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Coordination of root auxin with the fungus *Piriformospora indica* and bacterium *Bacillus cereus* enhances rice rhizosheath formation under soil drying

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Moderate soil drying (MSD) is a promising agricultural technique that can reduce water consumption and enhance rhizosheath formation promoting drought resistance in plants. The endophytic fungus *Piriformospora indica* (*P. indica*) with high auxin production may be beneficial for rhizosheath formation. However, the integrated role of *P. indica* with native soil microbiome in rhizosheath formation is unclear. Here, we investigated the roles of *P. indica* and native bacteria on rice rhizosheath formation under MSD using high-throughput sequencing and rice mutants. Under MSD, rice rhizosheath formation was significantly increased by around 30% with *P. indica* inoculation. Auxins in rice roots and *P. indica* were responsible for the rhizosheath formation under MSD. Next, the abundance of the genus *Bacillus*, known as plant growth-promoting rhizobacteria, was enriched in the rice rhizosheath and root endosphere with *P. indica* inoculation under MSD. Moreover, the abundance of *Bacillus cereus* (*B. cereus*) with high auxin production was further increased by *P. indica* inoculation. After inoculation with both *P. indica* and *B. cereus*, rhizosheath formation in wild-type or auxin efflux carrier *OsPIN2* complemented line rice was higher than that of the *ospin2* mutant. Together, our results suggest that the interaction of the endophytic fungus *P. indica* with the native soil bacterium *B. cereus* favors rice rhizosheath formation by auxins modulation in rice and microbes under MSD. This finding reveals a cooperative contribution of *P. indica* and native microbiota in rice rhizosheath formation under moderate soil drying, which is important for improving water use in agriculture.

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INTRODUCTION

Rice (*Oryza sativa* L.) is a major staple food crop for almost half of the global population [1]. Rice cultivation requires large quantities of water input [2, 3]. For instance, to produce 1 kg of rice, 1000–1500 L of irrigation water is needed in the field [4]. Changes in climate have increased the frequency of extreme weather events in the world, such as reduced rainfall [5, 6]. Thus, in order to ensure food security for a rapidly growing human population, it is important to identify beneficial irrigation management techniques to improve crop water-use efficiency [7]. Moderate soil drying (MSD) irrigation is a promising approach for reducing water inputs and maintaining or increasing crop yields [4, 8].

The rhizosheath is commonly used to describe the soil that is around the roots and can be bound by root hairs; in contrast, the rhizosphere is identified as the narrow area of soil influenced by root activity [9–11]. The rhizosheath has been observed in many major cereal crops, such as rice, wheat, maize, barley, oat, rye, sorghum and pearl millet [12–14]. Compared with the rhizosphere, the rhizosheath is the soil related to root hair cylinder volume and within a 0–0.5 mm radius of rice roots; however, the rhizosphere (a few millimeters wide) is the soil less related to root hair [10, 15]. Many factors can affect rhizosheath formation, including root hair, the soil structure and the mucilage secreted by roots or microbes [10, 16]. For example, barley mutant plants without root hair showed little or no rhizosheath formation [17]. Our previous study also found that rhizosheath can be formed in rice under MSD, but not under continuous flooding [18]. Furthermore, wheat cultivars with larger rhizosheath showed greater drought tolerance than cultivars with smaller rhizosheath [19]. Barley rhizosheath was positively correlated with plant phosphorus accumulation under phosphorus-deficiency stress [17]. For nitrogen, the rhizosheath of 42 plant species had higher nitrogen content than in the bulk soil [20]. In addition, the rhizosheath is also a trait of plant adaptation to soil acidity [21, 22]. Rhizosheath formation is connected with MSD as a drought adaptation in plants [12, 18],

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and the rhizosheath should be thought of as a mechanism for improving plant tolerance and root protection under drought stress [23–25].

Piriformospora indica (*P. indica*, DSM11827), a cultivable root-colonizing endophytic fungus of the order *Sebacinales*, can affect plant root growth via auxin production [26–28]. *P. indica* colonizes the roots of a broad host range and promotes their growth [29, 30], enhances nutrient uptake [31–33] and improves the host resistance to various biotic and abiotic stresses [34, 35]. For example, *P. indica* can alleviate the severity of infections by *Fusarium oxysporum*, *Verticillium dahlia* and Pepino Mosaic Virus in tomato [36–38], and protect tomato against salt stress [39]. In addition, the hormone-mediated signaling pathways of maize can be activated by *P. indica* under drought stress [40]. Our previous study showed that rhizosheath formation induced by MSD was connected with the root auxin response [12]. Thus, we hypothesized that *P. indica* may contribute to rice rhizosheath formation under MSD by modulating auxins. Furthermore, recent studies have suggested that fungi can interact with bacteria via the exudation of C-rich compounds (such as sugars, organic acids and amino acids) and the redistribution of soil water under drought conditions [41–44]. However, it is unclear whether *P. indica* can interact with native soil bacteria in rhizosheath formation under MSD.

In the present study, we examined rhizosheath formation in different rice varieties and auxin transport mutants inoculated with *P. indica* under MSD. RNA sequencing (RNA-seq) analysis with or without *P. indica* inoculation was performed. We further identified the microorganisms associated with *P. indica*, isolated candidate strains from the *Bacillaceae* family and finally investigated their role in rhizosheath formation. We propose that the use of *P. indica* would complement crop rhizosheath formation strategies under soil drying conditions. Our findings provide some evidence that the endophytic fungus interacts with a native bacterium in the soil resulting in the enhancement of rice rhizosheath formation and the improvement of water use under soil drying.

MATERIALS AND METHODS

Plant materials and fungal culture conditions

Three rice (*O. sativa*) varieties, ['Nipponbare' (NIP), 'Zhonghua11' (ZH11) and 'Zhonghan3' (ZH3)] and *OsPIN2* transgenic lines [*O. sativa* cv Hei-Jing2 (WT), *OsPIN2* mutant (*ospin2*) and three complementation lines with the *ospin2* background (C1, C2, and C3)] [45] were surface-sterilized using 1.5% (v/v) NaClO for 20 min, washed with double-distilled water three times, and grown for 3 d in 1/2 MS nutrient medium. Then, the seedlings were transformed to PNM nutrient medium, which contained five fungal discs with *P. indica* (*P. indica*; DSM11827; provided by Prof. Kaiwun Yeh from National Taiwan University). One to five rice plants were used per Petri dish and one fungal plague of 5 mm in diameter per rice plant was placed at a distance of 1 cm from the rice root [30]. Control treatments were conducted using media lacking *P. indica*.

Inoculation and root colonization with *P. indica* in rice

After 10 d co-cultivation with *P. indica*, the colonization of *P. indica* in rice roots was investigated using the marker gene *Pitef1* [46] (F, 5′-GATTCCTTCATCGCAGTCA-3′; R, 5′-TTGGTGGTGTCCATCTTGTT-3′) by PCR (Supplementary Fig. S1A). The PCR was performed as follows: 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, 72 °C for 10 min, and ending at 4 °C. The colonization of *P. indica* in rice roots was also investigated using GFP-labeled *P. indica*. After 10 d, the roots were observed under a Zeiss LSM710NLO confocal laser scanning microscope at the 488 nm laser line of an argon multiline laser. The genomic DNA was extracted from the samples using the FastDNA Spin Kit for Soil (MP Biomedicals) according to the manufacturer's instruction. Fifteen rice plants with or without *P. indica* inoculation were tested for the subsequent experiment.

