



# Prevalence of antibiotic resistance genes and bacterial pathogens along the soil–mangrove root continuum

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## ABSTRACT

Plants roots are colonised by soil bacteria that are known to be the reservoir of antibiotic resistance genes (ARGs). ARGs can transfer between these microorganisms and pathogens, but to what extent these ARGs and pathogens disseminate from soil into plant is poorly understood. Here, we examined a high-resolution resistome profile along the soil–root continuum of mangrove saplings using amplicon and metagenomic sequencing. Data revealed that 91.4% of total ARGs were shared across four root-associated compartments (endosphere, episphere, rhizosphere and unplanted soil). Rather than compartment-selective dynamics of microbiota, the resistome was disseminated in a continuous fashion along the soil–root continuum. Such dissemination was independent of underlying root-associated bacterial and fungal microbiota, but might be facilitated by a multiplicity of mobile genetic elements. As the multiple-drug resistant pathogens, *Vibrio vulnificus*, pathogenic *Escherichia coli* and *Klebsiella pneumoniae* consistently predominated across four compartments, indicating the potential dissemination of antibiotic pathogens along the soil–root continuum. Through deciphering the profile and dynamics of the root-associated resistome and pathogens, our study identified the soil–root continuum as an interconnected sink through which certain ARGs and pathogens can flow from soil into the plant.

## 1. Introduction

Soil is a rich reservoir of antibiotic resistance genes (ARGs) (Monier et al., 2011). These ARGs can disseminate across ecosystems and are acquired by pathogens capable of affecting human health (Nesme and Simonet, 2015). The past decade has seen an increasing interest in the soil-borne antibiotic resistome (comprising all of ARGs and their precursors) (Wright, 2007) and pathogens (Delgado-Baquerizo et al., 2020; Domínguez-Begines et al., 2020), as well as their dissemination risks via soil applications and ecological processes (Forsberg et al., 2012; Nesme and Simonet, 2015). Unraveling the migration traits of antibiotic resistance determinants from soils to their surrounding habitats is crucial, not only from a perspective of public health risk, but also to facilitate the development of adequate control measures. The soil–root interface is a metabolically interconnected ecosystem where beneficial soil-borne bacteria can selectively multiply within roots and modulate plant growth, while soil-borne pathogens are largely excluded (Hardoim

et al., 2008; Santhanam et al., 2015). Despite increasing recognition of ARGs' rapid proliferation in soil (Nesme and Simonet, 2015; Bengtsson-Palme et al., 2018), systematic field studies assessing the potential risks of soil resistome for plants, and tracking antibiotic resistance determinants along a soil–root continuum are limited.

The soil–root continuum comprises multiple spatially-separable microhabitats with distinct assemblages of highly active and coordinated microbiota (Bulgarelli et al., 2012; Edwards et al., 2015; Thiergart et al., 2020). An example is root-associated microbiota that is largely structured by immune systems of plants and rhizodeposits, which can improve nutrient bioavailability and improve stress tolerance of their plant host (Mendes et al., 2011; Berendsen et al., 2012; Bulgarelli et al., 2013). These unique interactions distinguish root-associated microbial communities from the surrounding soil biota. Furthermore, the niche differentiation of root-associated microbial communities highlights the critical gating role of the soil–root interface (Edwards et al., 2015). With widespread and highly transmissible ARGs among microbiota (Segawa

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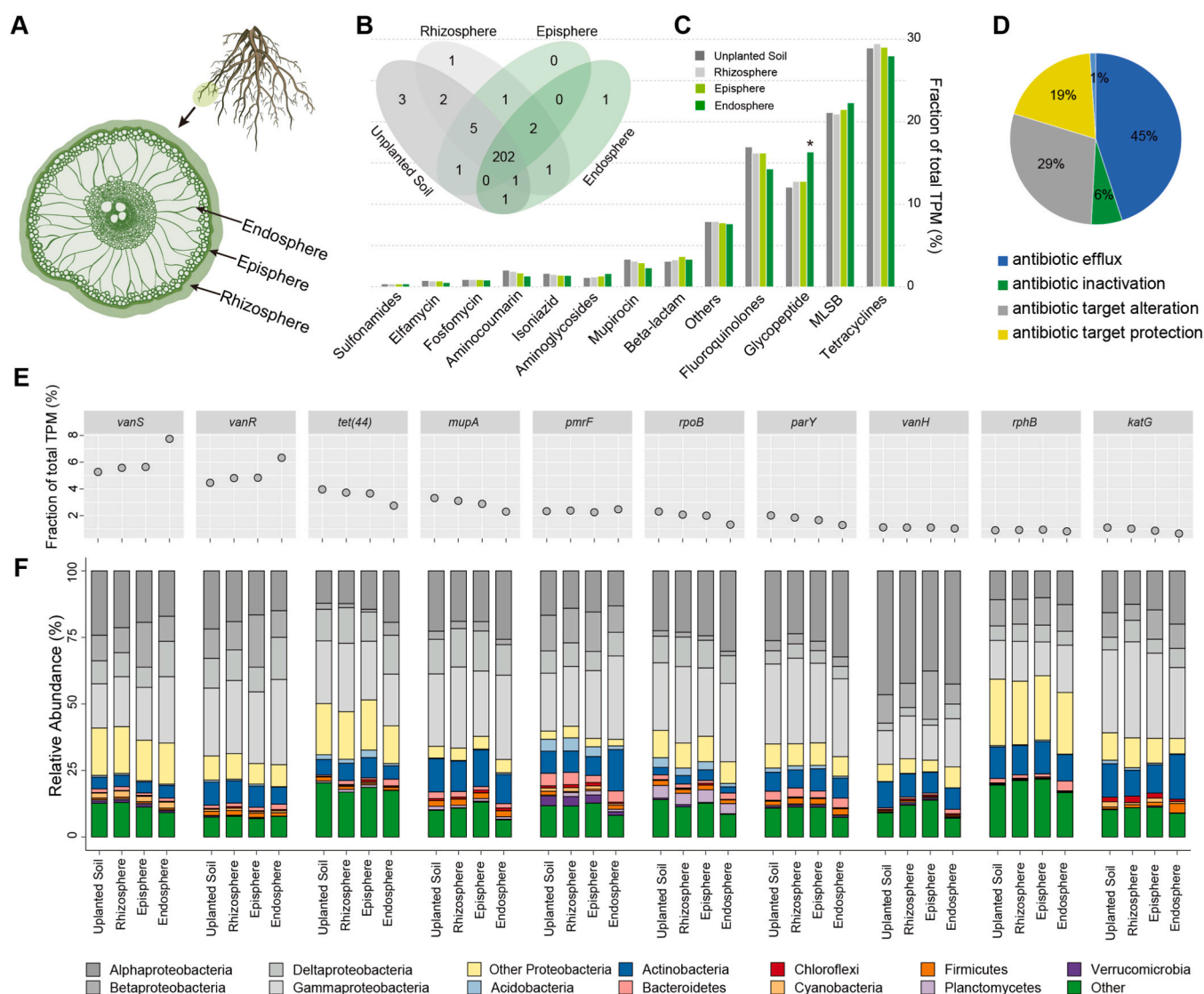
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et al., 2013; Berendonk et al., 2015; Chen et al., 2017a), there is an increasing interest in whether a large fraction of soil-borne ARGs in the soil-root continuum may enter and proliferate in the root interior, or if ARG dissemination is significantly restricted by the gating role of soil-root interface. Reliable information that accurately characterizes the resistome profile along the soil-root continuum is essential to define the pathways of ARG transmission, and to support treatment decisions and health risk assessment frameworks (Ashbolt et al., 2013).

