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Endophytic community of Pb-Zn hyperaccumulator *Arabis alpina* and its role in host plants metal tolerance

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Abstract

Aims: Endophytes benefit host plants by increasing biotic and abiotic stress tolerance. The aims of this study were to evaluate endophytic community (EC) of *Arabis alpina*, a Pb-Zn hyperaccumulator and investigate role of EC in host plants metal tolerance.

Methods: EC of *A. alpina* growing at Pb–Zn mining area was evaluated by Illumina MiSeq sequencing. Pot experiments were conducted for the role of EC in metal accumulation and tolerance of host.

Results: Fungal EC of shoots showed greater similarity to roots than to seeds; and Chao1 and Shannon indices for shoots and roots were significantly higher than for seeds. Inoculation of EC significantly improved host plants growth under multi-metal stress ($p<0.05$, T test). The shoot length, root length and dry biomass of the treatment were improved when compared with the control. EC inoculation significantly altered accumulation of Pb, Cd and Zn in plant tissues. Particularly decreased the accumulation of Pb ($p<0.05$) and Cd ($p>0.05$) in the shoots of the treatment.

Conclusions: Hyperaccumulator *A. alpina* growing in metals contaminated soils was colonized by a diverse assemblage of endophytic fungi, and the EC played a key role in increasing host plants metal tolerance.

Keywords: Hyperaccumulator, Endophytic community, High-throughput sequencing, Potentially toxic metals, Phytoremediation

Introduction

With the advent of rapid industrialization, urbanization and other anthropological activities such as mining, soils in many places have been contaminated with potentially toxic metals (PTMs). The high level of PTMs in the soil not only deteriorates soil health and changes soil microbial community structure, but also adversely affects plant essential element absorption and translocation, disturbs plant metabolism, and decreases plant growth and reproduction (Kidd et al. 2012; Etesami et al. 2018). Moreover, excess accumulation of PTMs in plants can be transferred to the next level in a food chain, thus affecting multiple levels in an ecosystem (Etesami et al. 2018). Metal pollution has become one of the major environmental problems in the world (Facchinelli et al. 2001; Solgi et al. 2012). To cope with soil metal contamination, phytoremediation is considered as the most promising technology for its simplicity, cost-effectiveness, sustainability and overall positive impact on the environment (Weyens et al. 2009a; Li et al. 2012a; Parmar and Singh 2015). However, application of phytoremediation in metal contaminated soils has some constraints, such as phytotoxicity, slowed plant growth, low biomass production, slow degradation of PTMs, limited contaminant uptake and evapotranspiration of volatile contaminants; therefore application of phytoremediation is limited in most circumstances (Gerhardt et al. 2009; Weyens et al. 2009b; Deng and Cao 2017). Microbe-assisted phytoremediation can effectively resolve these problems. Some microorganisms can effectively improve plant growth by transformation of nutrient elements, production of phytohormones, or provide iron to reduce the deleterious effects of metal contamination to plants (Rajkumar et al. 2010).

Endophytes are microorganisms that live inside internal tissues of the host plant without causing any symptom of disease. In the mutualistic relationship, the host plant allows the endophyte to survive and multiply inside its tissues while the endophyte provides various benefits to the plant, including improving its biotic and abiotic stress tolerance (Li et al. 2012a; Waller et al. 2005; Shahzad et al. 2017). In metal-contaminated environments, endophytes are found to be ubiquitous and some of them can improve host plants metals tolerance, enhance their metals accumulation (Deng et al. 2011; Li et al. 2012b; Shen et al. 2013; Yamaji et al. 2016). The possible mechanism involves enhancement of antioxidative systems, changing metal distribution in plant cells and detoxification of metals, etc. (Wang et al. 2016). However, almost all these results come from studies of 1-3 endophytes (Li et al. 2012b; Ma et al. 2016); while, in a natural ecosystem, endophytes may also interact with other endophytes and affect host plants as a unit (endophytic community). We supposed that the endophytic community as a unit benefits host plants metal tolerance and affects host metal accumulation under metal stress.

Arabis alpina is a Pb-Zn hyperaccumulator that grows naturally in Pb-Zn contaminated soils in Huize County, Yunnan Province, Southwest China (Zu et al. 2005). In our previous work, we have investigated the bacterial endophytic community of *A. alpina* and found that some bacterial endophytes can significantly enhance host plants growth under multi-metal stress (Sun et al. 2018). As a series of work, in the present study, we reported the fungal endophytic community of *A. alpina* recovered by Illumina MiSeq sequencing, and also the role of endophytic community (including fungal and bacterial endophytes) in host plants metal tolerance were evaluated through pot experiments.

Methods

Description of the site and sampling

The sampling site was situated in the town of Zhehai, Huize County, Yunnan Province, Southwest China (26°28′17″ N, 103°37′34″ E), where Pb–Zn mining has been carried out for more than 300 years. In this location there are many areas where soils are polluted with high levels of Pb, Zn, Cd; these soils support only sparse vegetation. *Arabis alpina* is one of the dominant species and has been reported to be a Pb-Zn hyperaccumulator (Zu et al. 2005).

A total of 15 healthy plants of *A. alpina* were randomly selected, each plant growing at least 30 m apart from another. The plants as well as seeds were collected. At the same time, adjacent soil samples were also collected from a depth of 5-10 cm. Each sample was placed separately into a sterile plastic bag, labeled and transported to the laboratory and further processed within 24 h in November 2014.

Culture-independent endophytic fungal community analysis

DNA extraction and PCR Amplification

Plants were washed with running tap water to remove surface debris and other contaminants, and then separated into roots and shoots. Five root and shoot segments from different plants, and 30 seeds were selected randomly and surface-sterilized by sequentially dipping into 0.5% sodium hypochlorite for 2 min, followed by 3 times washing with sterile distilled water, dipping into 70% ethanol for 2 min, rinsing 3X with sterile distilled water,

then drying on sterilized filter paper (Li et al. 2012b). The efficacy of the surface sterilization was checked by following the imprint method. Thereafter, the samples were homogenized in liquid nitrogen. The total genomic DNA was extracted taking approximately 0.2 mg of homogenized powdered samples using the MoBio PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, United States) following the manufacturer's protocol. Extracted DNA was verified by electrophoresis on a 1.5% (w/v) agarose gel. The qualified DNA samples were stored at -20°C for subsequent analyses.

Amplification of the fungal 18S ITS1 (internal transcribed spacer 1) gene region was performed using primer ITS1-F (5 '-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5 '-GCTGCGTTCTTCATCGATGC-3'), the amplification product was about 435 bp. All PCR reactions were carried out with a total PCR mixture of 50 µl, containing 30 ng of the genomic DNA as a template, 4 µl primer mixture, 25 µl NEB Phusion® High-Fidelity PCR Master Mix (New England Biolabs, United Kingdom) and rest milli Q water. PCR was performed under the following conditions: an initial pre-denaturation at 98°C for 3 min, followed by 30 cycles of denaturation at 98°C for 45s, annealing at 55°C for 45s, extension at 72°C for 45s, and a final extension at 72°C for 7 min. The PCR amplicons were verified on 1.5% (w/v) agarose gel. An equal volume of 1X loading buffer (containing SYBR green) along with PCR products were loaded on agarose gel and samples with amplicon bands in the range of about 450 bp were chosen for further analyses. To pool samples, barcoded amplicons were mixed in equimolar concentrations. Pooled barcoded amplicons were then purified using the Qiagen Gel Extraction Kit (Qiagen, Germany) and the non-specific products were removed.