Water treatments

After evaluating the colonization of *P. indica* using PCR analysis, uniform seedlings with or without *P. indica* inoculation, were transplanted into pots (12-cm diameter, 14-cm height) containing 1.8 kg dry soil. The soil used in the study was collected from a paddy rice field in the town of Huayang, Jiangxi Province, China (115°09′E, 28°32′N). The air-dried soil with mineral nutrients added was sieved through a 4 mm mesh to remove any coarse material and vegetative matter. Soil chemical factors are shown in Supplementary Table S1. For the water treatments, half of the pots were treated under well-watered (WW) conditions, where a water layer of 3–5 cm above the soil surface was maintained, and the other half were subjected to an MSD regime. For the MSD and severe soil drying (SSD) treatments, rice seedlings were irrigated every 3 d to 80% and 60% (w/w) of field capacity, respectively (Supplementary Fig. S2A–C). The WW, MSD and MSD with auxin efflux inhibitor 1-naphtaylphthalamic acid (NPA) were kept for 2 weeks. The experiments were conducted in the greenhouse under a 14 h light (26 °C)/10 h dark (22 °C), 60% (w/w) relative humidity, and a photosynthetic photon flux density of 300 mmol photons m^{-2} s^{-1}. Additional alkaline soil from Ronghuashan Town, Liaoning Province, China (122°86′E, 39°93′N) was used to repeat the above experiments to confirm the effect of the fungus and bacterium on rhizosheath formation under MSD.

Measurements of total root length, root hair length and rhizosheath weight

Under MSD, rice roots were carefully collected and shaken after the pots were disassembled. The soil, which tightly adhered to roots upon excavation, was identified as rhizosheath soil. The rhizosheath soil was recovered by washing the root gently in double-distilled water using plastic dishes [12, 14, 18]. The soil and rinsing water were then collected in a tray and dried at 105 °C for 3 d to measure the dry weight of the rhizosheath. An Epson Expression 1640 XL flat-bed scanner (Epson UK, London, UK) and WinRHIZO software (Regent Instruments, Quebec City, QC, Canada) were used to determine the total root length. The specific rhizosheath dry weight was calculated as the total rhizosheath soil dry weight per plant (mg) divided by the total root length (cm) [12, 18, 47]. Root hair length was measured following George et al. [17]. Photos were taken using a SMZ18 stereomicroscope and a DS-U3 camera (Nikon). Ten fully elongated root hairs were determined in each image using Image J software (National Institutes of Health). Four replicates for the rice plants were selected for the experimental measurement, and the experiments were repeated two times.

Assay of auxin concentration

For the measurement of auxin concentrations in the roots and *P. indica*, IAA was extracted from the *P. indica* inoculated roots and *P. indica*. Sample measurement of IAA was conducted by high-performance liquid chromatography (HPLC) according to Hou et al. [48]. Briefly, the fresh weight of the rice roots was determined, and the roots were frozen in liquid nitrogen. Then, the measurement of IAA was carried out using HPLC. The standard IAA sample was bought from Sigma-Aldrich (St Louis, MO, USA). For the measurement of IAA of *B. cereus*, the supernatant was obtained by passing through filter paper and then used to measure IAA by HPLC according to Yuan et al. [49].

Quantitative real-time PCR (qRT-PCR) and RNA-seq analysis

We harvested the roots to conduct RNA-seq and qRT-PCR analysis under MSD and WW with or without *P. indica* inoculation. Total RNA was isolated from the rice roots using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Primers for qRT-PCR are shown in Supplementary Table S2. The procedure was conducted according to Xu et al. [50]. For the RNA-seq analysis, RNA was qualified and quantified using a Nano Drop (Thermo Fisher Scientific) and BGISEQ-500 sequencer (BGI, Shenzhen, China). Sequencing libraries were generated according to the method of Zhang et al. [12]. Raw reads were analyzed and cleaned using SOAPnuk (v1.5.2). Gene expression that changed by more than 2-fold with an adjusted $P \le 0.01$ between root samples deemed as significantly different. The treatment groups included the uninoculated seedlings grown under WW (WW), seedlings inoculated with *P. indica* grown under WW (WW + *P. indica*), uninoculated seedlings grown under MSD (MSD), and seedlings inoculated with *P. indica* grown under MSD (MSD + *P. indica*). The RNA-seq results showed that over 1G of clean bases was obtained from each sample (Supplementary Table S3). In addition, over 97% of the clean reads could be aligned to the reference genome. Pathway enrichment analysis used Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. The sequencing dataset was deposited to the NCBI BioProject database under the accession number PRJNA732534.

Sample preparation and DNA extraction for bacterial community analysis

In order to investigate the bacterial composition and diversity of the rhizosheath and root endosphere under MSD and WW conditions with *P. indica* inoculation, we collected rhizosheath, root endosphere, and bulk soil samples from the additional three pots in the same rhizosheath formation experiment (Supplementary Figs. S3 and S4A). The different sample types used in this study are as follows: for MSD treatment, a 0.5-mm radius of soil around the root was collected as the rhizosheath soil [18] (Supplementary Fig. S3). Under the WW treatment, we selected the soil around the roots with the same radius (0.5-mm) of rhizosheath under MSD to consider as rhizosheath-like soil under WW owing to that rice is unable to form a rhizosheath under WW conditions. Details of the sample collection methods are described below. Briefly, the root was firstly removed from the pot, following which the soil around the root within a 0.5-mm radius was collected using a Vernier caliper, and other soil around the root was then removed using sterile tweezers. PBS-S buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4 (pH 7.0), and 0.02% [v/v] Silwet L-7) was used to wash the root with soil of both MSD and WW treatments in 50-mL tubes [51]. Then, the rhizosheath soil was collected from the washing buffer by centrifuging at $1500 \times g$ for 20 min. Bulk soil was taken from the pot without plant treatments. After collection, soil samples were immediately frozen in liquid nitrogen and stored at −80 °C. The root samples were surface-sterilized using 1.5% (v/v) NaClO for 15 min after they were removed from PBS-S buffer. After sterilizing with NaClO, they were washed with sterilized double-distilled water three times. Thereafter, the final wash water was volume onto Luria-Bertani (LB) medium to verify the efficacy of the sterilization [52]. Root samples were also stored at −80 °C. Total root and soil genomic DNA was extracted from 0.5 g sample using the Mag-Bind Soil DNA Kit (Omega Bio-Tek) following the manufacturer's instructions. The DNA quality and quantity were determined by gel electrophoresis and NanoDrop ONE spectrophotometry (Thermo Scientific, Waltham, MA, USA). Three biological repeats of each treatment were taken for 16S rRNA gene high-throughput sequencing (Supplementary Table S4).

16S rRNA gene high-throughput sequencing for bacterial community analysis

To determine the changes in the bacterial community composition of the rhizosheath and root endosphere under MSD and WW conditions, we amplified the V5–V7 region of the bacterial 16S rRNA gene using 799F/1193R primers [53]. These primers were selected because they were more suitable for 16S rRNA gene amplification sequencing for plant-associated bacterial communities [54, 55]. The PCR reactions were run in triplicate 50-μL mixtures containing 25 μL Phusion High-Fidelity PCR Master Mix with HF buffer (New England Biolabs), 3 μL of primers (10 μM), 10 μL template DNA, 6 μL double-distilled water, and 3 μL dimethyl sulfoxide. The PCR was performed as follows: 98 °C for 30 s; 25 cycles of 98 °C for 15 s, 58 °C for 15 s, and 72 °C for 15 s; and 72 °C for 60 s. The PCR products were extracted from 2% agarose gels, purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using Quanti-Fluor-ST (Promega, Madison, WI, USA) according to the manufacturers' protocols. Then, samples were sequenced on a MiSeq platform (Illumina, San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co. Ltd (Shanghai, China).

