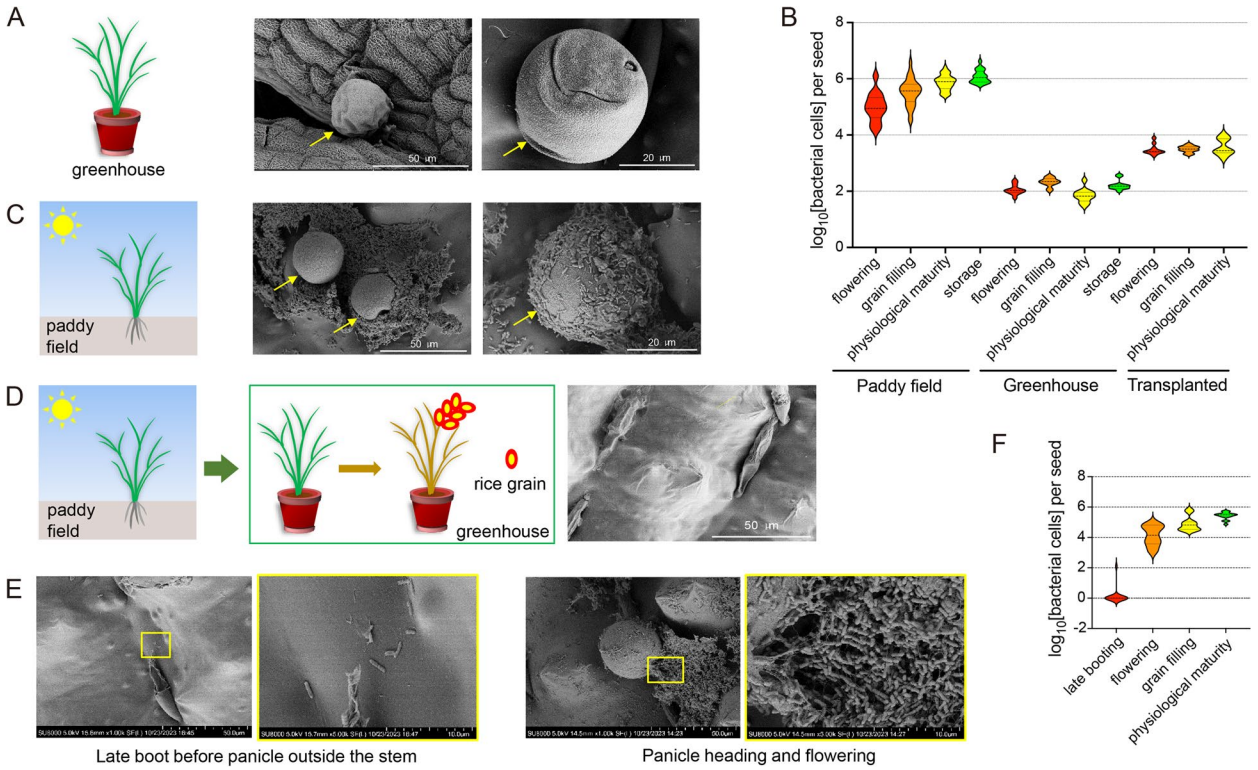

Fig. 3 Seed bacteria primarily originate through external routes during panicle heading and flowering. **A** Schematic diagram of rice cultivation under greenhouse conditions and SEM images showing absence of bacteria on panicle surfaces during heading and flowering stages. The yellow arrow indicates pollen. **B** qPCR analysis demonstrating varying bacterial abundance in internal grains during seed development under different cultivation conditions: greenhouse, paddy field, and plants transplanted from paddy field to greenhouse. **C** Schematic diagram of rice plant cultivation in paddy field, with SEM images revealing abundant bacteria on panicle surfaces during heading and flowering stages. The yellow arrow indicates pollen. **D** Schematic diagram of rice plants transplanted from paddy field to greenhouse before booting stage and cultivated until harvest. SEM images show absence of bacteria on panicle surfaces during heading and flowering stages. **E** SEM images illustrating that field-grown plants had minimal panicle surface bacteria at late boot stage (before panicle emergence), but abundant bacteria during heading and flowering. **F** qPCR analysis showing bacterial abundance in internal grains during seed development in paddy field. Note for B &F. Data were calculated from 12 seed samples per stage

it remains unclear why the transplanted plants harbored seed bacterial abundance tenfold higher than the greenhouse-growing plants.

We then used SEM to detect bacteria on the surface of the panicles during the late booting stage, just before the panicle becomes visible outside the stem. There are very few bacteria residing on the panicle surface during this stage (Fig. 3E). However, bacterial colonization increased significantly during the panicle heading and flowering stages (Fig. 3E). Q-PCR analysis corroborated these findings (Fig. 3F), indicating that seeds primarily acquire bacteria during the panicle heading to flowering stages.

To definitively identify the stages at which bacteria colonize the seeds, we created a synthetic bacterial community (SynCom) composed of six dominant seed-borne bacteria isolated from paddy field grains. The SynCom included three strains of the genus *Pantoea* and one strain each from *Pseudomonas*, *Sphingomonas,* and *Agrobacterium*. This SynCom reflected the major taxa found in grain bacterial communities (Fig. 2). The six strains were cultured to log phase and mixed in equal amounts at 10^8–10^9 CFU/ml. Under greenhouse conditions, this SynCom was then sprayed onto rice panicles at three different development stages: flowering, grain filling, and physiological maturity (Fig. 4A).

SynCom inoculation at the flowering stage yielded abundant bacterial colonization throughout seed development, with levels reaching 10^6–10^7 bacteria per seed at physiological maturity (Fig. 4B). However, SynCom application during grain filling or physiological maturity stages resulted in only a temporary increase in bacterial abundance in 3 days post-inoculation. Following this period, bacterial levels remained low (around 10^2 bacteria per seed) (Fig. 4B). These results further demonstrate that inoculation no later than the flowering stage is necessary for rice seeds to acquire bacteria from the external environments and establish their bacterial communities.