Mobile genetic elements (MGEs) including plasmids, integrons and insertion sequences act as facilitators in the ARG prevalence in various environments (Wright et al., 2008; Ciric et al., 2011). This is because microorganisms can capture ARGs housed on MGEs via horizontal gene transfer (HGT) (Martínez, 2008). Furthermore, the mobile resistome can easily spread among species including clinical pathogens, which deserve more public health concerns (Canton, 2009). However, little is known regarding the pool of ARGs, MGEs and pathogens in root-associated microbiome, as well as their dissemination along the soil-root continuum. As a result, it would be advantageous to provide a comprehensive picture regarding the fate and dissemination of root-associated

resistome by simultaneous analysis of both ARGs and MGEs.

In this study, mangrove roots were chosen since the mangrove ecosystem harbored a high abundance of antibiotic resistance microbes and served as a hot spot for discovering novel metabolites with antibacterial, antifungal, or antibiotic activity (Amrita et al., 2012; Cabral et al., 2016). We, here, applied metagenomic sequencing to evaluate and compare a high-resolution profile and the dynamics of antibiotic resistome along a soil-mangrove root continuum among four root-associated compartments (endosphere, episphere, rhizosphere and unplanted soil; Fig. 1A). SourceTracker analyses were conducted to determine the pathways of resistome along the soil-mangrove root continuum. Resistome profiles were also related to mangrove root-associated bacterial and fungal communities, as well as MGEs, to identify the factors that influence resistome dissemination in mangrove root ecosystems. Specifically, in the soil-mangrove root continuum, pathogenic bacteria that pose severe risks to public health were detected – as they are known to resist multiple classes of antibiotics (Martínez, 2009). Our study illustrates the profile and dissemination of antibiotic resistome and pathogens from soil to mangrove root, while enhancing our knowledge on the



**Fig. 1.** The profiles of antibiotic resistome were shared by four root-associated compartments in the soil-root continuum. (A) A representation of *Kandelia obovata* root cross-sections depicting the microbial communities sampled from rhizosphere, episphere and endosphere compartments. (B) Venn diagram depicting an extensively shared antibiotic resistome among four root-associated compartments. (C) Resistome profiles shown at the resistance class level for four compartments. TPM is calculated for ARGs; significantly different TPM values ( $P < 0.05$ ; two-tailed fisher exact test) are indicated by an asterisk. (D) Relative abundance of ARGs assigned to major mechanisms for antibiotic resistance. (E) and (F) Top 10 resistance determinants and their phylogenetic source in the soil-root continuum.

ecological importance of mangrove root-associated microbiota in regulating biotic stress.

## 2. Material and Methods

### 2.1. Site description and sample collection

Sampling sites were located at the Dianbai District (N 21°30'38.82'', E 111°0'37.27''), Maoming, Guangdong, China, where natural mangrove communities with an area of 150 ha were established in 1999 (Peng et al., 2016). With afforestation over seven years, Dianbai District has become one of the largest mangrove bases in China, greatly improving the coastal ecological environment (Huang, 2010). Mangrove communities at the Dianbai District were dominated by the native mangrove species (*Kandelia obovata*) (Peng et al., 2016), and our former investigation showed that this species performed better in maintaining long-term ecological effects than the alien mangrove species (Yu et al., 2020). *Kandelia obovata* was therefore applied as the test material for this study, and in April 2019, we collected three individual saplings of *Kandelia obovata* at an early fruiting stage. The samples were stored in a portable cooler at 4 °C and transported back to the laboratory within 24 h. In order to investigate the resistome profile in a soil-root continuum, the root-associated samples were fractionated into unplanted soil, rhizosphere, episphere and endosphere compartments. These were processed as described by Durán et al. (Durán et al., 2018). Briefly, unplanted soil was shaken off the roots to leave about 1 mm of soil around the roots. The remaining 1 mm of soil was removed using sterile water and was classed as the rhizosphere compartment. The clean roots were then washed three times to remove any remaining soil and placed in a 1 x tris-EDTA (TE) buffer in a 50 mL Falcon tube. Epiphytic samples were removed from root systems using extensive shaking in TE buffer supplemented with 0.1% Triton X-100. These washes were filtered through 0.22 µm pore size membranes and kept as the episphere compartment. Afterwards, roots were surface-sterilized for 1 min in 80% EtOH, followed by a second sterilization step for 1 min in 0.25% NaClO, to obtain the endosphere compartment. The four root-associated compartments' materials (unplanted soil, rhizosphere, episphere and endosphere) were stored at -80 °C until DNA extraction.