The raw 16S rRNA gene sequencing reads were quality-filtered by fastp version 0.20.0 [56]. Paired-end reads were processed in the following steps using USEARCH version 7.0 [57]: joining of paired-end reads, removal of barcodes and primers, filtering of low-quality reads, and finding non-redundant reads. For root endosphere samples, mitochondria- and chloroplast-assigned operational taxonomic units (OTUs) were removed. The OTUs were clustered with a 97% similarity cutoff [58] using UPARSE (version 11.0) [58]. The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier algorithm [59] against the SILVA (v138) [60] 16S rRNA gene database with a confidence threshold of 70%. The alpha-diversity (observed OTUs, Chao1, Shannon and Good's coverage) and beta-diversity analyses were performed by Mothur software [61] and the R vegan package (version 4.0.4). The rarefaction curves (based on OTUs) were calculated to access the sampling efficiency of samples. Liner discriminant analysis coupled with an effect size (LEfSe) analysis was conducted to search for significantly different ($p < 0.05$) taxa among the bulk soil, root endosphere and rhizosheath under MSD and MSD with *P. indica* inoculation. Ternary plots were conducted using the mean of the $\log_2$ transformed relative abundance values per compartment. The function "ternaryplot" was used to plot the point size of each OTU proportional to its weighted sum instead of the total relative abundance [51]. Venn diagrams were created to show the number of common or unique OTUs in multi groups using the R package Venn Diagram setting. All sequencing data were available in the NCBI Sequence Read Archive (SRA) database under accession number PRJNA739870.

Culturable *Bacillus* assay, *Bacillus* isolation and interaction assay

We measured the population densities of culturable *Bacillus* in the fresh bulk soil, rhizosheath and root endosphere samples, which was conducted using a standard 10-fold dilution plating assay following Wang et al. [62]. For quantification of the *Bacillus* population, three aliquots of dilution were pretreated at 80 °C for 10 min, and then plated on V8 *Bacillus*-semiselective medium (326 mL V8 juice, 33 g NaCl, 0.8 g glucose, 45 mg actidione and 22.5 mg polymyxin B per liter; the actidione and polymyxin B were filter-sterilized. In addition, the pH of the medium was adjusted to 5.2 before autoclaving) [63]. After incubation at 30 °C for 2 d, colony counting was performed to calculate the number of colony forming units (CFUs).

Then, the fresh rhizosheath soil or rice roots under MSD with *P. indica* inoculation were used for the isolation of putative *Bacillus*. The soil or root suspension was serially diluted and pretreated at 80 °C for 10 min, and plated on V8 *Bacillus* semiselective medium [55, 64]. After incubation at 30 °C for 2 d, the *Bacillus* colonies were randomly isolated from the selective plates based on colony morphology. A total of 239 candidate *Bacillus* strains were isolated with *P. indica* inoculation under MSD, out of which 129 *Bacillus* strains were from the rhizosheath and 110 *Bacillus* strains were from the root endosphere.

The interaction between *P. indica* and *Bacillus* was tested as described by Zhang et al. [65]. Candidate *Bacillus* strains were inoculated in 5 mL of V8 *Bacillus*-semiselective medium and incubated overnight at 30 °C and 200 rpm shaking. The OD of the bacterial cultures was adjusted to an OD_{600} of 0.1. Chlamydospores of *P. indica* were harvested from KM medium using tween water (0.002% Tween 20), scratched with a scalpel, and filtered through Miracloth (Calbiochem, Nottingham, UK) to remove mycelial fragments [66]. Then, the chlamydospore solution was centrifuged in a falcon tube at 3500 rpm for 7 min, and the supernatant was decanted to resuspend the chlamydospore containing pellet with 0.9% NaCl solution, which was sterilized in an autoclave. Next, 2.5 μL *P. indica* chlamydospore (containing 10^4 *P. indica* chlamydospores per μL) was inoculated in a diagonal row on the right side of a Petri dish. After 3 d, 2.5 μL of *Bacillus* was inoculated on the left side of the Petri dish with a bacterial inoculating loop, creating a V-shape of the inoculation sites with increasing proximity [67]. Plates with *P. indica* non-inoculation were used as controls. The plates were incubated at 30 °C for 3–5 d. We determined the diameters of candidate *Bacillus* strains on a line orthogonal to the line dividing the V-shape to select the strain interacting with *P. indica*. Then, we calculated the relative colony diameter of strains as the ratio of the strain diameter with *P. indica* inoculation versus the strain diameter under control conditions. Following the assessment of the interaction between *P. indica* and the entire *Bacillus* candidates, one strain (*Bacillus cereus*) isolated from the root endosphere displaying a strong interactive effect was selected for further study. The classification of the interactive isolate was determined by sequencing the 16S rRNA gene using primers 27F and 1492R in Sangon Biotech (Shanghai) Co., Ltd (Sequence accession: 2020023554). The sequencing data have been made available in the NCBI GenBank (MZ433237).

Growth assay of *P. indica* and *B. cereus*

Sixty grams of sterilized soil, which was sterilized three times by autoclaving and heat-incubation until completely dehydrated [68], was placed in a 300 mL tissue-culture container and inoculated with 0.4 g of *P. indica* mycelium [69] and 2 mL of *B. cereus* suspension at $OD_{600} = 0.5$ (~1.5×10^6 CFU ml^{-1}). The *B. cereus* strain was suspended in 10 mM $MgCl_2$ buffer [70]. The assay only addition of *B. cereus* without *P. indica* inoculation was performed as a control. The number of *B. cereus* cells in the soil was evaluated at 15 d after inoculation. For the quantification of the *Bacillus* population, three aliquots of dilution were pretreated at 80 °C for 10 min and then plated on the V8 *Bacillus*-semiselective medium. After incubation at 30 °C for 2 d, colony counting was used to calculate the abundance of *B. cereus* in the soil.

Rhizosheath formation assay with *P. indica* and *B. cereus* inoculation

The experiment was conducted in sterilized or non-sterilized soil under MSD. The sterilized soil was sterilized three times by autoclaving and