Illumina MiSeq high-throughput sequencing

Sequencing was completed by the Beijing Genomics Institute (Beijing, China). All purified amplicons were used to construct libraries. The final library concentrations and quality were checked using a Qubit 2.0 Fluorometer, Invitrogen and an Agilent Bioanalyzer 2100 system, respectively. Cluster was prepared using Illumina's TruSeq TruSeq PE Cluster Kit v3-cBot-HS, SR Cluster Kit v3-cBot-HS and qualified libraries were sequenced paired-end reads, with the PE250 (PE251+8+8+251) sequencing strategy using a ready-to-load reagent kit (TruSeq SBS Kit v3-HS, Illumina) on the Illumina HiSeq 2000 system following the manufacturer's recommendations.

Analysis of sequence data

The raw pyrosequencing data were obtained in FASTQ files along with sequencing quality files. The raw data was pre-processed as follows: (1) Sequence reads of below average quality of at least 20 over a 30 bp sliding window based on the phred algorithm were truncated, and reads having less than 75% of their original length were trimmed, as well as their paired reads were removed; (2) Reads contaminated by adapter (default parameter: 15 bases overlapped by reads and adapter with maximal 3 bases of mismatch allowed) were removed; (3) Reads with ambiguous base (N base), and their paired reads were removed; (4) Reads with low complexity (default: reads with 10 consecutive occurrences of the same base) were removed. For pooling library with barcode samples mixed, the clean reads were assigned to corresponding samples by allowing 0 base mismatch to barcode sequences with in-house scripts. The data processing results were listed in Supplementary Table S1. Paired-end reads from the original DNA fragments theoretically were merged using FLASH, which is a very fast and accurate analysis tool (Magoč et al. 2011). The detailed method was as follows: (1) Minimal overlapping length: 15 bp; (2) Mismatching ratio of overlapped region: ≤0.1 Paired end reads without overlaps

were eliminated. Removal of primer sequences, the forward and reverse amplification primers were mapped to the two end of tags, if 4 consecutive bases at the 3'-end of the primers matched completely with the tags, and the mismatched bases of the remaining primer was not more than 2, the tags were retained. The tags statistics were presented in Supplementary Table S2. The tags were clustered to OTU (Operational Taxonomic Unit) by scripts of software USEARCH (v7.0.1090) (Edgar et al. 2013). The details are as follows: 1) The tags were clustered into OTU with a 97% threshold by using UPARSE, and the OTU unique representative sequences were obtained; 2) Chimeras were filtered out by using UCHIME (v4.2.40); The ITS sequences were screened for chimeras by mapping to UNITE (v20140703), de novo chimera detection was done for 18S rDNA sequences; 3) All tags were mapped to each OTU representative sequences using USEARCH GLOBAL, then the tags number of each OTU in each sample was summarized to OTU abundance table. OTU representative sequences were provided taxonomical annotation by Naive Bayesian based classifier the Ribosomal Database Project (RDP) Classifier v.2.2, using 0.8 confidence values as cutoff (Wang et al. 2007). The databases used for species annotation were Silva (default): V119 (Quast et al. 2013) and UNITE (default): Version6 20140910 (Abarenkov et al. 2010). The OTUs were filtered by removing the OTUs that were not assigned to the target species. The filtered OTUs were used to downstream processing.

To indicate microbial diversity and richness in plant tissues, the α-diversity indices (including Chao1, Simpson, and Shannon indices) were quantified in terms based on OTU and taxonomic ranks. The indices were calculated by Mothur v1.31.2 (Schloss et al. 2009), and the corresponding rarefaction curve were drawn by R v3.1.1 software. The calculation formula of indices can be referred at http://www.mothur.org/wiki/Calculators. Among them, Chao1 indicate the minimum number of OTUs, Simpson diversity index indicate the richness of the communities, and Shannon index indicate some relation between number of OTU and number of individuals (Spellerberg and Fedor 2003; Akinsanya et al. 2015). Beta diversity was measured by Bray-Curtis calculated with QIIME v.1.7.0. For the differences in sequencing depth in different samples, normalization is introduced: Sequences was extracted randomly according to the minimum sequence number for all samples. Beta diversity heat map was drawn by 'aheatmap' package and clustering of was done through unweighted pair group method with arithmetic mean (UPGMA) and figure were drawn by R software.

The role of endophytic community in host plants metal tolerance

Pot experiments

To obtain sterile seedlings, 200 seeds of *A. alpina* were randomly selected and surface-sterilized as above, and then germinated in trays (10 days, 25 °C and relative humidity of 60%). The sterilized germination tray was filled with a mixture of Canadian sphagnum peat with perlite (7:3, v/v, Fafard, Canada) that had been autoclaved (121 °C, 15 psi for 15 min) 3 times. To maintain moisture the plants were watered every 3 days with sterilized water and fertilized weekly with Peter's General Purpose 20-20-20 fertilizer (Grace Sierra Horticultural Products, Milpitas, USA). About 60 days later, 78 uniform seedlings were selected and transplanted into the pots (10.0 × 4.5 cm; 3 seedlings/pot) containing 200 g autoclaved mixture of field soil with perlite (7:3, v/v). The pots were divided into two groups (GI and GII, 14 replicates for each group) and placed in a greenhouse at 24/20 °C day/night cycle, 60-70% relative humidity, and a photoperiod of 15 h (300 μE). During the culture period, the plants were watered with 25 ml of sterile distilled water every other day, and fertilized weekly with Peter's General Purpose 20-20-20 fertilizer.

To make the inoculation solution, freshly collected plants of *A. alpina* growing naturally from the same sampling site were surface-sterilized, and then 30g plant tissues (whole plant) were cut into pieces and homogenized with sterilized mortar and pestle, dissolved in 200 ml sterilized dH_2O and divided into two equal parts (A and B, and part B was sterilized at 121°C for 15 min). Afterwards, part A and part B were sprayed onto surfaces of GI (the treatment, endophytic community-inoculated, E+) and GII (the control, endophytic community-free, E−) until they were wet, respectively. Inoculation was done 3 times over 15 days (the 1st, 8th and 15th days after the transplanting). To confirm fungal endophytic community colonization into inoculated plants, 3 plantlets were randomly collected from GI and GII three days after the last inoculation, respectively, and were washed under running tap water and surface-sterilized as above. The plants were separated into shoots (above ground parts) and roots (underground parts). Further sample preparation and endophytic fungal community analysis were done as mentioned above. Fungal endophytic communities among natural growing plants, E+ plants, and E- plants were compared, and colonization was confirmed by identification of similar fungal endophytic communities in naturally growing plants (from field populations) and E+ plants; while E− plants showed a unique depauperate group of OTUs.

Effect of endophytic community on host plants growth

After 60 days of the pot experiments, the plants were harvested, washed with tap water and the height and root length of each plant were measured. Thereafter, the plants were separated into roots and shoots and oven-dried at 65°C until constant weight was achieved, and the dry weight of the plants was recorded.