### 2.2. DNA extraction of root-associated microbial communities

Approximately 0.5 g of unplanted and rhizosphere soil (each in triplicate) were used for DNA extraction by using the Power Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions with the modified sodium dodecyl sulfate extraction method (Zhou et al., 1996). The episphere and endosphere compartments' DNA was extracted using the Power Water DNA Isolation Kit (MoBio, Carlsbad, CA, USA) and the Power Plant DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA), respectively, after thorough grinding with liquid nitrogen. DNA quality was assessed using a Nano Drop ND-2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) based on 260/280 and 260/230 nm ratios (Tu et al., 2016). DNA samples of sufficient quality were stored at -80 °C for amplicon library preparation and metagenomic sequencing.

### 2.3. PCR amplification and sequencing of 16S rRNA and ITS1 gene amplicons

The V3-V4 region of bacterial 16S rRNA gene was amplified with the primer pair: forward primer, 5'-ACTCTACGGGAGGCAGCA-3'; reverse primer, 5'-GGACTACHVGGGTWTC TAAT-3' (Peng et al., 2017). The ITS1 region of fungal ITS gene was amplified with the primer pair: forward primer, 5'-CTTGGTCATTAGAGGAAGTAA-3'; reverse primer, 5'-GCTGCGTTCTTCATCG ATGC-3' (Blaalid et al., 2013). Both forward and reverse primers were tagged with Illumina adapter sequence, a primer pad, and a linker sequence. The PCR products were quantified

using a Quant-iT™ dsDNA HS Reagent and then pooled equally for each sample. Sequencing was performed using the HiSeq platform (2 × 250 paired ends; Illumina, San Diego, CA, USA) at the Biomarker Technologies Corporation (Beijing, China).

Raw sequences were first processed using Trimmomatic (Marc et al., 2012) and FLASH (Tanja and Salzberg, 2011). During the filtering, the sequences were trimmed with a moving window of 50-bp and a quality threshold score of 30 (Wu et al., 2015). After removing singlets, paired 16S rRNA amplicon sequences were clustered into operational taxonomic units (OTUs) by UPARSE (Edgar, 2013), based on a 97% pairwise identity using QIIME's (QIIME: Quantitative Insights into Microbial Ecology) (Caporaso et al., 2010) open reference OTU picking strategy with the Greengenes 16S rRNA database (v.13.5) as a reference (DeSantis et al., 2006). The sequences matching "Chloroplast" and "Mitochondria" were eliminated from the whole data set. ITS sequences were processed using ITSx (Bengtsson-Palme et al., 2013) and clustered at a 97% sequence identity by UPARSE (Edgar, 2013). Fungal OTUs were checked for chimeric sequences using the Uchime reference against a dedicated chimera detection database (Nilsson et al., 2015), which was based on the UNITE database for the molecular identification of fungi. The nucleotide sequences were deposited in SRA database under accession numbers PRJNA613787 and PRJNA613792.

To detect pathogens in four root-associated compartments, the 16S rRNA sequences for each sample were also processed and aligned via the 16SPIP pipeline against a human pathogen 16S rRNA sequence database, which consists of 29,258 sequences representing 346 bacterial species (Miao et al., 2017). We used the BWA-MEM algorithm for sequence alignment, and the sequence with similarity higher than 99% to reference sequences was identified as taxa of potential human health concern.

### 2.4. High-throughput sequencing and annotation of root-associated microbiota metagenomes

High-throughput sequencing of root-associated microbiota metagenomes was performed using Illumina HiSeq2500. Prior to DNA sequencing, a library was constructed using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's instructions. The raw reads containing three or more "N" or contaminated by adapters (> 15 bp overlap) were removed, and the filtered clean reads (about 11.8–12.5 Gb per sample) were used for metagenomic analyses. The metagenomic assembly was performed using Megahit (Li et al., 2015) in default mode. MetaGeneMark (v 2.10) was employed to predict genes from the assembled contigs, and redundancy was removed using CD-HIT Software (Fu et al., 2012).

The abundance and diversity of ARG families in the metagenomic contigs datasets were analysed by screening the antibiotic resistance protein families with the HMMER package (version 3.1b1) (Finn et al., 2011) against the Comprehensive Antibiotic Resistance Database (CARD 2020) (Alcock et al., 2020). A predicted open reading frames (ORFs) was annotated as an ARG according to its best BLASTn hit that had a nucleotide sequence identity above 90%, with a threshold e-value of 10<sup>-5</sup> (Kristiansson et al., 2011). We applied taxator-tk (version 1.3.3), which was designed to perform taxonomic analysis of assembled metagenomes (Dröge et al., 2015), to predict the bacterial origin of contigs and assign taxonomy of ARG-containing contigs at the phylum level. To characterise MGEs in the soil-mangrove plant continuum, metagenomes were searched for signatures of known integrons, insertion sequences and plasmids against INTEGRALL (1447 integrase genes and 8053 gene cassettes) (Moura et al., 2009), ISfinder (2578 sequences, 22 families of insertion sequences) (Siguiet et al., 2006) and NCBI RefSeq database (2408 plasmid genome sequences) (Pruitt et al., 2007). A contig was identified as an integron or insertion sequence if the BLAST hit (BLASTn, e-value cut-off: 10<sup>-5</sup>) had a sequence identity of > 90% (Kristiansson et al., 2011). The threshold of identified plasmids was determined as the BLAST hits (BLASTn, e-value cut-off: 10<sup>-5</sup>) with a nucleotide sequence

identity of > 95% (Kristiansson et al., 2011). In order to calculate the relative abundance of ARGs and MGEs in our metagenomic samples, the raw reads were mapped back to the scaffolds using bbmap v3.7.81 (Bushnell, 2014). The read counts for genes were subsequently normalized into transcripts per-million (TPM) counts. The TPM values could be applied to metagenomes to eliminate the effects of total read counts and gene lengths when the abundances of genes between samples were compared (Ribicic et al., 2018). The metagenomic data was deposited in the SRA database under accession number PRJNA613873.