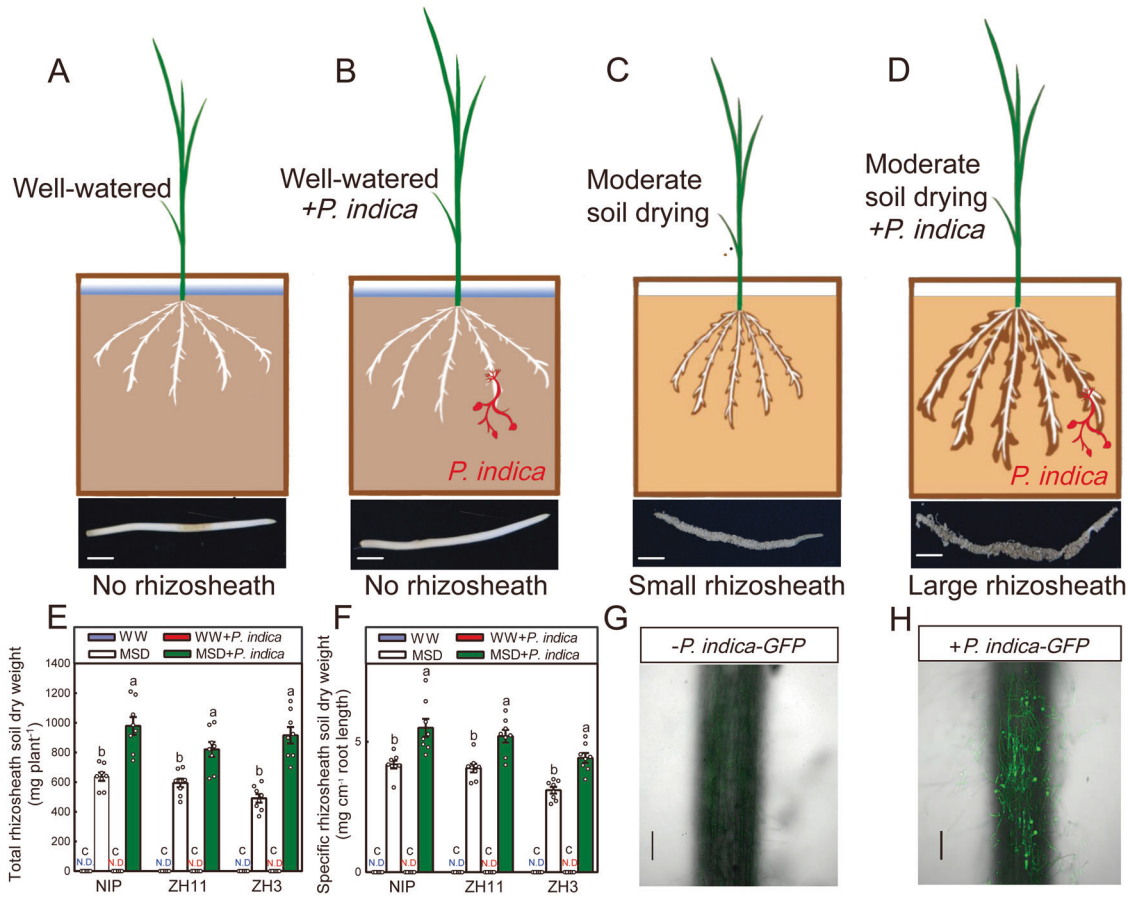


Fig. 1 Rice rhizosheath formation is increased under moderate soil drying (MSD) with *P. indica* inoculation compared to MSD alone. The non-inoculated and *P. indica*-inoculated rice seedlings (NIP rice) were cultured under well-watered (WW, **A**, **B**) and MSD (**C**, **D**) conditions. Rice seedlings (NIP rice) were able to form rhizosheath under MSD or MSD with *P. indica*-inoculation (MSD + *P. indica*), but not under WW or WW with *P. indica*-inoculation (WW + *P. indica*). Bar = 0.5 cm. The total rhizosheath soil dry weight (**E**) and specific rhizosheath soil dry weight (**F**) of three rice varieties (NIP, ZH11 and ZH3) under WW, WW + *P. indica*, MSD and MSD + *P. indica*. The specific rhizosheath dry weight was calculated as the total rhizosheath soil dry weight per plant (mg) divided by the total root length (cm). N.D. indicated that rhizosheath was not detectable. Data are the means ± SE ($n = 8$ replicates). Bars with different letters among different treatments are significantly different at $p < 0.05$ (ANOVA, Duncan's multiple range test). **G**, **H** Fluorescence microscopy of rice seedling (NIP rice) roots showing the presence of intracellular *P. indica* chlamydospores (spores are in green) in cortical cells using confocal microscopy with a superficial view. The image is an X- and Y-stack reconstruction. Rice roots without GFP-tagged *P. indica* (*P. indica*–GFP) inoculation were used as a control. Bar = 150 µm.

heat-incubation until completely dehydrated [68]. After the rice plants inoculated or not inoculated with *P. indica* were transplanted into sterilized or non-sterilized soil as described above, half of the seedlings were inoculated with *B. cereus* suspension to a concentration of 10^8 cells g^{-1} soil, while the uninoculated seedlings were water-treated with sterilized double-distilled water [71]. After 7 d of inoculation, the roots of some seedlings were subjected to MSD treatment for 2 weeks, as described above (Supplementary Fig. S4B). The root length, root hair length, and rhizosheath weight were then measured as described above. Four replicates of the rice plants were selected for each experimental measurement, and experiments were repeated two times.

Statistical analysis

Graphical representations were generated with GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). The means and standard error (SE) of the data were calculated. The overall mean differences between treatments were analyzed using ANOVA (Duncan's multiple range test) or Student's *t* tests, and *p* values less than 0.05 were considered statistically significant. For principal co-ordinates analysis (PCoA), PERMANOVA (Adonis function, 999 permutations) was used to analyze the bacterial community composition structure on the basis of the weighted-UniFrac distance. The Kruskal–Wallis (KW) sum-rank test was carried out to detect the features displaying significantly different abundances between assigned families in linear discriminant analysis coupled with an effect size (LEfSe) analysis.

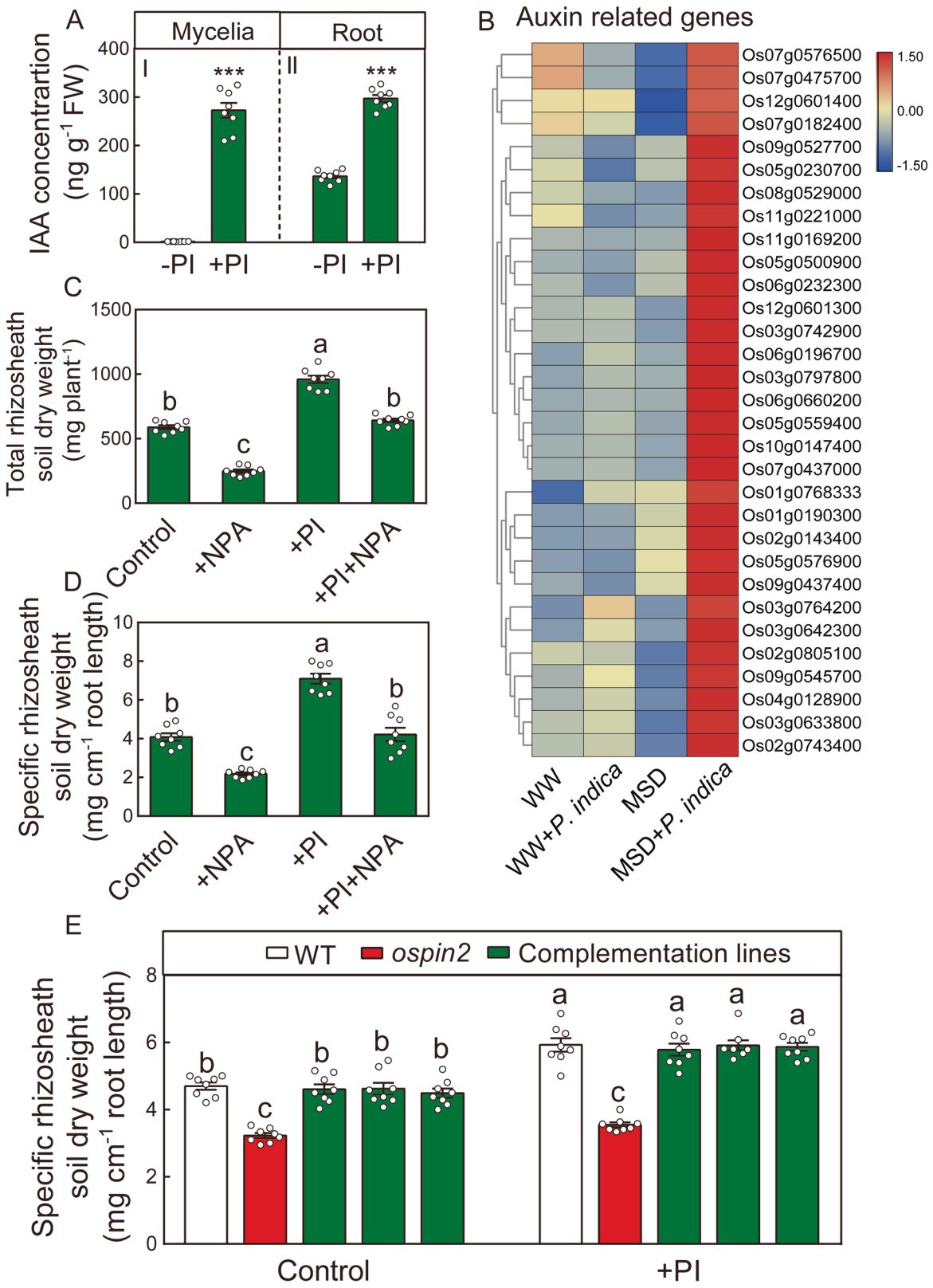
RESULTS

Moderate soil drying increased rice rhizosheath formation

Compared with WW, MSD increased the root dry weight and rhizosheath formation of wild-type NIP rice, while SSD decreased it (Supplementary Fig. S2A–C). Moreover, the exopolysaccharide (EPS) content of rhizosheath soil was significantly increased by 38% under MSD, compared with WW (Supplementary Fig. S2D). The sugars and amino acids of the root exudation were also significantly higher under MSD than that under WW (Supplementary Fig. S2E). Compared with WW, ethylene related genes expressions were also significantly enhanced under MSD (Supplementary Fig. S2F).