Effect of endophytic community on host plants metal accumulation

The plant samples dried above were crushed to fine powders with a mortar and pestle. The digestion was carried out according to the following procedure: 200 mg powdered sample was mixed with 5 ml HNO_3 (65% w/w) and kept overnight, then digested at 80-90°C for 30 min, 100-110°C for 30 min and 120-130°C for 1 h. After that, 1 ml H_2O_2 (30% w/w) was added and again digested at 100-110°C for 30 min, 120-130°C for 1 h, after cooling the samples were diluted to 50 ml with double distilled water. The collected soil samples were air-dried and sieved through 5 mm sieve followed by crushing with mortar and pestle. The digestion was carried out as per following procedure: 500 mg of soil was added to 4 ml HCl-HNO_3 3:1 (v/v) solution and kept overnight, then digested at 80-90°C for 30 min, 100-110°C for 30 min and 120-130°C for 1 h. After that, 1 ml $HClO_4$ was added and again digested at 100-110°C for 30 min, 120-130°C for 1 h, after cooling the samples were diluted to 50 ml with double distilled water. The concentration of metals in each sample was determined by flame atomic absorption spectrophotometer.

Translocation Factor (TF) or mobilization ratio of metals from roots to shoot has been estimated to determine relative translocation of elements from roots to aboveground parts of *A. alpina*. TF was calculated as follows: TF= $Sh_{(c)}/Ro_{(c)}$ where $Sh_{(c)}$ is concentration of metal element in the shoots and $Ro_{(c)}$ is concentration of metal element in the roots. Bioaccumulation factor (BAF) was calculated separately for the shoot and root of *A. alpina* inoculated with and without endophytic community. BAF was calculated as follows: $BAF_{(s)}= Sh_{(c)}/S_{(c)}$ and $BAF_{(r)}=Ro_{(c)}/S_{(c)}$, where $Sh_{(c)}$ and $Ro_{(c)}$ is the respective concentration of element in the shoot and root and $S_{(c)}$ is concentration of the element in the initial soil.

Statistical analysis

The SPSS (ver. 19) software was used for independent samples T test, alpha = 0.05, $p<0.05$ to confirm significant difference.

Results

Fungal endophytic community of *A. alpina*

The data processing results of Illumina MiSeq sequencing was listed in Supplementary Table S1. The high quality paired-end reads were combined to tags based on overlaps, and 227,872 tags were obtained in total with 25,319 tags per sample on average, and the average length is 269 bp. After the primer sequences were removed, 220,849 tags were remained in total with 24,538 tags per sample on average, and the average length is 227 bp- (Supplementary Table S2). Thus dataset developed consisted of 215,013 filtered high-quality and classifiable unique fungal ITS2 gene tags (Table 1). All sequences were clustered with the representative sequences, and >97% sequence identity cut-off was used; all tags of ITS2 region were classified at each level. In total, 144 different OTUs were detected from 3 tissues of *A. alpina*, belonging to 3 fungal phyla, 12 classes, 26 orders, 37 families, 45 genera and 51 species (excluding one unclassified group). The number of OTUs per sample ranged from 19 to 60 (Table 1), and the highest OTUs were recorded in shoots (94), followed by roots (90) and seeds (29) (Fig. 1). There were 34.02% OTUs shared between the shoots and roots, however, there were only 9.72% and 12.5% OTUs shared between shoots/roots and seeds, respectively (Fig. 1).

Ascomycota was found to be the most dominant phylum in all samples and it had a relative abundance of 89.247±2.073% in roots, 91.151±6.153% in shoots and 99.999±0.002% in seeds (Fig. 2a). Basidiomycota was also observed in all samples, but it was present at a very low relative abundance 0.224±0.052% in roots, 0.442±0.265% in shoots and 0.001±0.002% in seeds. Contrary to this, Zygomycota was only observed in the shoots and roots with a relative abundance of 0.056±0.082% and 0.046±0.04% respectively. At the genus level, *Tetracladium* was the most dominant endophyte in the roots (the relative abundance 3.138±1.767%) and shoots (16.935±4.130%), while, *Alternaria* (74.211±1.132%) was the dominant endophyte in the seeds (Fig. 2b). Although a total of 45 genera were annotated, there were 91.811±1.952%, 77.734±4.278% and 2.11±1.112% of the total OTUs that remained unclassified at genus level in the roots, shoots and seeds, respectively (Fig. 2b). The rarefaction curves of all samples tended to approach the saturation plateau (Fig. 3).

The heat map of the relative abundance of endophytic fungi (EF) indicated that the endophytic fungal community of shoots showed greater similarity to roots than to seeds. Similarly, Beta diversity indicated that the endophytic fungal community of the roots was more similar to shoots than seeds: The roots and shoots clustered to one group, in clear contrast to that of the seeds (Fig. 4). Fig. 5 showed that the α-diversity indices of the roots and shoots were similar, but differed from that of the seeds. The estimated Chao1 and Shannon index of shoots and roots were much higher than that of the seeds, and the highest Chao1 and Shannon index appeared in the shoots, followed by roots and least for the seeds. However, the Chao1 and Shannon indices of roots and shoots showed no significant differences ($P > 0.05$), whereas, Simpson indices of the roots and shoots were significantly different ($P < 0.05$).

The raw sequence data generated from this study have been deposited in National center for biotechnology information (NCBI) SRA (http:// www.ncbi.nlm.ni h.gov/sra) under accession number SRX4219218.

Effect of endophytic community on host plants growth

Pot experiments indicated that inoculation of plants with the endophytic community significantly improved host plants growth under multi-metal stress ($p < 0.05$) (Fig. 6). Compared with the controls, shoot length, root length and dry weight of endophytic community-inoculated plants were increased 38.32%, 41.88% and 85.37%, respectively (Fig. 6).

Effect of endophytic community on host plants metal accumulation

The metal concentration of soils and plants are shown in Table 2. Pre-planting soils and post-harvesting soils for all treatments and controls were heavily polluted by Pb, Zn, and Cd when compared to the 'Environmental Quality Standard for Soils' of China (GB15618-1995, grade III) (Supplementary Table S3). Similarly, concentrations of Pb, Zn, and Cd in both E+ and E- plant tissues exceeded the limiting values of those PTMs in food (Pb and Cd compared with GB2762-2012; Zn compared with GB13106-1991) (Supplementary Table S3). Overall, both the concentrations of Pb and Cd in E- soils were higher than those in E+ ($p > 0.05$). The concentration of Zn in E- soils was a little lower than that in E+ ($p > 0.05$). Compared with E-, in E+ plants, Pb decreased 53.09% in shoots ($p < 0.05$) and 6.21% in roots ($p > 0.05$); Cd decreased 2.24% in shoots ($p > 0.05$) but increased 5.48% in roots ($p > 0.05$); Zn increased 33.19% in shoots ($p < 0.05$), but decreased 20.39% in roots ($p > 0.05$) (Table 2).