## 2.5. Statistical analyses

Statistical enrichment of a given gene between two datasets was determined by pairwise comparisons using two-tailed Fisher's exact test, with confidence intervals at 99% significance and Benjamini–Hochberg correction ( $P < 0.05$ ). SourceTracker, a Bayesian approach, was used to attribute antibiotic resistome or microbial communities in an environmental sink to various potential sources, and to estimate the probability in which each source contributed to the sink communities (Knights et al., 2011). The unknown was the ARG or taxa that could not map to the input sources (Knights et al., 2011). The percentage value was derived from the statistical average of the SourceTracker results. Procrustes tests for correlation analysis between ARGs and microbial communities were performed in R3.6.0 (<https://www.rproject.org/>) with vegan (Oksanen et al., 2013). The significance was verified using the protest function to compare the  $M^2$  values with 9999 permutations. Hierarchical clustering analysis and 'fan' tree were performed with correlation distance and average-linkage method using the R package of 'pheatmap' (Szekely and Rizzo, 2005).

## 3. Results

### 3.1. An extensively shared antibiotic resistome along the soil–root continuum

The reservoir of ARGs in the root-associated microbiota within the four compartments was characterised by metagenomic analyses. Two hundred and twenty-one unique ARG types were detected, and majority of the detected ARGs (91.4%) were shared among the four compartments (Fig. 1B). These persistent genes were predominant in total resistome and encoded resistance to tetracyclines, MLSB (Macrolide-Lincosamide-Streptogramin B), glycopeptide, and fluoroquinolones (Fig. 1C). The main mechanisms of resistance were antibiotic efflux (45%), target alteration (29%) and protection (19%) (Fig. 1D). The ten most abundant ARGs in the soil–root continuum were *vanS*, *vanR*, *tet* (44), *mupA*, *pmrF*, *rpoB*, *parY*, *vanH*, *rphB* and *katG* (Fig. 1E). The dominance of ARGs resistant to vancomycins, tetracyclines and fluoroquinolones may result from the high concentration of their corresponding antibiotic residues in mangrove ecosystems (Li et al., 2016). Surprisingly, not only at the ARG level, but also across the entire resistance classes, the resistome patterns were highly similar across the four root-associated compartments, highlighting an extensively shared profile of antibiotic resistome along the soil–root continuum.

Next, we predicted microbial source phylum of each selected ARG in the soil–root continuum, which identified Proteobacteria and Actinobacteria as the most prevalent phyla inferred from resistance-conferring reads (Fig. 1F). Within the predominantly root-inhabiting Proteobacteria (Fig. S1), an over-representation of Alphaproteobacteria and Gammaproteobacteria were noted as potential microbial hosts of the antibiotic resistome (Fig. 1F). Consistent with the resistome profile in the soil–root continuum, the microbial phylogeny of the resistome also appeared consistent across the four compartments. This finding is evident especially with specific affiliations of *pmrF* and *rpoB* to Verrucomicrobia and Planctomycetes, whose occurrence and abundance were consistent across the four root-associated compartments.

To determine if there are ARG types responsible for the resistome

differentiation between compartments, we conducted the differential ARGs abundance analysis by using a likelihood ratio and two-tailed Fisher's exact test. Only a small subset of ARGs (15.4%) was more abundant in the endosphere than any other compartment (Fig. 2A). The analysis revealed their potential resistance to beta-lactam antibiotics (21.9%), aminoglycosides (16.6%), and fluoroquinolones (12.5%) (Fig. 2B). The endosphere-specific ARGs resulted in a clearly detached branch of the endosphere resistome that was distant to the other three compartment resistomes, as revealed in a 'fan' tree (Fig. 2C) and the hierarchical clustering (Fig. S2). However, most of the predominant ARGs in soil–root continuum were located around the center of ternary plots – with an almost equal contribution from each compartment (Fig. 2A). This indicates the dissemination of the dominant ARGs in the soil–root continuum.