Seed-borne bacteria establish abundant communities in the seedlings

To investigate the transmission of seed-borne bacteria to seedlings, we designed an axenic cultivation system for rice plants (Fig. 5A). Both caryopses and grains were surface-sterilized and grown for 10 days, followed by bacterial abundance quantification using qPCR. Seedlings grown from caryopses harbored a small number of bacteria [(0–10^4 bacteria per gram fresh weight (g^{-1} FW)] (Fig. 5B). In stark contrast, seedlings grown from grains exhibited significantly higher bacterial levels, reaching 10^6–10^7 g^{-1} FW in shoots and 10^7–10^8 g^{-1} FW in roots (Fig. 5B). These findings demonstrate the successful transmission of bacteria from the internal grain to the next generation seedling. The bacteria, if any in the internal caryopsis, cannot establish abundant bacterial communities in either the shoot or the root.

To further verify this transmission, a grain microbiota transplantation experiment was conducted. Surface-sterilized caryopses and grains were planted alternately in the axenic system. The results revealed a marked increase in the bacterial abundance of caryopses-derived seedlings when planted adjacent to grains. The bacterial population in the shoot reached 10^6–10^7 bacteria g^{-1} FW, while roots harbored around 10^8 bacteria g^{-1} FW, indicating the successful acquisition of bacteria from adjacent grains (Fig. 5B). This further confirms that the

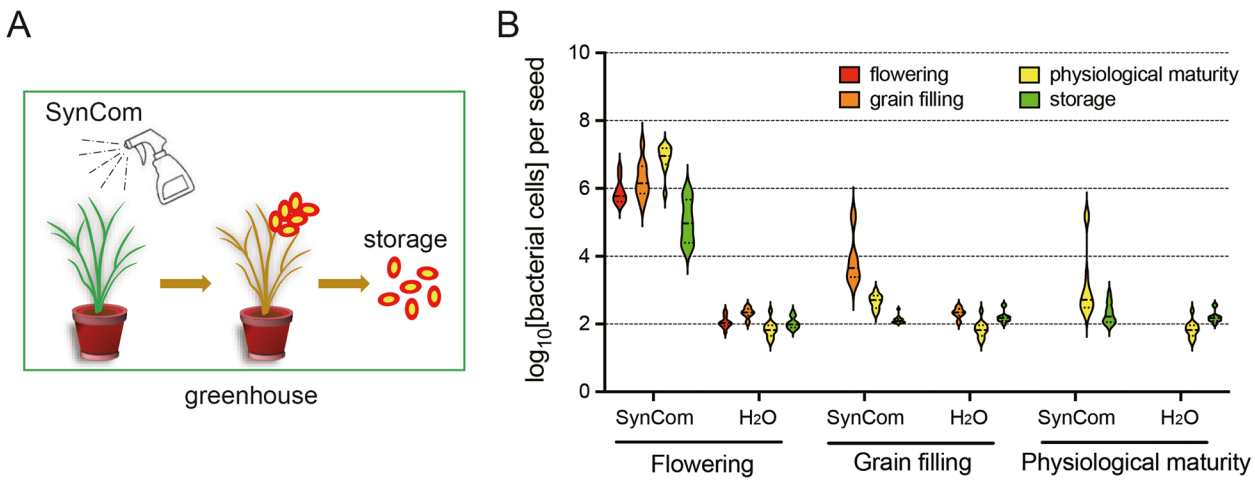

Fig. 4 SynCom colonizes rice seeds during panicle heading and flowering. **A** Schematic of SynCom inoculation at different seed development stages in the greenhouse. **B** qPCR showing impact of SynCom inoculation to the internal grain bacterial abundance during development. Data were calculated from 12 seed samples