The bioaccumulation factor (BAF) and translocation factor (TF) of Pb, Zn and Cd differed in E+ and E- plants (Fig. 7). The BAF of Pb was significantly lower ($p < 0.05$) in the shoots of E+ than E-, while it was only slightly lower ($p > 0.05$) in the shoots of E+ than E-. Similarly, the TF of Pb was also significantly lower ($p < 0.05$) in E+ than E-. The BAF of Zn was significantly higher ($p < 0.05$) in the shoots of E+ than E-, while it was significantly lower ($p < 0.05$) in the shoots of E+ than E-. The TF of Zn was significantly higher ($p < 0.05$) in E+ than E-. The BAF of Cd was lower in the shoots of E+ than E-, while it was higher in the shoots of E+ than E-, but the difference was not significant ($p > 0.05$). The TF of Cd was lower ($p > 0.05$) in the E+ plants than the E- plants.

Discussion

Several factors affect the composition and abundance of endophytes in plants; factors include altitude, humidity, precipitation, temperature, plant community, and host species and plant tissue (Huang et al. 2008; Arnold 2007; Novas et al. 2007). The soil properties, bioavailable metal concentrations in soil and *A. alpina* plant samples from the field are shown in Table 3. In the present study, a total of 144 OTUs were detected from three parts of *A. alpina*, but the composition and abundance of endophytic fungi (EF) in different plant tissues showed significant differences (Table 1, Fig 2). The highest number of OTUs was found in the shoots, then, followed by the roots and seeds (Fig. 1). Consistent with this, the Chao1 and Shannon indices of EF in the seeds were distinctly lower than those of the roots and shoots. These results are consistent with previous findings that the shoots and roots possessed higher diversity of EF than seeds (Vega et al. 2010). Similarly, Qin et al. (2016) also found that seed fungal endophytes of *Suaeda salsa* exhibited lower species richness.

There were 34.02% OTUs shared between the shoots and roots, however, there were only 9.72% and 12.5% OTUs shared between shoots/roots and seeds, respectively. The heat map of relative abundance of EF also

indicated that the fungal endophytic community of shoots showed greater similarity to roots than to seeds (Fig. 2). Similarly, the α-diversity indices of roots and shoots were more similar than that of the seeds (Fig. 5). Beta diversity heat map and cluster tree also supported these results (Fig. 4a, b). Previous studies also found that the number and diversity of seed endophytes are often lower than that of the other plant parts (Vega et al. 2010; Ganley and Newcombe 2006). One hypothesis is that the host plant may increase the fitness of next generation through the selection of certain seed endophytes (Hardoim et al. 2012). Truyens et al. (2013) studied culturable seed endophytes of *Arabidopsis thaliana* for several generations exposed to cadmium and without cadmium, and their results also supported the hypothesis: the selected seed endophytes could improve subsequent germination and early seedling development. Similarly, *Cladosporium cladosporioides* isolated from seeds of a coastal plant *Suaeda salsa* with superior halo-tolerance significantly improved host seed germination and other plant growth in saline soil conditions (Qin et al. 2016).

All of the OTUs were annotated to 3 phyla, 12 classes, 26 orders, 37 families, 45 genera and 51 species. Among them, Ascomycota was found to be the most common EF in all of the three plant tissues, while Basidiomycota and Zygomycota were detected at very low relative abundances (Fig. 2a). This is consistent with the previous finding that Ascomycota was the dominant group of EF in many plant species from various environments (Gazis and Chaverri 2010; Peršoh et al. 2010; Khan et al. 2017). At the genus level, *Tetracladium* was the most dominant endophyte in the roots (3.14%) and shoots (16.94%), while, *Alternaria* (74.21%) was the most dominant endophyte in the seeds (Fig. 2b). *Tetracladium* is an environmentally ubiquitous fungal genus which has evolved mechanisms to adapt and prevail under diverse conditions (Sati et al. 2009; Letourneau et al. 2010; Almario et al. 2017). Miersch et al. (1997) found that *T. marchalianum* from a copper-mine stream showed strong copper resistance. *Alternaria* has been reported as a seed endophyte in various plant species (Fisher and Petrini 1992; Orfali et al. 2017; Shearin et al. 2018). Shearin et al. (2018) found that the seed endophyte *Alternaria* can increase seed germination and seedling growth. Most of EF in *A. alpina* belong to classes Sordariomycetes, Dothideomycetes and Leotiomycetes of Ascomycota. The microbial community of the plant growing in the multi-metal-contaminated natural environment is very complex, and it may contain some new species of EF. Due to sequence information lacking in the database, all of the EF could not be annotated to genus/species level (Fig. 2b).

In pot experiments, it was found that E+ and E- soils were highly contaminated with Pb, Zn and Cd (Table 2). Similarly, the concentrations of Pb, Zn, and Cd in both E+ and E- plant tissues far exceeded limiting values. However, when compared with E- plants, the shoot length, root length and dry biomass of E+ plants increased 38.32%, 41.88% and 85.37%, respectively (Fig. 6). These results demonstrated that under multi-metal stress, the inoculation of endophytic community significantly promoted the growth of *A. alpina*. Moreover, the endophytic community-inoculation also influenced Pb, Cd and Zn accumulation in the plant tissues. Particularly, the accumulation of Pb ($p<0.05$) and Cd ($p>0.05$) in the shoots of E+ were decreased, while, Zn accumulation ($p<0.05$) in the shoots of E+ were increased when compared with E- (Table 2). In the E+ plants, the BAF (shoot) decreased for Pb ($p<0.05$) and Cd ($p<0.05$) as compared to the E- plants, which can be due to decreased translocation of both these metals from roots to the shoots (Fig. 7). On the contrary, in E+ plants the BAF(shoot) increased ($p < 0.05$) for Zn as compared to the E- plants. This resulted due to increased translocation of Zn in E+ plants than the E- plants from roots to the shoots, thus accumulation of Zn decreased in the roots of the E+ plants than the E- plants (Fig. 7, Table 2). It has been suggested that Cd toxicity may be prevented by Zn

application, which probably increases antioxidant enzymes activities, and competes with Cd to bind to –SH groups and membrane proteins (Wu and Zhang 2002; Street et al. 2010; Singh et al. 2016). The antagonistic and synergistic effects of Cd and Zn were also found in other works (Street et al. 2010; Aravind and Prasad 2005).

Previous studies have demonstrated that endophytes can improve host plants growth and change metal accumulation (Li et al. 2011; Ma et al. 2011). However, these results mainly come from the study of 1-3 endophytes (Li et al. 2012a; Ma et al. 2016). While, in a natural ecosystem, single isolate likely does not function separately, but interacts with microbes. The function of an entire endophytic community should have greater impact on hosts than a single or a few endophytic species. In the present study, our results indicated that inoculation of plants with the endophytic community significantly improves host plants growth and changed PTMs accumulation into the host. Further, *Acremonium, Alternaria, Cladosporium, Colletotrichum* etc. that have relatively higher abundance in natural growing *A. alpina* were also observed to internally colonize the inoculated plants (Supplementary Table S4 and S5). This confirms successful colonization of the endophytic community in the treatment. In a recent study, we found that *A. alpina* was also colonized by various bacterial endophytes, and some of them can significantly improve host plants growth under multi-metal stress (Sun et al. 2018). The improved metal tolerance of the E+ plants can be the synergistic effect of both endophytic fungi and bacteria. Future experiments will be necessary to determine how fungal endophytes interact with bacterial endophytes, as well as how single endophyte interacts with other members of the endophytic community. Studies that evaluate the impacts of entire endophytic communities may enable eventual development of applications that take full advantage of a plant's microbiome to enhance plant growth, stress tolerance and crop yields.