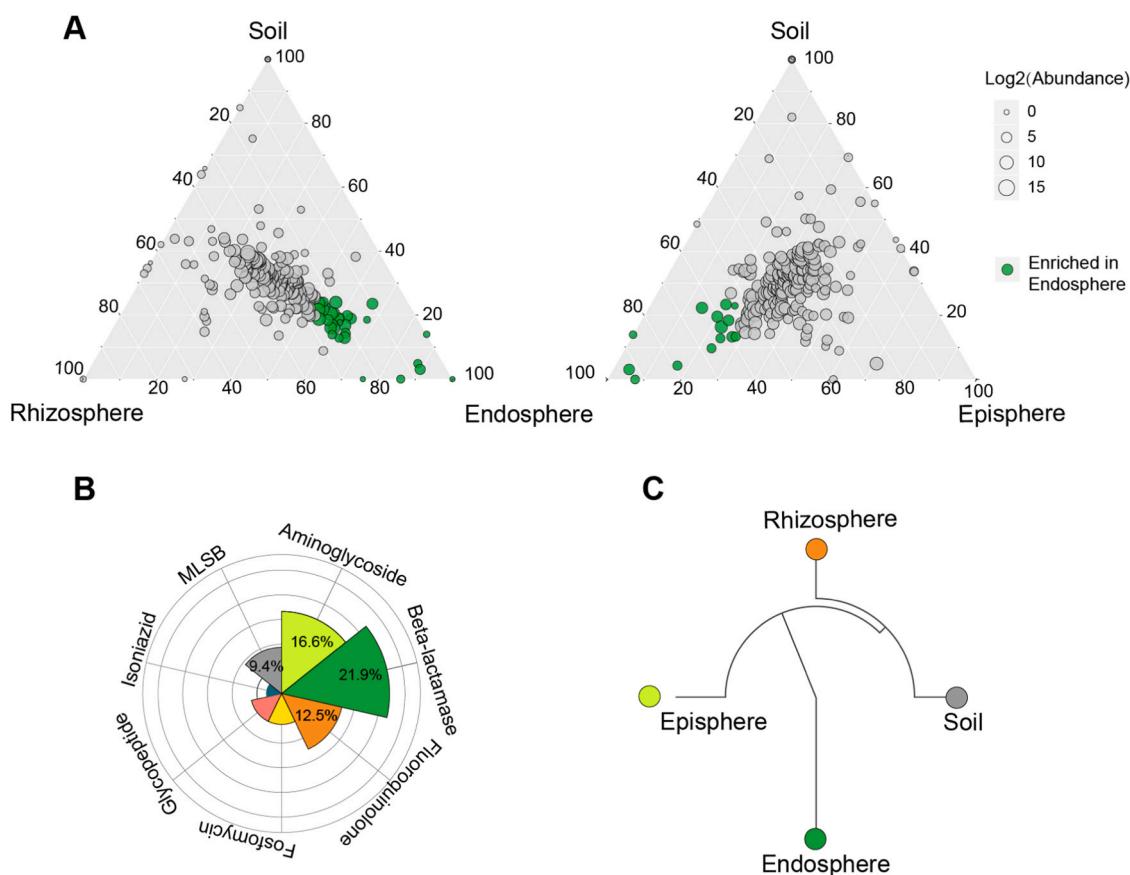
### 3.2. A compartment-continuous but microbiota-independent dissemination of resistome along the soil–root continuum

SourceTracker was used to study the proportion of endosphere resistome and microbial communities derived from exterior compartments. Data indicated a dissemination of antibiotic resistome along the soil–root continuum (Fig. 3A). The dissemination was continuous from soil to plant, where the vast majority of the ARGs in the interior compartment (~61.6%) occurred in the neighbouring exterior compartment. Taking the endosphere as an example, 51.4% were derived from the episphere while the unknown only contributed to 2.6%. However, such compartment-crossover of the resistome was inconsistent with the compositional shifts of the root-associated microbial community (Fig. 3B and C). The majority of the endosphere microbial community (54.3% for bacteria and 33.0% for fungi) were derived from rhizosphere compartment, but there were no episphere-derived bacteria and only traces of episphere-derived fungi (1.3%). Compared to the resistome, the microbial community in endosphere were derived from a larger proportion (69.5%) of the unknown source. These findings revealed that rather than compartment-selective dynamics of root-associated microbiota (Bulgarelli et al., 2012), antibiotic resistome displayed a spatially-continuous and relatively stable dissemination along the soil–root continuum. This further implied a considerably lower influence of the soil–root interface on the dynamics of antibiotic resistome, as opposed to its influence on microbial communities.

To investigate whether the composition of the underlying root-associated microbiota dictates the resistome, we performed a post-hoc procrustes analysis (Peres-Neto and Jackson, 2001) by computing and visualising the structural correlations of resistome with bacterial and fungal communities. Bray-Curtis distances calculated from resistome abundance metrics at the resistance class level displayed no significant correlations with bacterial OTUs (sum of squares  $M^2 = 0.1232$ – $0.4271$ ,  $P > 0.05$ , 9999 permutations) and fungal OTUs ( $M^2 = 0.0781$ – $0.3826$ ,  $P > 0.05$ , 9999 permutations) (Fig. 3D), inferred from 16 S rRNA and ITS sequence data. This indicates that the persistent resistome dissemination along the soil–root continuum was independent of microbial phylogeny.

### 3.3. The dissemination of resistome were accompanied by a multiplicity of MGEs in the soil–root continuum

To investigate the underlying mechanism of ARG disseminations, we characterized the occurrence and abundance of MGEs including plasmid, integron and insertion sequences along the soil–root continuum. Metagenomic analyses show the average ratio of integron, insertion sequence and plasmid to the total ARGs abundance in the soil–root continuum was 34:1, 6:1, and 134:1, respectively (Fig. 4A). As with ARGs, MGEs exhibit a similar profile across the four root-associated compartments. For each compartment, Class 1 integrase gene *intI1*, IS3 and pMaq22A.1p were the predominant integron, insertion sequence



**Fig. 2.** Resistome differentiation between compartments at the ARG level. (A) Ternary plot of all ARGs. Each circle represents one ARG. The size of each circle represents its log2 relative abundance. The position of each circle is determined by the relative contribution of the indicated compartments to the total resistome abundance. Green circles denote ARGs significantly enriched in the endosphere compartment ( $P < 0.05$ ; two-tailed fisher exact test). (B) Relative abundance of ARGs significantly enriched in the endosphere compartment at the resistance class level. (C) Bray-Curtis similarity-based 'fan' tree showing resistome composition for four root-associated compartments.