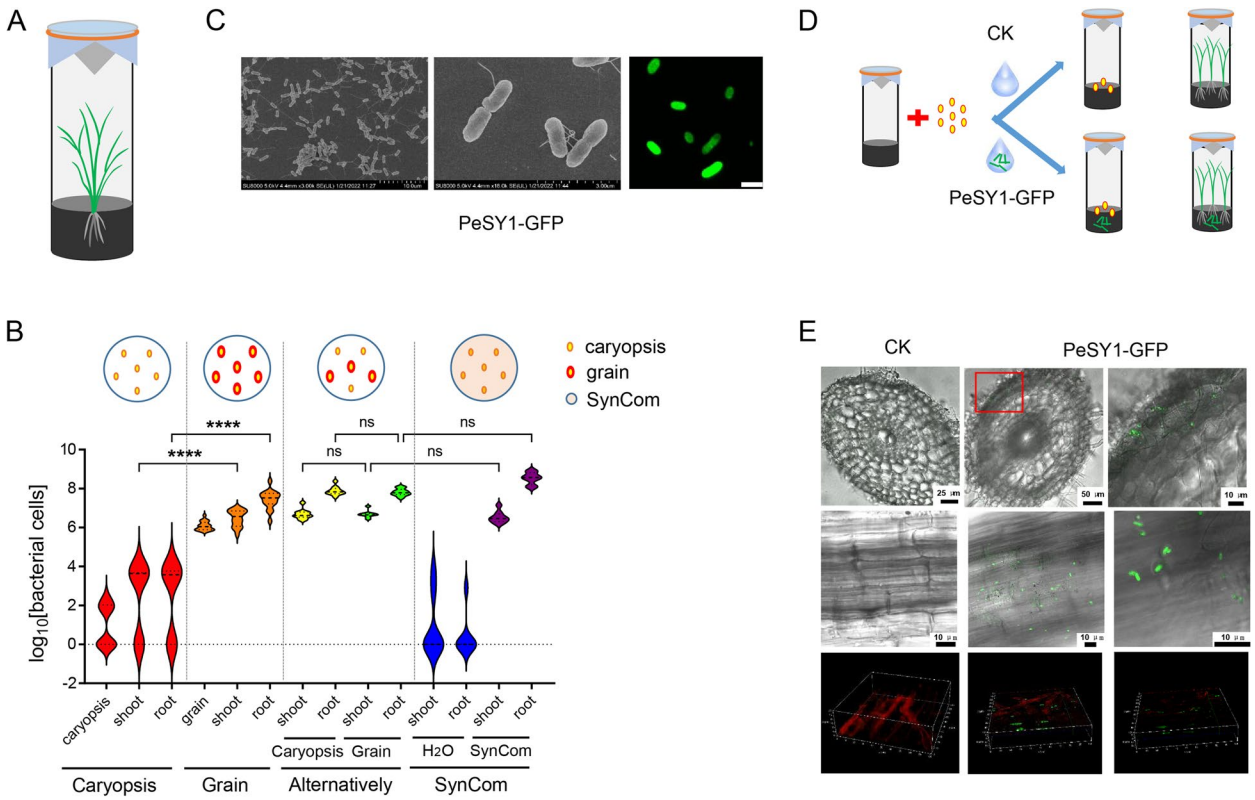

Fig. 5 Seed-borne bacteria spread to next-generation seedlings upon germination. **A** The axenic system for rice cultivation. The entire device was autoclaved, and the rice caryopses and grains planted inside were surface-sterilized. **B** qPCR showing bacterial abundance in internal caryopsis (caryopsis), internal grain (grain), endosphere of seedling shoot (shoot) or root (root). Seedlings germinated from grain or caryopsis cultivated with grain or SynCom contain abundant bacteria within shoots and roots, while seedlings from sterilized caryopsis contain few bacteria. The circles indicate planting methods: surface-sterilized caryopsis on axenic medium (left), surface-sterilized grain on axenic medium (middle), alternate planting of caryopsis and grain on axenic medium (middle), and surface-sterilized caryopsis on SynCom-containing medium (right). Bacterial abundance in caryopses and grains calculated with $\log_{10}$[bacterial cells]/seed, in seedlings with $\log_{10}$[bacterial cells]/g fresh weight. ns, not significant; ****, $p < 0.0001$. Data from 12 seed samples. **C** SEM or confocal laser scanning microscope (CLSM) showing GFP-labeled *Pantoea eucrina* SY1 (PeSY1-GFP). Scale bar, 3 μm. **D** Inoculation schema of PeSY1-GFP into rice plants under axenic conditions. **E** PeSY1-GFP colonization in longitudinal and cross-sections of seedling roots. Images examined at 14 days after planting using a Leica TCS SP8 confocal microscope

seed-borne bacteria can be transmitted to the seedlings from the internal grains via the external environments.

Furthermore, surface-sterilized caryopses were planted in the axenic system. Prior to planting, 40 mL of the SynCom suspension (10^6 CFU/mL) was added to the axenic cultivation system containing approximately 50 mL of soil. Ten days after planting, qPCR analysis revealed that the bacterial abundance in seedling shoots and roots is at levels comparable to those found in germinated grains (Fig. 5B). These findings indicate that externally inoculated seed bacteria can be successfully acquired by rice seeds to establish a robust bacterial community.

Finally, to visualize the spread of bacteria from seeds to seedlings, we introduced an *eGFP* gene into one of the seed-borne bacterial strains, *Pantoea eucrina* SY1 (Fig. 5C). This strain was designated PeSY1-GFP (Fig. 5C). We utilized SEM and confocal laser scanning microscope (CLSM) to observe the distribution of PeSY1-GFP in seedlings. The strain was inoculated into the soil of the axenic system, and surface-sterilized caryopses were planted (Fig. 5D). SEM confirmed the absence of bacteria on the surface of shoots and roots of the seedlings cultured in the axenic system, verifying bacteria-free conditions (Fig. S2). In contrast, PeSY1-GFP inoculation resulted in bacterial colonization on the surface of both seedling shoots and roots (Fig. S2). Notably, CLSM revealed the presence of PeSY1-GFP not only on the root surface but also within the internal spaces (Fig. 5E).

Bacterial communities adapt and diversify from seed to seedling

16S rRNA gene sequencing was performed to investigate the dynamic of bacterial communities in rice grains, seedling shoots, and seedling roots. Venn analysis revealed that 19 out of 20 OTUs from rice grains successfully

colonized the seedling shoots or roots (Fig. 6A), indicating most seed-borne bacteria successfully established themselves in the seedlings. While overall taxonomic composition remained similar, the relative abundance of specific genera displayed remarkable changes. Notably, dominant grain bacteria like *Pantoea* and *Pseudomonas* declined significantly in seedlings, while *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Stenotrophomonas*, *Sphingomonas*, *Acidovorax*, and *Aureimonas* emerged as the major members (Fig. 6B, Fig. S3A). Ace index values indicated that community richness was unchanged from seeds to seedlings (Fig. 6C). However, Shannon index values revealed that seed microbiome diversity was significantly lower than in seedling organs (Fig. 6D). This denotes that although community richness was maintained from seeds to seedlings, community diversity increased markedly. PCoA based on Bray–Curtis distances further confirmed significant divergence in community structure between the three compartments (Fig. 6E and S3B-G). Here, PC1 captured the highest variance between grains and seedlings, while PC2 reflected the distinction between seedling shoots and roots.

To gain deeper insights into functional shifts, the BugBase algorithm predicted high-level phenotypes in the different microbiome samples. Remarkably, 6 phenotypes were significantly different between the grain and seedling microbiomes. The grain microbiome exhibited a higher proportion of stress-tolerant, biofilm-forming, potentially pathogenic, facultatively anaerobic, and mobile elements-containing bacteria compared to the seedling microbiome, which showed a higher proportion of Gram-negative and aerobic bacteria (Fig. 6F and S4).