The endophytic fungus (*P. indica*) further increased rice rhizosheath formation under moderate soil drying

To investigate the effects of *P. indica* on rice rhizosheath formation, rice plants with and without *P. indica* inoculation were grown under WW and MSD conditions. We found that *Pitef1* was amplified in the PCR-reaction from the root samples inoculated with *P. indica* (Supplementary Fig. S1A). Furthermore, *P. indica*-chlamydospores, exhibiting green fluorescence, had formed after colonization (Fig. 1G, H). Then, the total root length and root hair lengths of NIP, ZH11 and ZH3 were increased by 9.6–21% and



51–108% under WW with *P. indica* inoculation, compared to WW alone (Supplementary Fig. S1B, D). Compared to MSD alone, the total root lengths and root hair length in NIP, ZH11, and ZH3 were 6–32% and 53–92% greater under MSD with *P. indica* inoculation, respectively (Supplementary Fig. S1C, E). Rice seedlings were able to form rhizosheath under MSD and MSD with *P. indica* inoculation (MSD + *P. indica*), but not under WW, or WW with *P. indica* inoculation (WW + *P. indica*) (Fig. 1A–D). In addition, the total rhizosheath soil dry weight of NIP, ZH11 and ZH3 increased by ~38–86% under MSD with *P. indica* inoculation compared to MSD

Fig. 2 Auxin response of rice root is important for rhizosheath formation with *P. indica* inoculation under moderate soil drying (MSD). **A** IAA concentration of *P. indica* mycelia (I) or rice roots (II) without *P. indica* (−PI) and with *P. indica* (+PI) under axenic conditions. Data are the means ± SE ($n = 8$ replicates). Significant difference between with (+PI) and without *P. indica* (−PI) inoculation under WW or MSD were analyzed in figures (**D**, **E**) using the Student's *t* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **B** Differentially expressed genes related to auxin in rice roots under WW, WW with *P. indica* inoculation (WW + *P. indica*), MSD, and MSD with *P. indica* inoculation (MSD + *P. indica*). **C**, **D** The effect of NPA (an auxin efflux inhibitor) on rice rhizosheath formation with or without *P. indica* inoculation. The total rhizosheath soil dry weight (**C**) and specific rhizosheath soil dry weight (**D**) of NIP rice seedlings with non-inoculation (−PI), NPA (+NPA), *P. indica* inoculation (+PI), *P. indica* and NPA treatments (+PI + NPA) under MSD. Data are the means ± SE ($n = 8$ replicates). Bars with different letters are significantly among different treatments at $p < 0.05$ (ANOVA, Duncan's multiple range test). **E** Auxin efflux carrier *OsPIN2* is required for rice rhizosheath formation with or without *P. indica* inoculation under MSD. The specific rhizosheath soil dry weight of rice seedlings (WT, auxin efflux carrier *OsPIN2* mutant *ospin2* and its complementation lines) with non-inoculation (−PI) and *P. indica* inoculation (+PI), under MSD. Data are the means ± SE ($n = 8$ replicates). Bars with different letters are significantly among different treatments at $p < 0.05$ (ANOVA, Duncan's multiple range test).

alone (Fig. 1E). A 31–37% increase in the specific rhizosheath soil dry weight was observed in NIP, ZH11, and ZH3 plants grown under MSD with *P. indica* inoculation compared to MSD alone (Fig. 1F). Moreover, the tiller number, total shoot dry weight, total root dry weight and total root length of rice inoculated with *P. indica* were significantly higher than those of the non-inoculated rice under field conditions (Supplementary Fig. S5A–D).

Rice root auxin transport was involved in increasing rice rhizosheath formation with *P. indica* inoculation under moderate soil drying

P. indica can affect plant growth by producing auxin [26]. Here, the IAA concentration (auxin) of the *P. indica* mycelia was about 270 ng g^{-1} (Fig. 2A-I). And, the IAA concentration of the rice roots was increased by ~119% with *P. indica* inoculation compared to the uninoculated plants (Fig. 2A-II). To better understand the possible physiological differences resulting from *P. indica* inoculation under WW and MSD, we performed transcriptomic comparisons of the seedlings grown under WW and MSD with or without *P. indica* inoculation. When comparing the MSD samples with the WW samples, there were 1623 significantly up-regulated genes and 1092 significantly down-regulated genes (Supplementary Fig. S6A). There were 3785 differentially expressed genes when comparing MSD and MSD with *P. indica* (Supplementary Fig. S6A). Moreover, KEGG enrichment analysis showed that the plant hormone signal transduction pathway (including the auxin signaling pathway) was enriched under MSD + *P. indica* compared with MSD alone (Supplementary Fig. S6B). And, previous study showed that *P. indica* increased auxin signaling in the root of Chinese cabbage [30]. Thus, auxin related differentially expressed genes were further analyzed (Fig. 2B). The gene expression of auxin efflux carrier component (*OsPIN2*, Os06g0660200) was enhanced under MSD with *P. indica* inoculation. Compared to MSD, qRT-PCR revealed that the gene expression of *OsPIN2* (an important gene for auxin efflux) in rice root was increased by 2.2-, 2.3- and 3.9-fold with *P. indica* inoculation, *B. cereus* inoculation, or a combination of *P. indica* and *B. cereus* inoculation under MSD, respectively (Supplementary Fig. S6C).

Under MSD with the addition of auxin efflux inhibitor 1-naphtaylphthalamic acid (NPA), the total rhizosheath soil dry weight, specific rhizosheath soil dry weight and root hair length were significantly lower than those under MSD alone (Fig. 2B, C, Supplementary Fig. S7A). Similarly, the total rhizosheath soil dry weight, specific rhizosheath soil dry weight and root hair length under MSD with inoculation of *P. indica* and NPA were also decreased compared to the treatment of MSD with inoculation of *P. indica* (Fig. 2B, C, Supplementary Fig. S7A).

Auxin efflux carrier *OsPIN2* was required for rhizosheath formation with *P. indica* inoculation under moderate soil drying

To further understand whether auxin efflux was involved in rhizosheath formation under MSD with *P. indica* inoculation, we examined rhizosheath formation with *P. indica* inoculation under MSD using the auxin efflux carrier *OsPIN2* mutant *ospin2*, complemented *OsPIN2* lines and WT rice. Compared to MSD alone, the specific rhizosheath soil dry weight was increased by 21–25% in WT and complemented lines with *P. indica* inoculation under MSD, while that of *ospin2* was only increased by ~9% (Fig. 2E). Furthermore, MSD with *P. indica* inoculation increased root hair length of 20–33% in the WT and complemented rice lines, compared to MSD alone (Supplementary Fig. S7B). However, no significant difference in root hair length was recorded in *ospin2* between MSD and MSD with *P. indica* inoculation (Supplementary Fig. S7B).