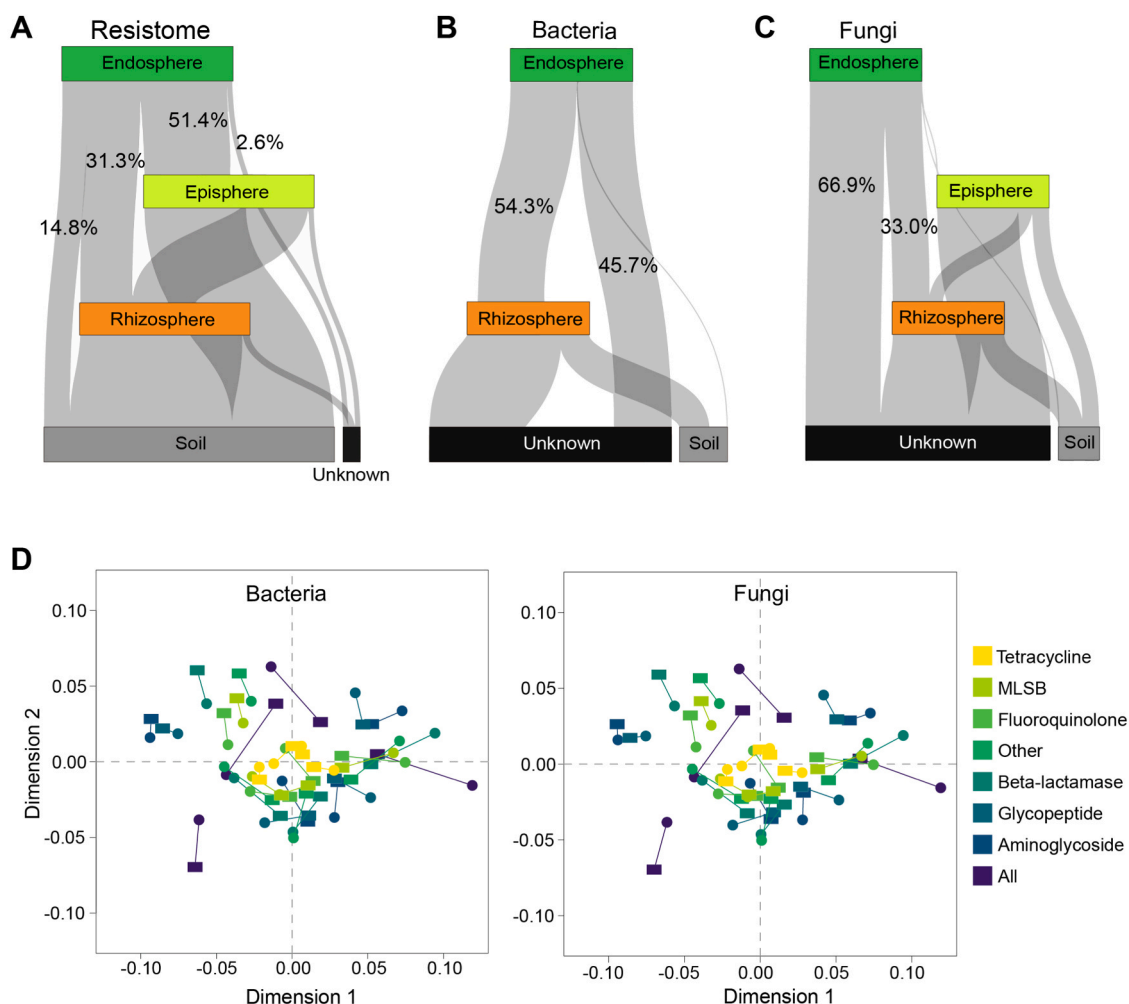
and plasmid, respectively (Fig. 4B). These MGEs are known to be associated with ARGs in many ecosystems (Mahillon and Chandler, 1998; Wang et al., 2015; Stedtfeld et al., 2017), making them a common mechanism for HGTs (Heuer et al., 2011). Besides, we also observed the differentiation of MGE types across four root-associated compartments. For instance, the plasmid abundance in endosphere is much higher than other compartments (Fig. 4A), and *Leptolyngbya boryana* NIES-2135 plasmid3 tends to be enriched in endosphere compartment (Fig. 4B). As the main component of Insertion sequences (IS), both IS3 and IS21 family members have active horizontal transmission (Mahillon and Chandler, 1998), and they have lower abundance in endosphere than that in three peripheral compartments ( $P < 0.05$ ; two-tailed fisher exact test) (Fig. 4B).

In order to assess the potential for ARGs to be transferred among microbiota in soil-root continuum, ARG-like tags were extracted from all sequencing data sets and aligned against the databases of integrons, insertion sequences and plasmids. The results demonstrated that 12.4% and 27.8% of ARG-like tags in soil-root continuum was also assigned to the integrons and plasmids, respectively (Fig. S3). Although no ARG-like tags were assigned to the insertion sequences, Bray-Curtis distance calculated from abundance metrics of resistome has a significant correlation with insertion sequences (sum of squares  $M^2 = 0.06573$ ,  $P < 0.05$ , 9999 permutations) (Fig. S4). Together, our study for the first time explored the MGE profiles across the soil-plant continuum, while identifying high levels of MGEs potentially facilitating the dissemination of resistome via HGTs.

### 3.4. Prevalence and flux of pathogens along the soil-root continuum

There is increasing evidence of antibiotic resistance transfer to pathogens (Hinnebusch et al., 2002; Blake et al., 2003; Forsberg et al., 2012), however, along the soil-root continuum, the profile and dynamics of soil-borne pathogens with potential human health risks have not been established. In the current study, we identified 31 clinically relevant pathogens, and their sum of the abundance was 0.08%, 0.57%, 0.62% and 0.21% in the total bacterial community of unplanted soil, rhizosphere, episphaere and endosphere, respectively. The most abundant pathogenic bacterium was *Vibrio vulnificus*, which consistently predominated across four root-associated compartments (Fig. 5A). This naturally occurring estuarine bacterium is a major concern in aquatic environments and human health (Baker-Austin et al., 2009), since it could show multiple-antibiotic resistance to ampicillin, penicillin and tetracycline due to misuse of antibiotics to control infections in aquaculture production (Elmahdi et al., 2016). Moreover, pathogenic *Escherichia coli* and *Klebsiella pneumoniae* that are multidrug-resistant and extensively regarded as threats to public health (Fischbach and Walsh, 2009), were also consistently detected across four root-associated compartments (Fig. 5B).