Since most seedling bacteria originate from seeds (Fig. 6A), we further compared bacterial profiles between rice seeds and leaves throughout seed developmental stages: flowering, grain filling, and maturity. Under paddy field conditions, leaves and seeds acquire their microbiota through different routes. 16S rRNA gene sequencing revealed distinct bacterial compositions between seeds and leaves at each stage (Fig. S5A-D). Both Ace and Shannon indices were significantly different between seeds and leaves at each stage, denoting divergent community richness and diversity (Fig. 6G-H). Moreover, 30% of leaf bacteria genera were not shared with seeds (Fig. 6I). This represents substantial differences in taxa between plant leaves and developing seeds.

Comparing phenotype differences during seed development, we found seeds throughout their development harbored a higher proportion of stress-tolerant, biofilm-forming, potentially pathogenic, facultatively anaerobic, and mobile element-containing bacteria, while leaves consistently harbored more Gram-negative, aerobic, and Gram-positive bacteria (Fig. 6J and S5E-F). These results largely mirror those obtained from the seeds vs. seedlings comparison (Fig. 6F and J).

These findings collectively demonstrate significant reshaping and diversification of bacterial community composition and function during the seed-to-seedling transition. This dynamic adaptation likely reflects the bacterial response to changing environmental conditions faced within the developing plant.

Discussion and conclusion

This study elucidates the distribution and composition of bacterial communities associated with rice seeds, and reveals the dynamics of their acquisition, maintenance, and transmission between generations (Fig. 7). We clarified that while rice grain contains abundant bacteria residing in the interspace between the caryopsis and glumes, the caryopsis itself is not the main reservoir. Therefore, the entire grain structure is essential for harboring the seed-borne bacterial community. Grains mainly acquire bacteria during panicle heading and flowering. The bacterial taxonomic composition remains relatively stable throughout seed development and storage, while population sizes vary by several folds. Upon germination, these seed-borne bacteria can spread into different organs of the newly formed seedlings. Depending on the shoot or root environments, the bacterial community structures are reshaped.

It is not conclusively confirmed whether all rice caryopses contain endobacteria. However, it is clear that even if present, these endobacteria are unable to form abundant bacterial communities in germinated seedlings. Regarding the relationship between seed-borne bacteria and seedling bacterial communities, previous

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Fig. 6 16S rRNA gene sequencing to decipher bacterial community composition and structure from seeds to seedlings. **A** Venn diagrams showing shared OTUs among seed and seedling organ microbiomes. **B** Relative abundance of bacterial taxa at genus level; taxa > 0.1% shown. **C** and **G** Bacterial community richness shown by the ACE analysis. **D** and **H** Bacterial community diversity shown by the ACE analysis. **E** PCoA of bacterial communities in rice grains and offspring seedlings. Significant difference by ANOSIM. **F** BugBase prediction of microbiome phenotypes comparing seeds and seedling organs. **I** Venn diagrams showing shared genera among seed and leaf microbiomes during development. Sequenced seed microbiome includes 5 stages with 5 samples per stage, leaf microbiome includes 4 stages with 5 samples per stage. **J** BugBase-predicted different microbiome phenotypes among grain and leaf samples during development by Kruskal–Wallis H test. ***, $p < 0.001$. SF and LF, seeds and leaves at flowering; S7d and L7d, S15d and L15d, S24d and L24d, and S40d, seeds and leaves at 7, 15, 24, and 40 days after pollination