P. indica inoculation increased the abundance of *Bacillus* in the rhizosheath and root endosphere under moderate soil drying

To investigate whether the *P. indica* affected rhizosheath or root endosphere microbiota, 16S rRNA gene amplicon sequencing was conducted to analyze the composition of bacterial community in rhizosphere, rhizosheath, root endosphere and bulk soil under MSD and WW (Supplementary Fig. S8D). The gradually flattening rarefaction curves and over 99.1% Good's coverage showed that the sequencing depth was adequate for capturing the microbial communities (Supplementary Fig. S8E; Supplementary Table S5). Principal coordinate analysis (PCoA) revealed that *P. indica* inoculation had significant effects on the composition of bacterial communities (based on weighted-UniFrac distance) in the root endosphere under MSD and WW conditions, however, no significant effects on the rhizosheath microbiome (Fig. 3C, D). Venn diagrams showed that *P. indica* inoculation impacted the bacterial OTUs number in root endosphere under WW and MSD condition (Supplementary Fig. S8A–C). The alpha-diversity (based on Chao and Shannon index) of the endosphere microbiomes significantly increased with *P. indica* inoculation compared to non-inoculation under MSD conditions, but no significant difference in rhizosheath microbiomes (Supplementary Fig. S8F, G). Moreover, the influence of *P. indica* on bacterial composition of root endosphere was different between MSD and WW conditions (Fig. 3A, B). For example, the relative abundance of the phylum *Firmicutes* significantly increased (from 8.3% to 41.6%) in the root endosphere inoculated with *P. indica*, whereas that of phylum *Proteobacteria* in the root endosphere (from 90.8% to 51.3%) significantly decreased under MSD (Fig. 3A). These phylum taxonomies changes were mainly due to the increased abundance of the families *Bacillaceae*, *Sporolactobacillaceae* and *Ruminococcaceae* or the decreased abundance of the families *Burkholderiaceae*, *Mitochondria*, *Enterobacteriaceae* and *Xanthomonadaceae* (Fig. 3B). Under WW, a considerable decrease in *Burkholderiaceae* (from 64.2% to 19.4%) and a significant increase in *Mitochondria* (from 13.5% to 58.1%) were observed in the root endosphere with *P. indica* inoculation compared with the non-inoculation treatment (Fig. 3B). Together, these results showed that *P. indica* played an important role in the assembly of the bacterial community of the root endosphere under MSD and WW.

P. indica inoculation significantly increased the abundance of the family *Bacillaceae*, belonging to phylum *Firmicutes*, in the

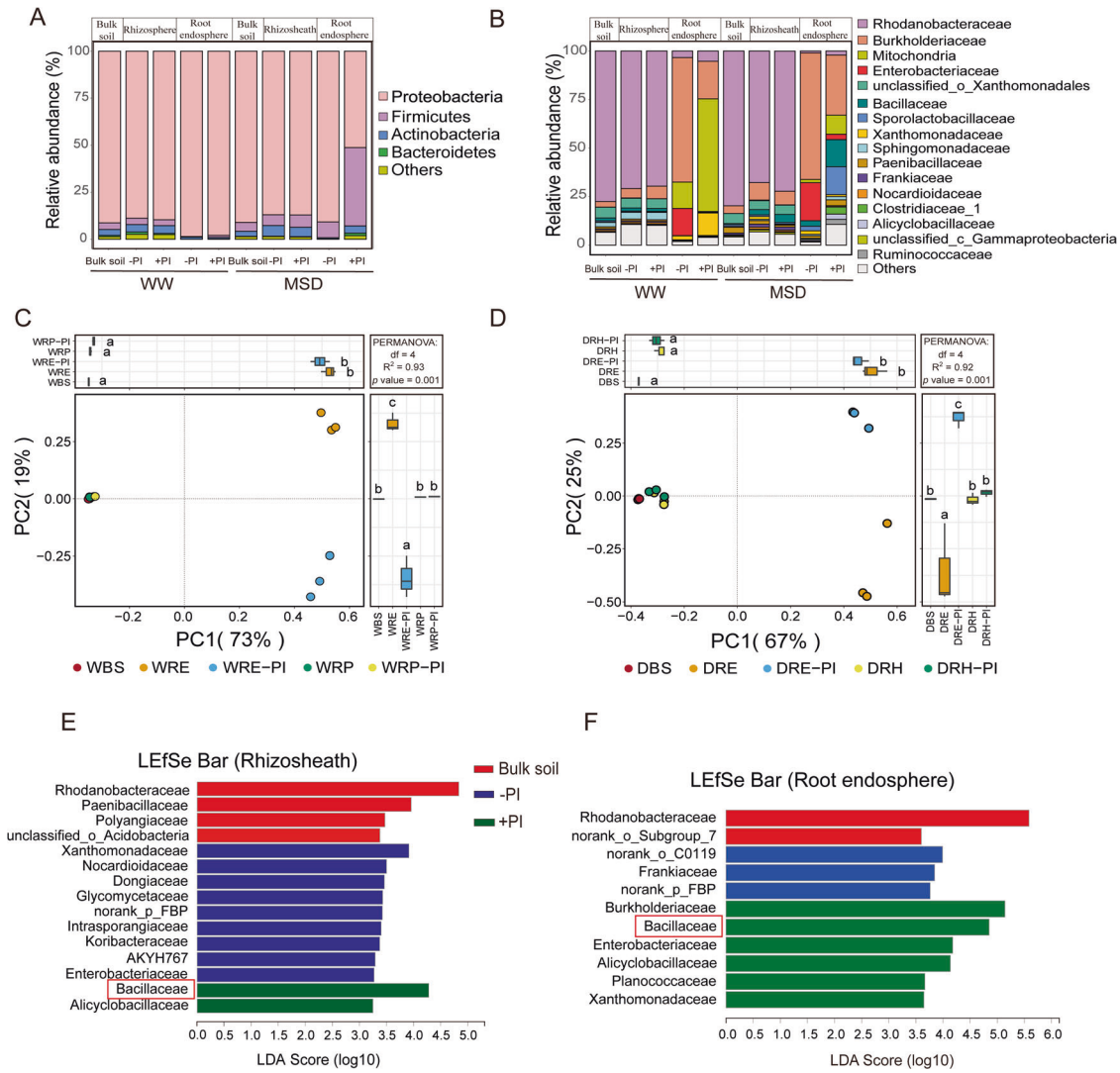


Fig. 3 The *Bacillaceae* family, which harbors many plant growth-promoting rhizobacteria, may be associated with rice rhizosheath formation under moderate soil drying (MSD) with *P. indica* inoculation. A, **B** The phylum level and family level relative abundance showed >1% relative abundance of all sequences in samples from different root-system compartments (root endosphere and rhizosheath or rhizosphere) of NIP rice with non-inoculation (−PI), *P. indica* inoculation (+PI) under well-watered (WW) and MSD conditions. **C**, **D** Principal-coordinate analysis (PCoA, based on weighted-UniFrac distance) showed the changes in composition of bacterial microbiomes in the bulk soil, rhizosheath (under MSD)/rhizosphere (under WW) and root endosphere of non-inoculated or *P. indica* inoculated under WW (**C**) or MSD (**D**). PERMANOVA (Adonis function, 999 permutations) was used to test differences in the bacterial community composition between treatments in principal co-ordinates analysis (PCoA). Linear discriminant analysis (LDA) for discriminating bacterial taxon (family level) that showed significant differences in abundances among bulk soil, rhizosheath with non-inoculation (−PI), and rhizosheath with *P. indica* inoculation (+PI) (**E**; LDA score ≥ 3.2) and among bulk soil, root endosphere with non-inoculation (−PI) and root endosphere with *P. indica* inoculation (+PI) (**F**; LDA score ≥ 3.5) under MSD conditions. Significance test $p < 0.05$. WBS, bulk soil under WW; WRP, rhizosphere soil of NIP rice under WW with non-inoculation; WRP-PI, rhizosphere soil of NIP rice under WW with *P. indica* inoculation; WRE, root endosphere of NIP rice under WW with non-inoculation; WRE-PI, root endosphere of NIP rice under WW with *P. indica* inoculation; DBS, bulk soil under MSD; DRH, rhizosheath soil of NIP rice under MSD with non-inoculation; DRH-PI, rhizosheath soil of NIP rice under MSD with *P. indica* inoculation; DRE, root endosphere of NIP rice under MSD with non-inoculation; DRE-PI, root endosphere of NIP rice under MSD with *P. indica* inoculation.