Conclusions

A. alpina, a Pb-Zn hyperaccumulator from metal contaminated sites, harbored diverse fungal endophytes. A total of 144 fungal OTUs belonged to 3 phyla, 12 classes, 26 orders, 37 families, 45 genera and 51 species were detected in *A. alpina*. Ascomycota was the dominant phylum in roots, shoots and seeds. The fungal endophytic community of shoots showed greater similarity to roots than to seeds. *Tetracladium* was the dominant endophyte in roots and shoots, while, *Alternaria* was the dominant endophyte in seeds. The Chao1 and Shannon indices for fungal endophytes in the shoots and roots were significantly higher than those indices for the seeds. The total endophytic community is benefit to host plants growth under multi-metal stress, and altered accumulation of Pb, Cd and Zn in the plant tissues, particularly decreased the accumulation of Pb and Cd in the shoots. The study finds future application prospects in the area of enhancing the phytoremediation potential of hyperaccumulators.

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498

499 **Table 1** The unique tags and α diversity of endophytic fungi from *A. alpina* (distance < 0.03)

Sample name	Sample ID/Replicate	Tag number	Observed number of OTU	α diversity		
				Chao1	**Shannon**	**Simpson**
	FR1	21597	60	60.5	1.788505	0.340206
Root	FR2	23045	52	53.5	1.099915	0.567329
	FR3	21893	49	49	1.575964	0.413927
	FS1	24344	57	57	1.633157	0.330019
Shoot	FS2	24402	51	51.25	1.602738	0.299424
	FS3	24611	54	55.5	1.507252	0.365824
	FZ1	24742	19	19	0.799709	0.578958
Seed	FZ2	25375	20	20.75	0.737291	0.599711
	FZ3	25004	20	23	0.784011	0.602816

500 Note: FS: Shoot, FR: Root, FZ: Seed and 1, 2, 3 represent the replicate number of each individual sample
501

502 **Table 2** Concentration of potentially toxic metals in soils and *A. alpina* plant samples

	PTMs concentration* (mg/kg)								
	Root			Shoot			Soil		
	Pb	**Zn**	**Cd**	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**	**Cd**
E+	249.69 ± 1.31 a	5954.85 ± 8.66 a	24.85 ± 0.18 a	46.48 ± 0.55 a	13551.39 ± 777.16 a	16.16 ± 0.68 a	2302.08 ± 202.79 a	34871.10 ± 734.61 a	13.73 ± 0.56 a
E-	266.23± 4.47 a	7480.15±26.70 a	23.56±0.15 a	99.09 ± 10.52 b	10174.16 ± 922.59 b	16.53 ± 1.08 a	2718.75 ± 136.22 a	34215.29 ± 1285.78 a	15.02 ± 0.55 a
Soil							2250.00 ± 31.25	31739.03 ± 453.55	13.73 ± 0.17

503 Note: E+ and E-, the plants inoculated with and without endophytic community, respectively. *Mean ± SE, n =
504 3. The difference between the different letters in the experimental group and the control group showed
505 significant difference ($P < 0.05$).

506

507 **Table 3** The soil properties, bioavailable metal concentrations in soil and *A. alpina* plant samples from the field

	Field soil	Root	Shoot
Bioavailable Pb	355.95 ± 13.52	27.37±0.17	98.9±0.54
Bioavailable Zn	2042.68 ± 53.78	408.03±26.11	502.19±4.02
Bioavailable Cd	1.39 ±0.06	10.28±0.32	10.95±0.23
pH	6.59		
Organic Matter	115.68±5.05		
Total N	1.58±0.26		
Total P	1.01±0.09		
Total K	6.12±0.64		
Hydrolyzable N	84.07±8.9		
Available P	6.9±4.24		
Available K	268.83±19.66		

508 Notes: Values are mean ± standard deviation (n=3)
509

510

Figure legends

Fig. 1 Shared and tissue-specific OTUs. Venn diagrams representing the distribution of OTUs in the shoots (FS), roots (FR) and seeds (FZ) of *A. alpina* (Values summarize OTUs in all three replicates)

Fig. 2 The taxonomic composition distribution and Log-scaled percentage heat map showing relative abundance in different samples at **a** phylum level and **b** genus-level (FS: Shoot, FR: Root, FZ: Seed and 1, 2, 3 represent the replicate number of each individual sample)

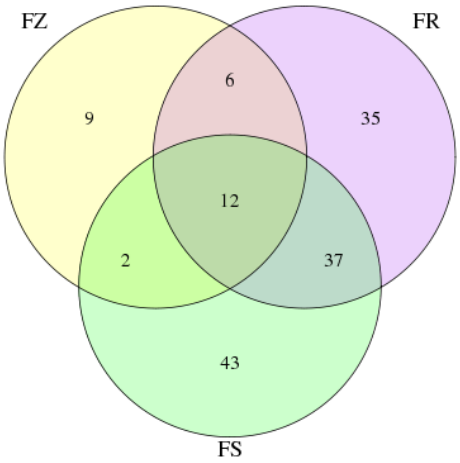
Fig. 3 Sample-based rarefaction analysis (FS: Shoot, FR: Root, FZ: Seed and 1, 2, 3 represent the replicate number of each individual sample)

Fig. 4 Beta diversity heat map **a** UPGMA hierarchical cluster tree **b** showing Bray Curtis of the different samples (FS: Shoot, FR: Root, FZ: Seed and 1, 2, 3 represent the replicate number of each individual sample)

Fig. 5 Box-plot diagrams showing Alpha diversity indices among different groups of samples (FS: Shoot, FR: Root, FZ: Seed; n=3)

Fig. 6 Impact of endophytic community on shoot and root length, and total dry weight of *A. alpina* under metal stress (Mean±SD, n=13); * indicates significantly different ($P < 0.05$)

Fig. 7 Bioaccumulation factor [BAF] (Shoot and Root) and translocation factor [TF] of **a** Pb, **b** Zn, **c** Cd in the plants inoculated with endophytic community (E+) and not inoculated (E-) plants of *A. alpina* (Mean±SD, n=3); variation between E+ and E- were indicated by *$P < 0.05$; ** $P < 0.005$; # $P > 0.05$