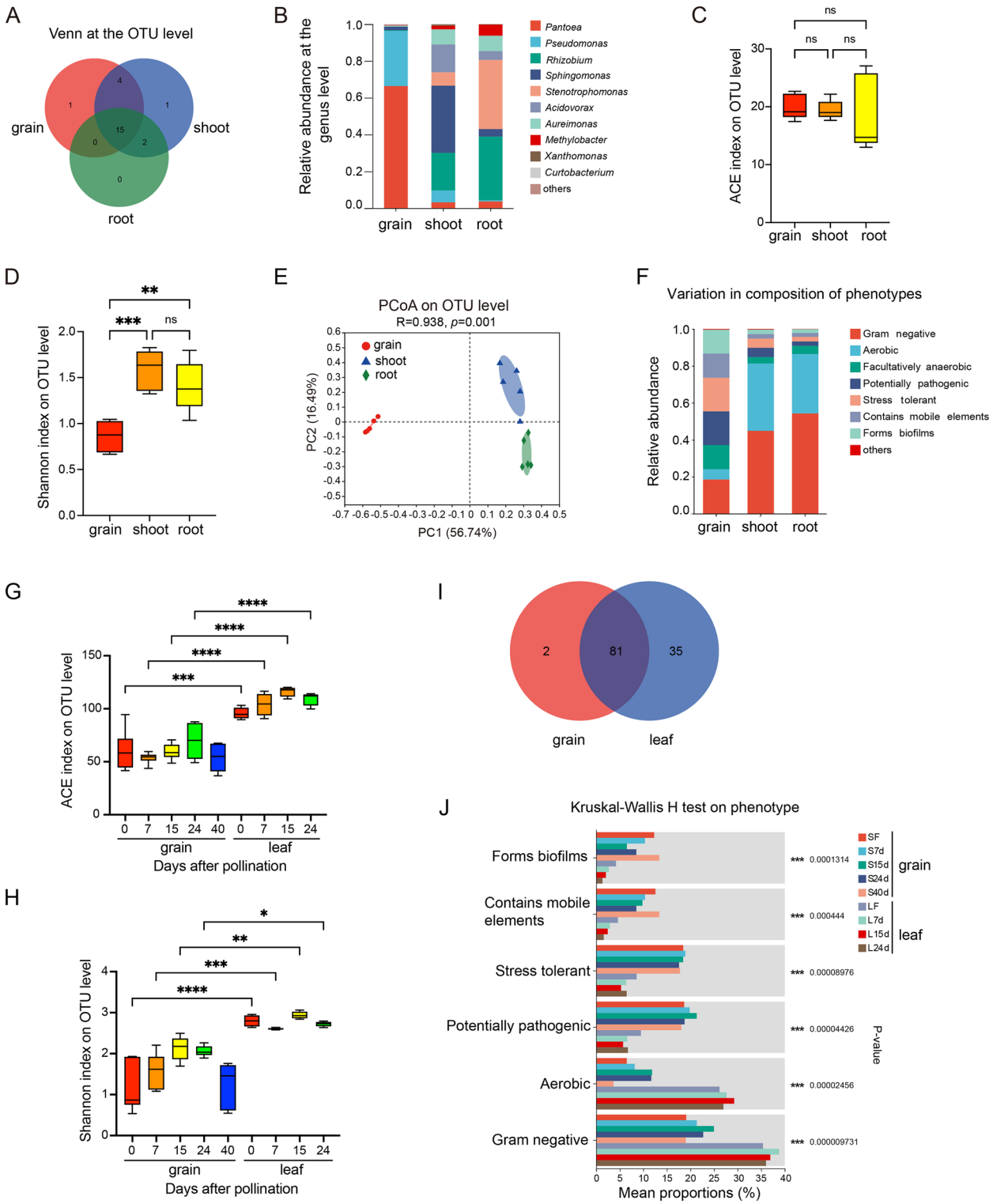

Fig. 6 (See legend on previous page.)

studies have shown some differing results. For example, surface-sterilized wheat seeds did not yield any colonies, yet colonies were recovered from seedlings derived from surface-sterilized seeds grown under axenic conditions. Five days of growth increased the endophyte densities to a range between 8.0×10^3 and 1.6×10^5 CFU g^{-1} FW

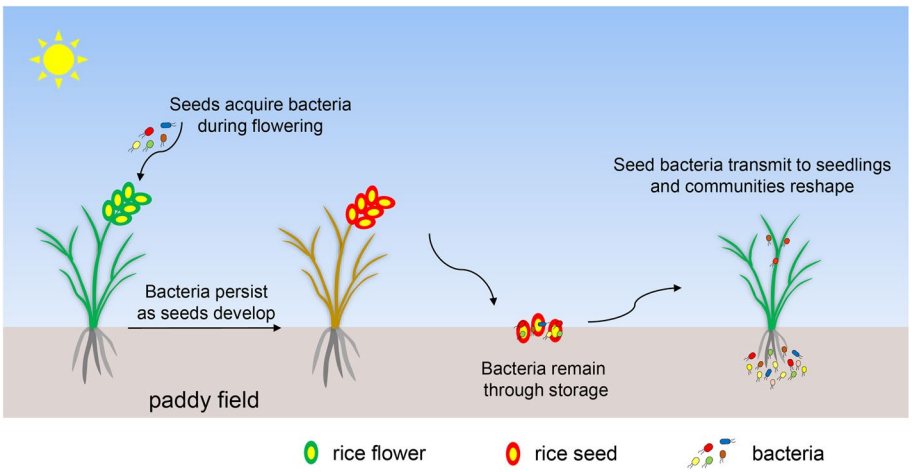

Fig. 7 Predicted model for acquisition of seed-borne bacteria during panicle heading and flowering, persistence through seed development and storage, and spread to seedlings reshaping communities in different organs

[33]. The endophytes from maize kernels increased to 10^1–10^2 CFU g^{-1} FW and 10^5–10^8 CFU g^{-1} FW in day-2 and day-7 seedlings, respectively, indicating significant amplification of seed-associated bacteria during plant development [34].

Multiple lines of evidence confirm the external origin of seed-borne bacteria and demonstrate how the planting environment and timing of bacterial inoculation are critical factors for the acquisition and establishment of seed-borne bacterial communities. 1) External origin: Under greenhouse conditions where seeds are not inoculated with bacteria, seeds remain essentially bacteria-free (Fig. 3A-B). This indicates that potential internal-transmitted bacteria are incapable of establishing significant seed-borne communities. This finding strongly suggests the external environment as the primary source of seed-borne bacteria. 2) Inoculation during panicle heading and flowering: Spraying the SynCom onto panicles at the flowering stage results in successful bacterial acquisition and establishment of robust seed-borne communities (Fig. 4). This directly demonstrates the efficacy of external inoculation in shaping the seed-borne microbiome. 3) Field-grown seeds: Seeds grown under natural field conditions consistently harbor abundant bacterial communities (Figs. 1, 2, 3 and 4), suggesting that specific environmental factors present in the field are crucial for bacterial acquisition. 4) Limited inoculation window: SynCom inoculation after flowering fails to establish significant seed-borne microbiota (Fig. 4B). This indicates a specific developmental window of opportunity exists for successful bacterial colonization, emphasizing the importance of timing external inputs.

It is interesting to investigate whether seeds contain core bacterial taxa and whether these pioneer taxa drive priority effects influencing the assembly of plant microbiota. In this study, through 16S rRNA gene sequencing analysis, we found rice grains at different developmental stages contained similar bacterial compositions, with the genera *Pantoea*, *Pseudomonas,* and *Sphingomonas* showing high relative abundance (Fig. 1G, 2C, and S5B). These results are consistent with previous studies [5, 6], suggesting rice plants may exert significant selection on the seed microbiota. In a previous study analyzing bacterial communities from 99 cultivated rice varieties by 16S rRNA gene sequencing, 15 generalist core taxa were detected to be shared among all samples collected from rice seeds, root endospheres, and rhizospheres. Seven out of these core taxa were annotated as *Pantoea*, *Pseudomonas,* and *Sphingomonas* [31]. These three genera have also been identified as core taxa in other plants' seed bacterial communities such as *Salvia miltiorrhiza*, maize, bean, *Triticum* spp., and *Brassica* spp. [35, 36]. Therefore, although the seed microbiota composition is affected by plant species, genotype and seed development stage [4], some core taxa at low taxonomic levels appear to exist across seed microbiota of various plants.