rhizosheath (from 2.7% to 4.1%) and root endosphere (from 2.9% to 14%) compared to the non-inoculation treatment under MSD (Fig. 3B). We also found that the family *Bacillaceae* was strongly enriched in the rhizosheath and root endosphere under MSD using the linear discriminant analysis (LDA) effect size tool (LEfSe) analysis (Fig. 3E, F, Supplementary Fig. S8H). Furthermore, the abundance of genus *Bacillus* (belonging to family *Bacillaceae*) was 1.5 and 20 times higher in the rhizosheath and root endosphere with *P. indica* inoculation compared to the non-inoculation treatment under MSD, respectively (Fig. 4A–C). However, there was no significant difference in the relative abundance of *Bacillus* in the rhizosphere and root endosphere with and without *P. indica* inoculation under WW (Supplementary Fig. S9A, B). *P. indica* also significantly increased the abundance of genus *Bacillus* in root endosphere and rhizosheath using colony counting (Supplementary Fig. S10A). Together, these results showed that *P. indica* inoculation increased the abundance of *Bacillus* in the rhizosheath and root endosphere under MSD.

P. indica and *B. cereus* greatly promoted rice rhizosheath formation under moderate soil drying

To further explore which *Bacillus* species co-works with *P. indica*, 239 candidate *Bacillus* isolates were randomly isolated based on semiselective medium. After that, we found that *B. cereus* showed

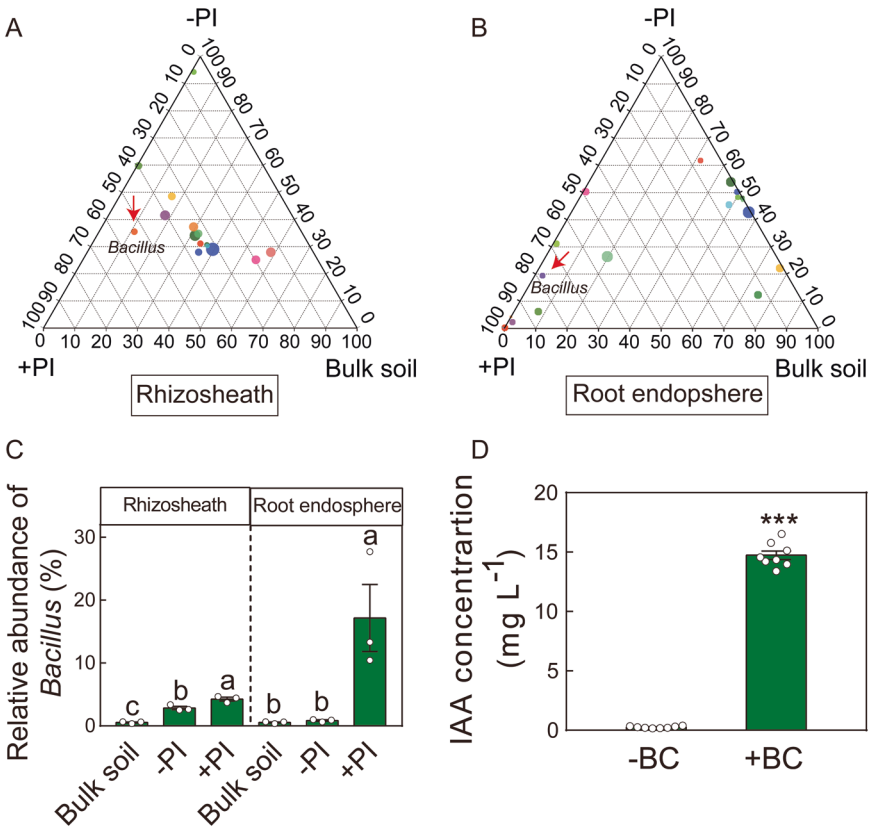


Fig. 4 The relative abundance of native *Bacillus cereus* is promoted by the *P. indica* in rhizosheath or root endosphere, and this *Bacillus cereus* can produce high concentration of auxin. Ternary plot of the bacterial operational taxonomic units (OTUs) in the rhizosheath (**A**) and root endosphere (**B**) with non-inoculation (-PI), *P. indica* inoculation (+PI) and bulk soil under moderate soil drying (MSD). Each circle with color represents different OTUs (relative abundance > 1%), and *Bacillus* is indicated with red arrows. The size of each circle represents its relative abundance. The position of each circle is determined by the contribution of the OTUs from each of the treatments to the total relative abundance. **C** Bar plot showing the relative abundance of the reads classified as *Bacillus* (OTU1256) in the rhizosheath and root endosphere with non-inoculation (−PI), *P. indica* inoculation (+PI) and bulk soil under MSD. Data are the means ± SE. Bars with different letters are significantly different among different treatments at $p < 0.05$ (ANOVA, Duncan's multiple range test). **D** IAA concentration produced by *Bacillus cereus*. –BC, medium without *Bacillus cereus*; +BC, medium with *Bacillus cereus*. Data are the means ± SE ($n = 8$ replicates). The asterisk (***) is shown significantly different between without and with *Bacillus cereus* at $p < 0.001$ (Student's *t* test).

the highest relative colony diameter among the candidate isolates in the contact-assay (Supplementary Fig. S10B; Supplementary Table S6). Nextly, *P. indica* also significantly enhanced the relative diameter of *B. cereus* in volatile organic compounds interaction assay (Supplementary Fig. S10C). Further, the abundance of *B. cereus* was significantly increased with *P. indica* inoculation in sterilized soil, compared with non-inoculation conditions (Supplementary Fig. S10D). Together, the results showed that *P. indica* enhanced the abundance of *B. cereus*.

The rice plants with *P. indica* and *B. cereus* co-inoculation showed the highest root IAA concentration compared to *P. indica* inoculation or *B. cereus* inoculation alone (Supplementary Fig. S11A). To assess the promotional effect of *P. indica* and *B. cereus* on rhizosheath formation, WT, *ospin2* and the complemented line rice, which were inoculated with *P. indica*, *B. cereus*, a combination of *P. indica* and *B. cereus*, or the uninoculated control, were treated under MSD using non-sterilized or sterilized soil (Supplementary Fig. S4B). Compared to the non-inoculation treatment, the rhizosheath formation of the WT and complemented line was significantly increased with *P. indica* inoculation, *B. cereus* inoculation and co-inoculation of *P. indica* and *B. cereus* under MSD using non-sterilized and sterilized soil, while the rhizosheath formation of *ospin2* was not significantly different (Fig. 5). In addition, the rhizosheath of the WT and complemented line rice treated with the combination of *P. indica* and *B. cereus* was significantly higher than the treatment with *P. indica* or *B. cereus* separately. However, no significant difference in the rhizosheath formation was found in the *ospin2* mutant with identical treatments (Fig. 5). Furthermore, inoculation of both *P. indica* and *B. cereus* also significantly promoted rice rhizosheath formation under MSD using the additional alkaline soil (Ronghuashan Town, Liaoning Province, 122°86′E, 39°93′N) (Supplementary Fig. S11B).