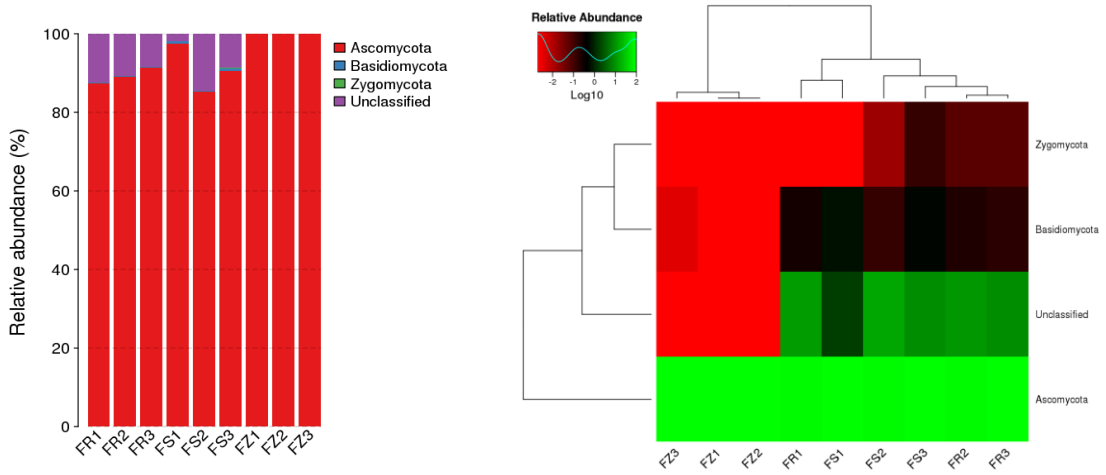


530

531 **Fig. 1**

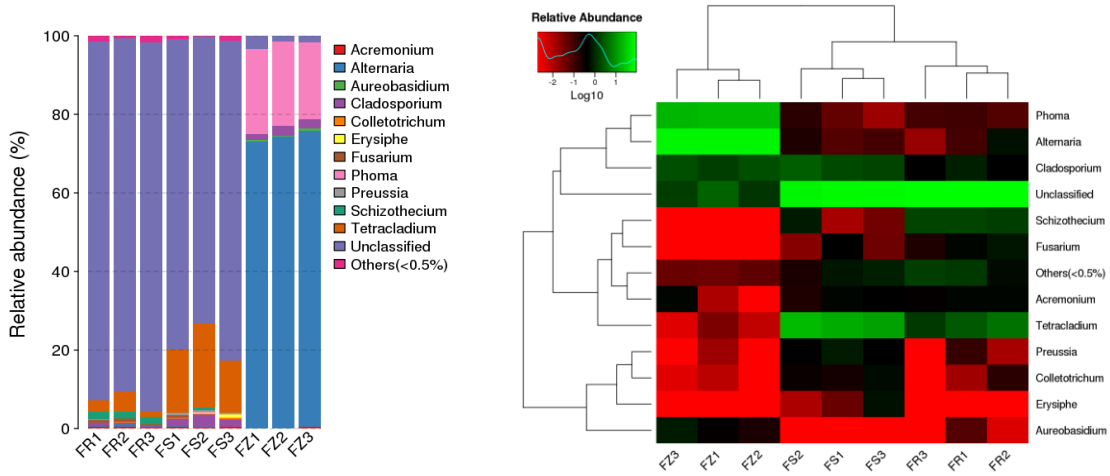
532

533



534

535 **a**



536

537 **b**

538

539 **Fig. 2**

540

541

542

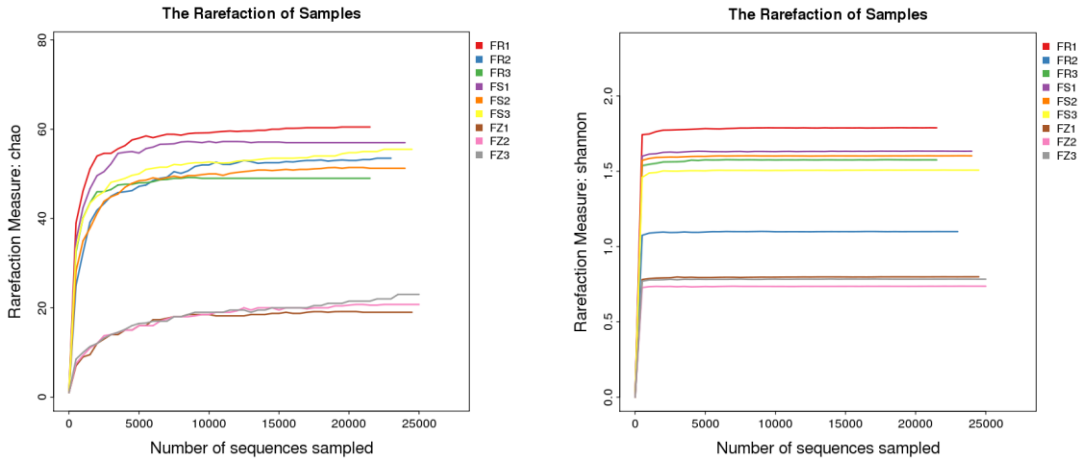
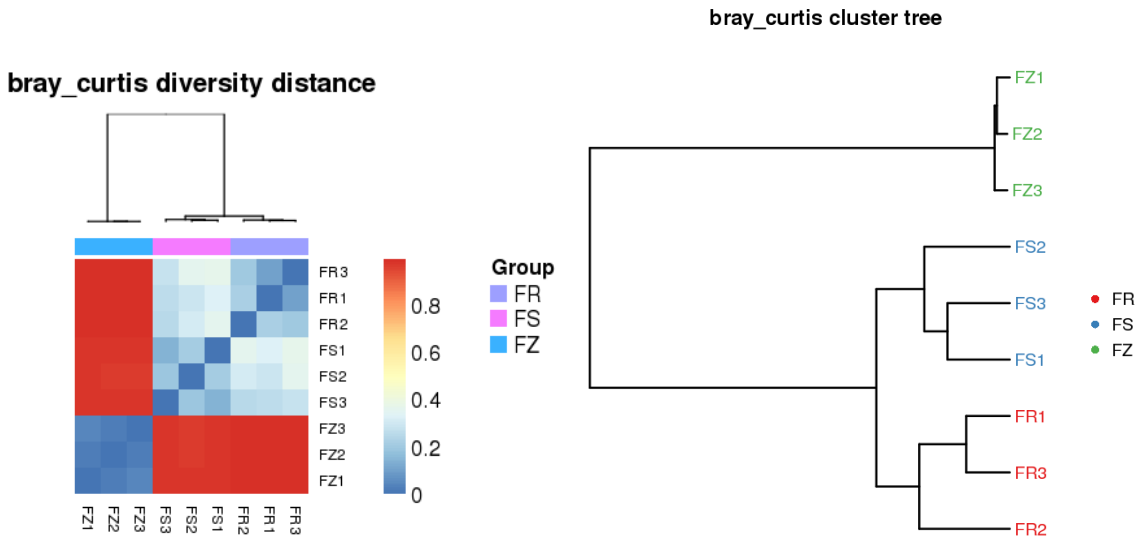

The Rarefaction of Samples
Rarefaction Measure: chao
Number of sequences sampled
FR1
FR2
FR3
FS1
FS2
FS3
FZ1
FZ2
FZ3
The Rarefaction of Samples
Rarefaction Measure: shannon
Number of sequences sampled
FR1
FR2
FR3
FS1
FS2
FS3
FZ1
FZ2
FZ3