As a niche-specific microhabitat with less freely available nutrition and high osmotic pressure [25], seeds can impose strong selective pressures on their microbial inhabitants [4]. Studies have also revealed certain shared characteristics among endophytic bacteria residing in seeds, including tolerance to high osmotic pressure, endospore formation capabilities, and amylase activity – traits not typically found in other plant microhabitats [25]. BugBase predicted 6 significantly different organism-level microbiome phenotypes between seeds and seedlings (Fig. 6 and S4-S5), representing environment-dependent spread of bacteria from seeds to seedlings.

Moreover, BugBase predicted all 7 phenotypes as significantly different between developing seeds and leaves, with difference patterns largely mirroring the seed vs. seedling comparison (Fig. 6 and S4-S5). This further supports tight linkage between microbiome phenotypes and tissue environments. Internal grains contained more facultatively anaerobic bacteria, while both seedlings and leaves contained more aerobic bacteria (Fig. 6 and S4-S5). This may be because seeds primarily depend on anaerobic respiration during development and storage, while leaves support more aerobic bacteria due to atmospheric oxygen exposure. Seedling roots also harbored more aerobic bacteria given the shallow soil depth during germination. The higher abundance of biofilm-forming bacteria residing on the caryopsis-glume interspace may facilitate colonization and protect bacteria from desiccation and harsh conditions. The functions of these differential phenotypes warrant further investigation.

The extent to which the seed microbiota contributes to the plant microbiota community composition has been less investigated. Most studies have found that the seed microbiota contributes to root endosphere community composition, but the covariation between the seed and endosphere communities tends to be relatively weak [31]; notably, most seed-borne bacterial taxa are absent from root samples [4, 26, 37]. One hypothesis is that during seed germination, small population sizes and abundant inactive or dormant bacteria struggle to adapt to the rapidly changing habitat, fail to efficiently colonize new niches, and thus minimally impact the plant microbiota assembly [4, 38]. Finding that rice leaves in the reproductive phase harbor microbiome phenotypes similar to those of seedlings germinated from surface-sterilized grains under axenic cultivation lends some support for plant tissue as a determinant of microbiome phenotypes. Using an axenic cultivation system whereby microbiota originate solely from planted seeds, we demonstrated that seed-borne bacteria can spread to various seedling tissues, though with dramatically altered relative abundances.

Conclusions

Using rice seeds as a model system, we have elucidated the major niches of the seed bacterial community, revealed the dynamics of community acquisition, maintenance, and transmission between generations, and demonstrated dramatic reshaping of the community in seedling tissues. To our knowledge, this is the first study to establish an axenic cultivation system to quantify bacterial dispersal and transmission in plant seeds. These novel findings substantially improve our understanding of microbial community dynamics in plant ecosystems. Further investigation of beneficial microorganisms in crops will enable optimized management practices to promote plant health, an essential step toward improving agricultural sustainability.

Materials and methods

Rice seeds and cultivation

The seeds of six rice varieties—three japonica (Longjing 31, Jijing 88, and Kongyu 131) and three indica (Huanghuazhan, Shuangkangmingzhan, and 9311)—were harvested from paddy fields in Lingshui, Hainan and Wuqing, Tianjin. Additionally, Nipponbare seeds were harvested from Shunyi, Beijing. These harvested seeds were all kept in the dark and stored in dry conditions until their DNA was extracted. The Nipponbare seeds planted in Shunyi rice paddy fields were collected at 0, 7, 15, 24, and 40 days after pollination (DAP) and stored at -80 °C immediately after collection until DNA extraction. The 40-DAP seeds were mature, harvested and stored in the dark under dry conditions until DNA extraction. All these rice seeds are listed in Supplemental Table 1.

The rice plants in the field were planted in paddy fields, including Lingshui, Wuqing and Shunyi, with over 10 years rice planting history. The greenhouse for rice planting is a room with a glass roof. Rice is planted in cement ponds with a dimension of 2 m length, 1.5 m width, and 1 m height. The light source is sunlight, and the greenhouse is naturally ventilated by skylights. Rice plants were watered with tap water through a water pipe directly into the cement pond. Rice plants transplanted from paddy field to the greenhouse were grown in Shunyi, Beijing for the entire vegetative growth stage, then were transplanted to the greenhouse right before the booting stage and finished subsequent productive development until seed maturity in the greenhouse.

Surface sterilization of rice seeds and DNA extraction from plant tissues

Rice grains, caryopses, and the seed accessory structures were placed into a 50-ml Falcon tube, washed in 70% ethanol for 1 min, and then washed in 1.5% sodium hypochlorite (mixed in ddH_2O) on a shaking platform at 30 rpm for 40 min, and then washed four times in sterile ddH_2O. The ethanol, sodium hypochlorite, and sterile ddH_2O were all handled in a biological safety cabinet.

After surface sterilization, four rice grains, caryopses, or seed accessory structures were added to a 2.0 ml centrifuge tube and ground into a fine powder using a SPEX Sample Prep (Geno Grinder, USA). Separately, shoot and root samples of offspring seedlings were collected at two and three weeks after planting in the axenic system. The root samples were washed in sterile ddH_2O to remove any attached soil. Both the shoot and root samples were cut into pieces, put into 2.0 ml centrifuge tubes,

and ground to a fine powder using a SPEX Sample Prep. DNA was extracted according to a previously described method [30]. DNA was dissolved in ddH_2O and stored at -20 °C until further analysis.