SourceTracker was used to elucidate whether these pathogens fit the replenishment process of soil-borne resistome along the soil-root continuum. The soil-borne pathogens served as the potential sources to quantify their contribution to the assembly of antibiotic-resistant pathogens in the latter three compartments. Different with the continuous drift of the whole resistome, the majority of endosphere pathogens (67.0%) were derived from the unknown source and 21.9% were from



**Fig. 3.** Dynamics of the antibiotic resistome and microbial communities along the soil-root continuum. (D) Procrustes analyses showing no significant correlation between resistome composition and bacterial community (left), and between resistome composition and fungal community (right). SourceTracker analyses illustrating the proportion of endosphere resistome (A), bacterial community (B), and fungal community (C) derived from exterior compartments.

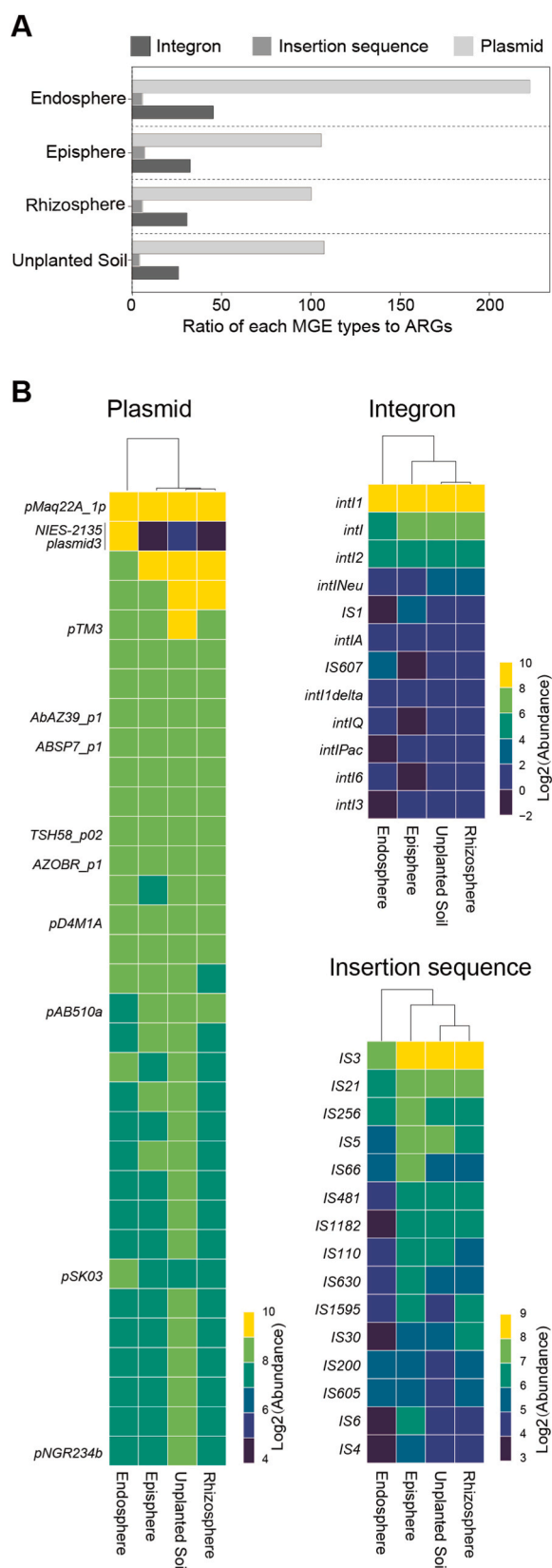
the soil. Interestingly, the episphere compartment had no detectable contributions for the assembly of endosphere pathogens. This is evident, as we observed a higher proportion of pathogens and an over-representation of *Acinetobacter lwoffii*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Afpia broomeae* in episphere (Fig. 5A). One possible explanation for such pattern is that pathogens can be attracted by specific compounds released from plant tissues (such as acetosyringones, vanillic acids), which facilitated the germination, chemotaxis and directional growth of pathogens towards the plant roots (Cho and Winans, 2005; Wu et al., 2008). Together with the observation of shared dominant pathogen taxa across four root-associated compartments, our data demonstrate that the boundary of soil-root interface exerts a quantitative control on the flux of pathogens from soil to plant.

#### 4. Discussion

Early studies have demonstrated the vast functional capabilities of the root-associated microbiota for plant performance in response to biotic and abiotic stressors (Hartman et al., 2017; Fitzpatrick et al., 2018). So far, our recognition of the fate and dissemination of the antibiotic resistome and pathogens across the root-associated microbiota is still limited. This study represents, to the best of our knowledge, the first attempt to monitor and predict the assembly of overlapping antibiotic resistome and pathogens across four compartments of a soil-root continuum, namely: the unplanted soil; rhizosphere;

episphere; and endosphere. Moving from the outside toward the internal sections of the mangrove root, each compartment was observed to contain a large subset of the resistome from the neighbouring exterior compartment. This points to a continuous fashion by which the resistome was disseminated along the soil-root continuum. Combined with the fact that 91.4% of total ARGs were shared across four root-associated compartments, our findings provide strong evidence for previously common assumptions that ARGs would transfer from soil to plant via rhizosphere (Chen et al., 2017b; Chen et al., 2019).