Fig. 3



549

550 **a** **b**

551 **Fig. 4**

552

553

554

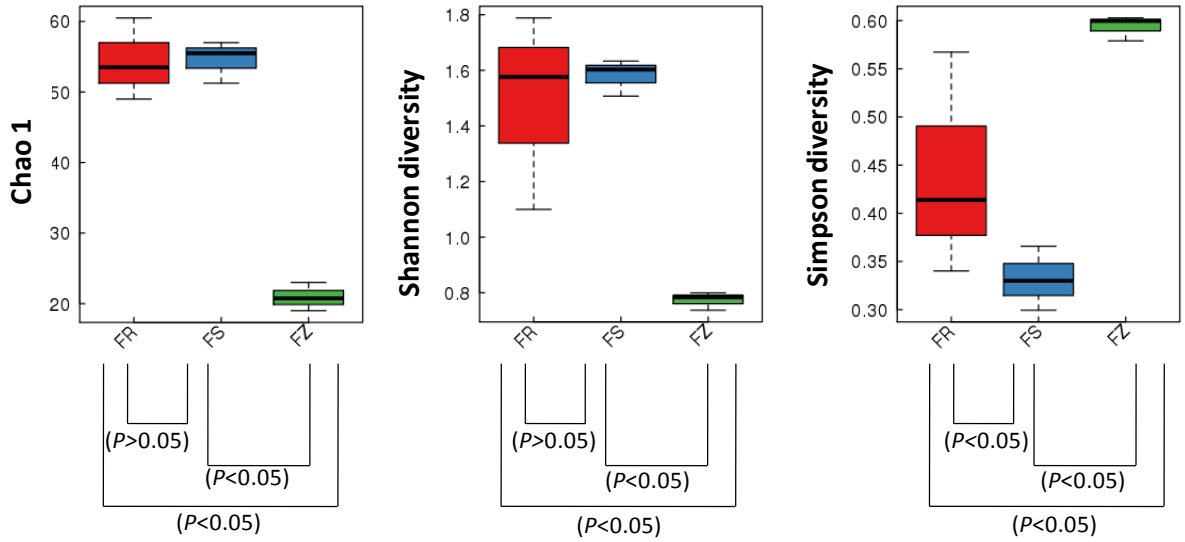
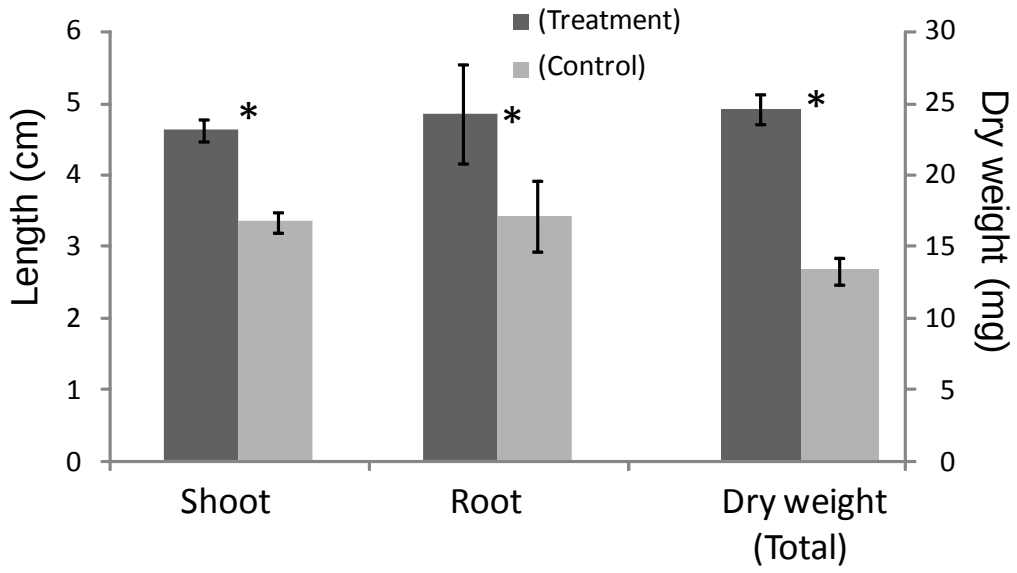

Chao 1
60
50
40
30
20
FR
FS
FZ
(P>0.05)
(P<0.05)
(P<0.05)
Shannon diversity
1.8
1.6
1.4
1.2
1.0
0.8
FR
FS
FZ
(P>0.05)
(P<0.05)
(P<0.05)
Simpson diversity
0.60
0.55
0.50
0.45
0.40
0.35
0.30
FR
FS
FZ
(P<0.05)
(P<0.05)
(P<0.05)