Quantitative PCR to determine bacterial abundance in rice tissues

The primer pair 322F-A (5'-ACGGHCCARACTCCTAC GGAA-3') and 796R (5'-CTACCMGGGTATCTAATC CKG-3') were used to amplify the bacterial 16S rRNA gene from the plant's whole genomic DNA to determine the abundance of bacteria in rice tissues [30]. Based on the standard curve of bacterial abundance in rice tissues [30], quantitative PCR (qPCR) was performed to determine the absolute bacterial abundance of rice tissues, including shoots, roots, caryopses, grains, and seed accessory structures. SYBR-Green qPCR (25 µl) was performed according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

16S rRNA gene amplification

The primer set 322F-A/796R was used to amplify the bacterial 16S V_3-V_4 region [30]. PCR mixtures were made in a volume of 30 µl containing 200 µM dNTPs, 0.2 µM each primer, 2 U Platinum Taq DNA polymerase (Invitrogen, USA), and 1.5 mM $MgCl_2$. The concentration of the template DNA depended on the sample type, with 120 ng/30 µl for rice seed and shoot DNA and 30 ng/30 µl for root DNA. The following PCR conditions were used: initial denaturation at 94 °C for two min, followed by 34 (for seed and shoot) or 30 (for root) cycles consisting of denaturation (94 °C for 30 s), annealing (56 °C for 30 s), extension (72 °C for 30 s), and a final extension step at 72 °C for five min. Each sample was amplified in triplicate. The PCR products were run on a 1% agarose gel to ensure successful amplification. No visible amplification was observed from the negative control (no template added).

Illumina next generation sequencing

The primer barcode sequences were synthesized by Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). PCR products were purified and amplicon libraries were constructed at Majorbio. Paired-end (2 × 300 bp) sequencing was conducted using the Illumina MiSeq PE300 platform. The paired reads were merged into single sequences based on a 10 bp overlap. 16S rRNA gene sequences were processed in QIIME 1.9.1 (http://qiime.org/install/index.html). Sequences with quality scores < 20 were discarded [39]. Tags were merged with Fast Length Adjustment of Short Reads (v. 1.2.11) [40]. All samples had > 30,000 effective sequences. Effective tags were clustered into 97% identity operational taxonomic units (OTUs) using Usearch program v. 7.0.1090 [41]. All samples had coverage index > 0.97. Representative sequences for each OTU were selected by UPARSE [42]. OTUs aligned to chloroplast and mitochondrial sequences were removed during clustering. OTUs with ≥ 5 sequences in at least 3 samples were retained. Taxonomic classification of representative sequences was performed using the Ribosomal Database Project's classifier in QIIME with the default parameters [43].

Statistical analysis

All bioinformatics analyses of 16S rRNA gene sequencing were performed on the online platform Majorbio I-Sanger Cloud Platform (http://cloud.majorbio.com). To reveal the richness and diversity of the bacterial communities in different amplicon libraries, both ACE and Shannon indices were calculated using Mothur (https://mothur.org/wiki/calculators/) [44]. Student's *t-test* was performed to compare the values of the alpha diversity indices ($P < 0.05$). To visualize the beta diversity pattern of bacterial communities, principal coordinate analyses were conducted using the prcomp() function based on the Bray–Curtis distance algorithm [45]. Analysis of similarity (ANOSIM) was performed to test the significance of bacterial community dissimilarity. Venn diagrams at various taxonomic levels were generated using the lapply() function. Considering the different levels of sample difference in the microbial community size, each study contained 12 to 32 sample replicates, and each seed (caryopsis or grain) sample contains 3–4 caryopses or grains. To predict high-level phenotypes presenting in our microbiome samples, BugBase [46] was performed using the 16S rRNA sequencing data. BugBase analysis focused on seven common traits for most prokaryotic organisms, including Gram-negative and Gram-positive delineation, biofilm formation, pathogenic potential, presence of mobile elements, oxygen utilization, and stress tolerance. We applied the non-parametric Kruskal–Wallis test to compare the results from BugBase.

Scanning electron microscope (SEM)

After surface sterilization, the rice tissues were cut into several pieces under axenic conditions to expose the internal profile. For sample fixation, the tissue pieces were washed twice with ddH_2O, fixed in 4% paraformaldehyde at 4 °C for 3–7 days, and then washed with ddH_2O three times for 7, 8, and 9 min each. The fixed samples were dehydrated in a graded ethanol series (50%, 70%, 85%, 95%, and 100%; 16 min immersion at each concentration). Overall, 100% ethanol dehydration was repeated 3 times. After dehydration, the specimens were critical-point dried with liquid CO_2 (Leica EM CPD300;

Leica, Hanau, Germany) and sputter coated with gold–palladium (E-1045 ion sputter; Hitachi, Tokyo, Japan). The samples were observed under a Quanta200 scanning electron microscope (FEI, Hillsboro, OR, USA).

Axenic culture system and grain microbiota transplantation system

The flower nutrient soil and vermiculite are mixed well in a ratio of 1: 3. Mixed soil was put into a pot and the pot was sealed with a filter membrane. The entire device was autoclaved at 121 °C for 60 min. When rice seed was planted, the entire operation process was conducted in a biosafety cabinet, and all relevant instruments were disinfected in advance. Sterile distilled water was added to moisten the mixed soil, then the surface-sterilized grain or caryopsis were sown in the soil. Finally, the pot was sealed again, the entire device was placed in a climate incubator, and the seedlings were cultured at 25°C in a 16-h light/8-h dark cycle.

Microbiota was transplanted from grain to the caryopsis in the above axenic culture system. After surface sterilization, rice caryopses and grains were alternately sown in the axenic system. Four caryopses and four grains were cultured in each pot. The distance between each caryopsis/grain and the adjacent grains/caryopses is about 1 cm. The grain microbiota transplantation system was placed in a 25°C climate incubator and cultured in a 16 h light/8 h dark cycle.