DISCUSSION

P. indica can enhance rice rhizosheath formation under moderate soil drying

Rice can form rhizosheath under MSD, but not under WW [18], which is different from dryland-farmed crops. Rhizosheath formation under MSD was significantly higher than that under severe soil drying (SSD), which suggested that MSD was better for rice rhizosheath formation (Supplementary Fig. S2C). Thus, in this study, we investigated the effect of *P. indica* on rice rhizosheath formation and identified the factors governing rice rhizosheath formation under MSD. Furthermore, the quantity and quality of root exudates can be affected by drought [72]. Carbon in root exudates can provide energy for soil microbiota [14]. In the present study, the sugars and amino acids of the root exudates, which were related to rhizosheath formation [24], were increased under MSD, compared to WW (Supplementary Fig. S2E). Exopolysaccharide (EPS) produced by bacteria significantly enhanced

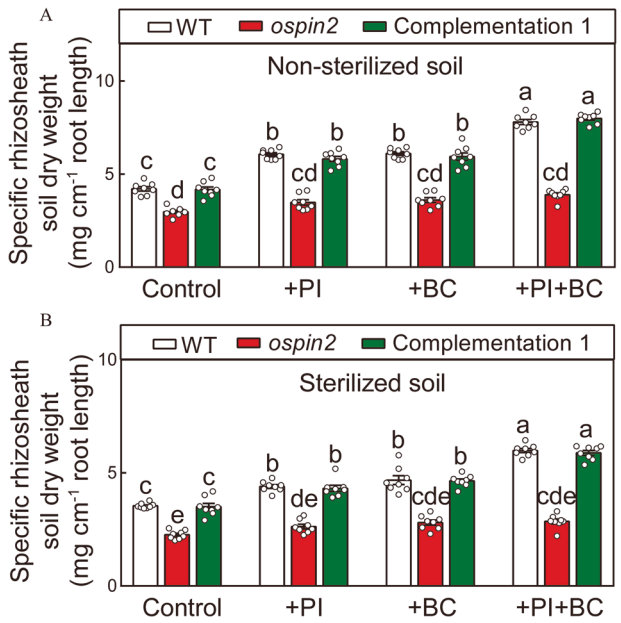


Fig. 5 The rice rhizosheath formation is improved under moderate soil drying (MSD) conditions via co-inoculation with *P. indica* and *Bacillus cereus* by involving auxin efflux carrier *OsPIN2* gene in non-sterilized soil or sterilized soil. The specific rhizosheath soil dry weight of rice seedlings (WT, auxin efflux carrier *OsPIN2* mutant *ospin2* and complementation 1) with non-inoculation (Control), *P. indica* inoculation (+PI), *Bacillus cereus* inoculation (+BC), or *P. indica* and *Bacillus cereus* co-inoculation (+PI+BC) in non-sterilized soil (**A**) or sterilized soil (**B**) under MSD. Data are the means ± SE ($n = 8$ replicates). Bars with different letters are significantly different at $p < 0.05$ (ANOVA, Duncan's multiple range test).

the root-adhering soil per root tissue [73]. Sunflower inoculation of an EPS-producing strain significantly increased root-adhering soil per root mass ratio under drought conditions [74, 75]. Here, the content of rhizosheath EPS under MSD was significantly higher than that of WW (Supplementary Fig. S2D). The results suggest that moderate soil drying can increase rhizosheath formation by regulating root exudation quality and soil EPS extraction.

A previous study suggests that the hyphae of *arbuscular mycorrhizal* fungi show a minor involvement in rhizosheath formation [76]. *P. indica*, which can produce auxin and be involved in root development [26, 30], significantly increased rice rhizosheath formation in two different soil types (acid soil or alkaline soil) under MSD (Fig. 1F, Supplementary Fig. S11B). The probable reason for this finding is that *P. indica* can increase the rice root hair growth to search for water under MSD via auxins modulation, which also provides an important physical framework for rice rhizosheath formation [10, 17, 77].

Auxins are important for the *P. indica*-promoted rhizosheath formation in rice under moderate soil drying

P. indica-induced stimulation of root architecture changes is involved with auxin metabolism or signaling in roots [26, 30]. Rice root IAA concentration was enhanced after *P. indica*-inoculation (Fig. 2A, Supplementary Fig. S11A), which is consistent with results in Chinese cabbage [29] and barley [78]. Further, the rice roots co-inoculated with *P. indica* and *B. cereus* showed the highest IAA concentration (Supplementary Fig. S11A), which suggests that auxins can be produced by the rice plant, fungi and bacteria after inoculation. Auxin plays an important role in root growth for searching of water under MSD [12, 79]. The relative expression of the auxin efflux carrier gene *OsPIN2* in the rice roots was significantly increased with *P. indica* inoculation under MSD, compared to MSD alone (Fig. 2B, Supplementary Fig. S6C), suggesting that auxins are important for rhizosheath formation. Moreover, ethylene related genes were also significantly increased under MSD (Supplementary Fig. S2F), which is consistent with our previous study [18]. In this study, we focused on auxins and rhizosheath formation with *P. indica* inoculation under MSD. Plants tend to rearrange their root architecture in search of water by mediating root auxin under drought conditions [80]. Compared to WW, the relative expression of some auxin related genes under MSD was also significantly increased (Fig. 2B).

When auxin transport was inhibited by NPA (an auxin efflux inhibitor), the total rhizosheath soil dry weight and specific rhizosheath soil dry weight was significantly decreased (Fig. 2C, D). The probable reason for this decrease was the low root hair length under NPA treatment (Supplementary Fig. S7B), which is similar to the findings in our previous study [12]. Numerous studies have shown that *PIN2* is mainly asymmetrically localized at the upper side in the epidermis of the root meristem and elongation zones [81, 82], which in turn results in the suppression of root hair growth. When rice plants (WT and complementation rice) were inoculated with *P. indica*, rhizosheath formation significantly increased, but not in *ospin2* because auxins are not sensed (Fig. 5). The results suggested that auxins are important for *P. indica*-promoted rhizosheath formation in rice under MSD.

The fungus *P. indica* can co-work with the native soil bacterium *Bacillus cereus* for rice rhizosheath formation under moderate soil drying

Bacteria-fungi interactions are common in soil environments [83, 84], and their interactions are significant drivers of important ecosystem functions and services [84]. These interactions allow the respective partners to utilize sparse carbonaceous nutrients, which is beneficial for either or both of the partners [85–87]. Moreover, bacterial communities have a positive effect on plant rhizosheath formation [11, 13]. Here, the relative abundance of *Bacillus* was significantly increased in the rhizosheath and root endosphere with *P. indica* inoculation under MSD, compared with MSD alone (Fig. 4A–C). This is probably because fungal exudates are a source of nutrients for bacteria in the mycosphere under drought stress [42, 65, 88]. *B. cereus* grew toward *P. indica* and grew more in the presence of *P. indica* under sterilized soil and Petri plate conditions (Supplementary Fig. S10C, D), which suggests that *P. indica* may interact with *B. cereus*. Furthermore, rhizosheath formation in WT and the *OsPIN2* complemented rice line inoculated with *P. indica* under non-sterilized soil conditions was significantly higher than under sterilized soil conditions (Fig. 5B). The WT and *OsPIN2* complemented rice line with a combine of *P. indica* and *B. cereus* inoculation showed the greatest rhizosheath formation under MSD (Fig. 5). The results suggest that the fungus *P. indica* can co-work with the native soil bacterium *Bacillus cereus* for rice rhizosheath formation under MSD.

In conclusion, we demonstrate that the cooperation of *P. indica* (fungus) and *B. cereus* (bacterium) can contribute to rhizosheath formation in rice plants under MSD through the involvement of the auxin efflux carrier *OsPIN2* (Supplementary Fig. S12). These results improve our understanding of how plant roots integrate microbial action into drought responses, which will help to identify novel research avenues for improving plant adaptation to changing climate.

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AUTHOR CONTRIBUTIONS

WX and FX planned and designed the research. FX, YZ, JL, LS, XZ, JY, KW, XW, and YD conducted most of the experiments. FX, HL, YZ, MY and CL analyzed the data. FX, HL, CR, JZ, KY and WX wrote the article. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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