Fig. 5

559

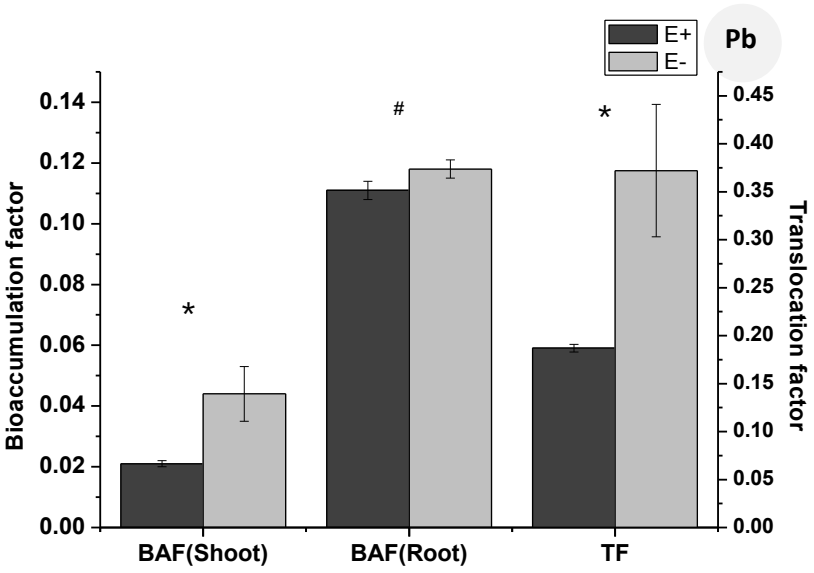


560

561 **Fig. 6**

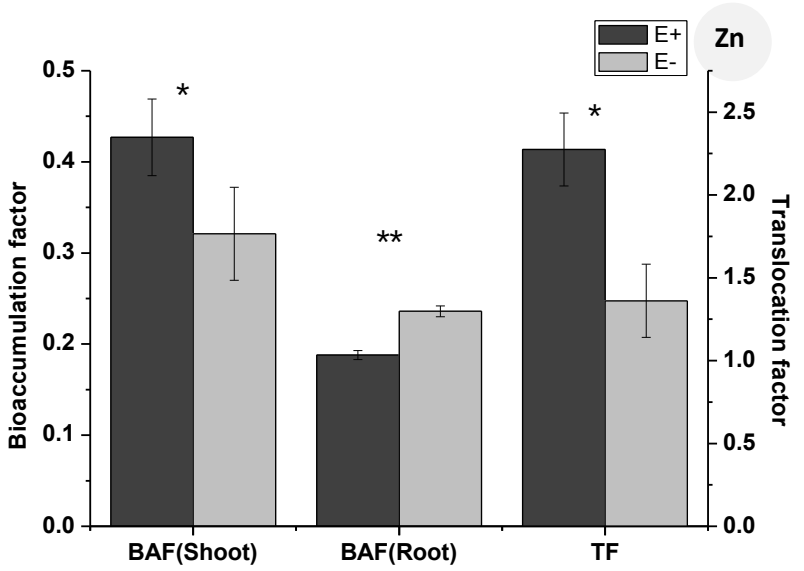
562

563



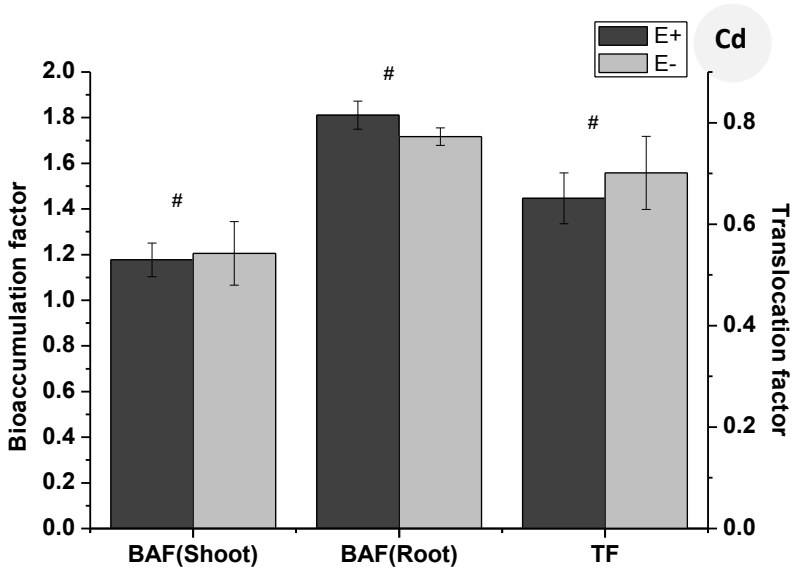
564

565 **a**



566

567 **b**



568

569 **c**

570 **Fig. 7**

Supplementary Table S1 Data statistics

Sample	Sample ID / Replicate	Reads length (bp)	Raw Data (Mbp)	Adapter (%)	N Base (%)	Ploy Base (%)	Low Quality(%)	Clean Data (Mbp)	Data Utilization Ratio (%)	Raw Reads	Clean Reads	Read Utilization Ratio (%)
Root	FR1	248:246	13.34	0	0.182	0.012	5.97	12.39	92.85	27013*2	25148*2	93.1
	FR2	244:244	12.81	0	0.031	0.006	1.109	12.54	97.91	26245*2	25745*2	98.09
	FR3	247:246	14.91	0	0.186	0.028	15.148	12.49	83.78	30242*2	25398*2	83.98
Shoot	FS1	250:244	12.76	0	0.065	0.006	1.182	12.48	97.77	25840*2	25312*2	97.96
	FS2	249:244	12.67	0	0.057	0.004	1.021	12.42	98	25707*2	25239*2	98.18
	FS3	248:244	12.72	0	0.04	0.011	1.313	12.43	97.69	25863*2	25316*2	97.89
Seed	FZ1	249:243	12.61	0	0.03	0.003	1.197	12.36	98.03	25627*2	25163*2	98.19
	FZ2	248:243	12.85	0	0.072	0.004	0.987	12.6	98.09	26162*2	25702*2	98.24
	FZ3	247:243	12.61	0	0.055	0	1.013	12.38	98.16	25740*2	25300*2	98.29

Note: FS: Shoot, FR: Root, FZ: Seed and 1, 2, 3 represent the replicate number of each individual sample

Supplementary Table S2 Tags statistics

Sample	Sample ID / Replicate	Total Pairs Read number	Connect Tag number	Connect Ratio (%)	Average length and SD	Tags without Primer	Tag Utilization Ratio (%)	Average length and SD
Root	FR1	25148	25049	99.61	270±17	24507	97.45	228±17
	FR2	25745	25702	99.83	262±13	23173	90.01	220±12
	FR3	25398	25345	99.79	268±18	24085	94.83	225±16
Shoot	FS1	25312	25223	99.65	265±14	24451	96.6	223±13
	FS2	25239	25102	99.46	268±14	24446	96.86	226±13
	FS3	25316	25311	99.98	265±15	24837	98.11	222±14
Seed	FZ1	25163	25161	99.99	275±11	24836	98.7	233±10
	FZ2	25702	25689	99.95	276±11	25444	99	234±10
	FZ3	25300	25290	99.96	276±10	25070	99.09	234±10

Note: FS: Shoot, FR: Root, FZ: Seed and 1, 2, 3 represent the replicate number of each individual sample

579 **Supplementary Table S3. Environment quality standard of China**

	Pb	Zn	Cd
Food (mg kg $^{-1}$, dry weight)	0.3 [c]	20 [d]	0.2 [c]
Soil (mg kg $^{-1}$, dry weight)	500 [e]	500 [e]	1 [e]

580 [c] The standard of 'national food safety standards' (GB2762-2012).
581 [d] The standard of 'tolerance limit of zinc in foods' (GB13106-1991).
582 [e] The standard of 'environment quality standard for soils'(GB 15618-1995), grade III, pH > 6.5.
583

584 **Supplementary Table S4. Relative abundance of endophytic fungi in the roots of field plants and treated**
585 **plants**

	Relative abundance (Mean±SD)	
	FR	TR
Unclassified	91.779±1.985	21.360±17.149
Tetracladium	3.136±1.763	0.031±0.053
Schizothecium	1.813±0.023	0.000±0.000
Others(<0.5%)	1.330±0.548	1.231±0.588
Cladosporium	0.587±0.279	27.705±10.330
Fusarium	0.480±0.257	0.089±0.154
Acremonium	0.459±0.083	43.831±28.712
Alternaria	0.259±0.349	0.151±0.148
Colletotrichum	0.062±0.096	0.001±0.002
Preussia	0.052±0.078	0.003±0.005
Penicillium	0.029±0.025	0.228±0.070
Coniothyrium	0.006±0.010	0.235±0.247
Volutella	0.005±0.008	0.260±0.437
Peziza	0.003±0.003	3.299±2.455
Erysiphe	0.000±0.000	0.077±0.065
Sarocladium	0.000±0.000	1.114±1.774
Simplicillium	0.000±0.000	0.386±0.244

586 Note: n=3; The fungi of which abundance is less than 0.5% in all samples were classified into 'others'; F: Field
587 plants, T: Treatment (Inoculated plants), R: Root
588
589

Supplementary Table S5. Relative abundance of endophytic fungi in the shoots of field plants and treated plants

	Relative abundance (Mean±SD)	
	FS	TS
Unclassified	77.744±4.285	24.444±6.615
Tetracladium	16.929±4.142	0.000±0.000
Cladosporium	2.297±0.747	6.564±1.219
Others(<0.5%)	0.875±0.422	0.172±0.051
Preussia	0.543±0.174	0.000±0.000
Acremonium	0.393±0.155	65.254±5.658
Colletotrichum	0.389±0.169	0.000±0.000
Schizothecium	0.269±0.425	0.001±0.002
Erysiphe	0.236±0.356	0.515±0.473
Fusarium	0.177±0.246	0.014±0.012
Alternaria	0.132±0.079	0.033±0.027
Penicillium	0.015±0.026	1.279±1.005
Coniothyrium	0.000±0.000	0.000±0.000
Peziza	0.000±0.000	1.261±0.850
Sarocladium	0.000±0.000	0.071±0.029
Simplicillium	0.000±0.000	0.385±0.124
Volutella	0.000±0.000	0.007±0.012

Note: n=3; The fungi of which abundance is less than 0.5% in all samples were classified into 'others'; F: Field plants, T: Treatment (Inoculated plants), S: Shoot