Preparation of synthetic community (SynCom)

Nipponbare rice grains, harvested from paddy fields, were surface-sterilized. Subsequently, in a biosafety cabinet, they were separated into caryopses and seed accessory structures. These components were then individually placed on three different culture media, Nutrient Agar (1% peptone, 0.3% beef paste, 1.5% agar, 0.5% sodium chloride, pH 7.0), Reasoner's 2A Agar (0.05% proteose peptone, 0.05% casamino acids, 0.05% yeast extract, 0.05% glucose, 0.05% soluble starch, 0.03% dipotassium phosphate, 0.005% magnesium sulfate, 0.03% sodium pyruvate, 1.5% agar) and Tryptic Soy Broth (1.7% pancreatic digest of casein, 0.3% peptic digest of soybean, 0.5% sodium chloride, 0.25% dipotassium phosphate, 0.25% glucose, 1.5% agar). The samples were cultured for 7 days at 28 °C. Six bacterial strains were isolated from surface-sterilized rice grains, including 3 strains annotated to *Pantoea* and other 3 strains respectively annotated to *Pseudomonas*, *Sphingomonas,* and *Agrobacterium*. Each bacterial strain was cultured in Tryptic Soy Broth (TSB) medium until the OD_{600} was between 0.6 and 0.8. After collecting the bacteria by centrifugation, each bacterial strain was washed twice with ddH_2O and resuspended in ddH_2O at $OD_{600}=1.0$. The six bacterial strains were then mixed in equal proportions to create the SynCom of the rice grain bacteria. When spraying the SynCom on rice grains at different seed developmental stages in the greenhouse, the bacterial concentration of the SynCom was 10^8–10^9 CFU·ml^{-1}. When the SynCom was inoculated into the axenic cultivation system with the sowing of rice caryopses, the bacterial content of the SynCom was 10^6–10^7 CFU per seed. To achieve this bacterial content, the $OD_{600}=1.0$ suspension was serially diluted to 10^6 CFU/mL. Subsequently, 40 mL of this bacterial suspension was added to the axenic cultivation system containing approximately 50 mL of soil. Finally, eight seeds were planted into the inoculated soil.

eGPF-labelling for visualizing bacterial strains using a confocal laser scanning microscope (CLSM)

An *eGFP* gene was cloned into the plasmid pEHLYA2-SD, and the recombinant plasmid was transformed into *P. eucrina* SY1 to create the eGFP-labelled *P. eucrina* SY1 strain, named Pe-SY1-eGFP. To examine the colonization of Pe-SY1-eGFP, the bacteria were cultured to the log-phage stage, washed twice with ddH_2O, and resuspended in ddH_2O at $OD_{600}=1.0$. The bacterial suspension was inoculated into the axenic cultivation system with rice seedlings. After co-incubation, the rice roots were collected and rinsed three times with sterile ddH_2O to remove the surface-attached bacteria. CLSM was carried out to observe the seedling samples. To create cross-sections, the roots were cut into small segments and deposited on silylated glass slides (Sigma cat. no. S4651; St. Louis, MO, USA). The samples were examined using a Leica TCS SP8 confocal microscope. The eGPF-labelled bacteria were detected with the 488 nm for green channels.

Abbreviations

PCR	Polymerase chain reaction
CFU	Colony forming unit
SEM	Scanning electron microscopy
DAP	Days after pollination
OTU	Operational taxonomic units
PCoA	Principal coordinate analysis
OD	Optical density
SynCom	Synthetic community

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-01978-8.

Supplementary Material 1: Fig S1. Culture-dependent experiments showing bacteria between caryopsis and glumes. A. Surface-sterilized grain contains bacteria internally (left), not from the internal accessory structures (middle) or internal caryopsis (right). B. Surface-sterilized grain separated into caryopsis and accessory structures, both contain culturable bacteria.

Supplementary Material 2: Fig S2. SEM showing colonization of *Pantoea eucrina* SY1 on surfaces of germinated seedling organs. Images examined

at 14 days and 28 days after planting using a Quanta200 scanning electron microscope.

Supplementary Material 3: Fig S3. A. Different genera among bacterial communities of rice seeds (grain) and offspring seedling organs (shoot, root) by Kruskal-Wallis H test. *, $p<0.05$, **, $p<0.01$. B-G. PCoA of bacterial communities at species (B), genus (C), family (D), order (E), class (F), and phylum (G) levels. Significant differences by ANOSIM.

Supplementary Material 4: Fig S4. BugBase-predicted different microbiome phenotypes among seeds and seedling organs by Kruskal-Wallis H test. **, $p<0.01$.

Supplementary Material 5: Fig S5. A. Relative abundance of bacterial taxa in rice seeds or leaves during development at genus level. Taxa > 0.1% shown. B-C. Relative abundance at genus (B) and phylum (C) levels. Taxa > 0.1% shown. D. PCoA of bacterial communities in seeds and leaves during development. Significant differences by ANOSIM. E-F. BugBase prediction of microbiome phenotypes between seeds and leaves during development. B-D and F, seeds represent 5 stages (0, 7, 15, 24, and 40 DAP), 5 samples per stage. Leaves represent 4 stages (0, 7, 15, and 24 DAP), 5 samples per stage.

Supplementary Material 6: Supplemental Table 1. The rice seeds used in this study.

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Authors' contributions
L.C. and L.Z. designed the research. L.C., J.Y., H.B., and J.Z. performed the research. L.C. and L.Z. analysed data. L.Z., Y.H. and R.F. guided the experimental design. L.Z., Y.H. and R.F. conceptualized, administered, and supervised the study. L.C., L.Z. and R.F. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Data availability
The original sequencing data reported in this paper have been deposited in the National Microbiology Data Centre (NMDC) Sequence Read Archive (accession no. NMDC10017892).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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