Our early study has revealed a strictly selective role of soil-mangrove root interfaces in shaping the root-associated microbial community structure, especially for the fungal community, via an amplification-selection model (Zhuang et al., 2020). However, in this study, our SourceTracker analyses showed that the vast majority of the ARGs in the interior compartment (~61.6%) occurred in the neighbouring exterior compartment (Fig. 3a), suggesting that a considerably lower influence of the soil-root interface on the dynamics of antibiotic resistome than on microbial communities. In line with our findings, previous studies have determined that soil derived ARGs are important sources of vegetable resistome (Chen et al., 2017b; Zhang et al., 2019), and indicated that these ARGs might transfer upward from root endophyte to leaf endophyte through the interior of lettuce tissue (Zhang et al., 2019). Through examining the relation between root-associated resistome and microbiome, our study further determined that soil-borne ARGs can be disseminated into plant via root in a compartment-continuous but



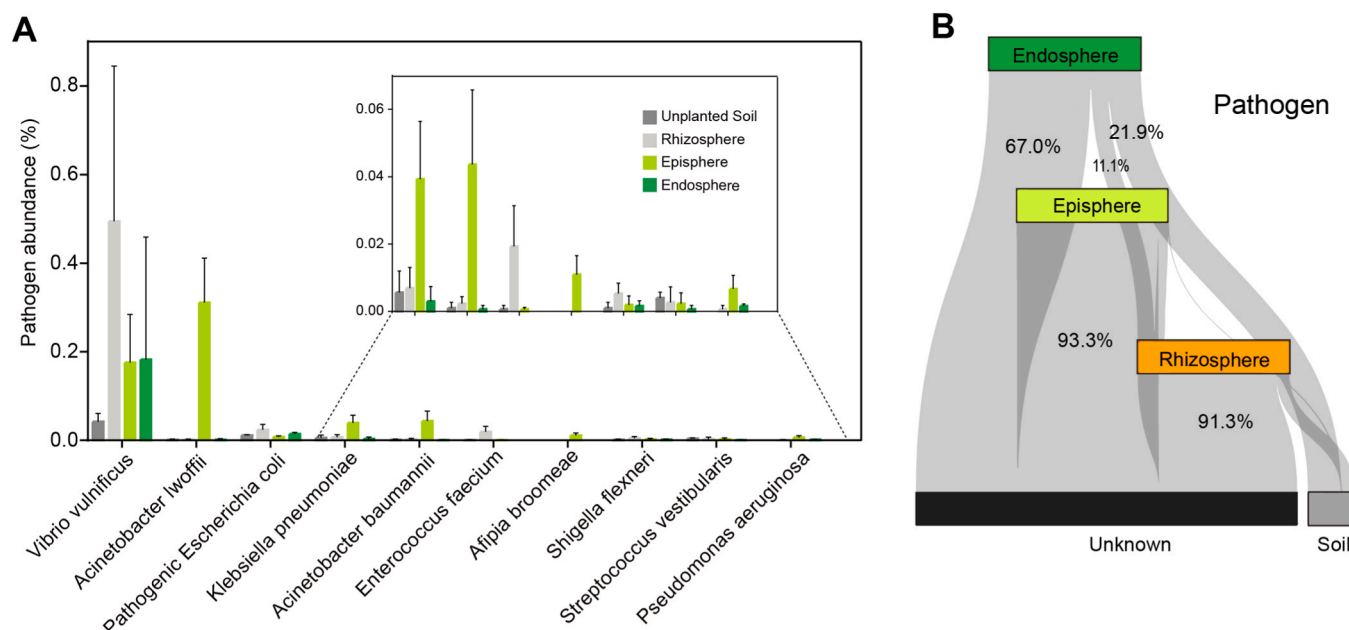
**Fig. 4.** Abundance of the mobile genetic elements (MGEs) along the soil-root continuum. (A) The ratio of integrons, insertion sequences and plasmids to resistome abundance in four compartments. Abundances were obtained via BLAST against INTEGRALL, ISfinder and NCBI Plasmid Genome Database. (B) Heatmaps depicting the log2 abundance of integrons, insertion sequences and plasmids along the soil-root continuum.

microbiota-independent manner. Undoubtedly, this raises an emergent health concern regarding the potential risks of soil-borne resistome migration to plant tissue and even to human food chain, which needs further investigations.

There are two possible modes for persistent dissemination of antibiotic resistome along the soil-root continuum. The first is the transmission of soil-borne microorganisms that carry ARGs into their plant host in a horizontal manner via the environment (Reinhold-Hurek and Hurek, 2011; Van der Meij et al., 2018). Such transmission would be less prominent in this study, as the soil-root interface acts as the transitional boundary for root microbiome assembly – there were no episphere-derived bacteria and only traces of episphere-derived fungi for the assembly of endosphere microbial community (Fig. 3b and c). The second is the direct transmission of ARGs between the soil-borne and the root-associated microbiota. For this mode of transmission, HGT plays a key role in the dissemination of antimicrobial resistance in microbial ecosystems (von Wintersdorff et al., 2016). This may be the primary transmission path for the persistent dissemination of antibiotic resistome along the soil-root continuum in our study. Firstly, the persistent resistome dissemination along the soil-root continuum was independent on microbial phylogeny, based on our correlation-based procrustes analyses (Fig. 3D). Secondly, we found that four root-associated compartments contained the high levels of MGEs (Fig. 4). Gene *int11* was the most ubiquitous integrone among resistant bacteria and is generally considered important in the emergence and wide dissemination of ARGs (Wang et al., 2015). IS3 family member, predominating in insertion sequences, was assumed to have active horizontal transmission (Mahillon and Chandler, 1998). Thirdly, it was acknowledged that the rhizosphere is a hot spot for HGTs due to cells clusters, biofilms formation, enhanced nutrient input and water fluxes (Van Elsas et al., 2003). In the present study, because of the limited information of HGT events, future studies are needed to provide a more direct evidence of HGT along the soil-root continuum.

It was reported that most ARG-carrying plant microbes are fortunately non-pathogenic (Zhang et al., 2011). However, in this study, we observed that four root-associated compartments consistently carried multiple-drug resistant pathogens, including *Vibrio vulnificus*, pathogenic *Escherichia coli* and *Klebsiella pneumoniae* that were extensively regarded as threats to public health (Baker-Austin et al., 2009; Fischbach and Walsh, 2009). This raises an emerging concern that the ARGs prevalent in the soil-root continuum were likely to spread to human pathogens or opportunistic human pathogens via HGT. Consistent with our concern, a previous study has determined the widespread dissemination and exchange of antibiotic resistance genes between soil microbes and human pathogens, and proposed a shared mechanism of HGT between soil and pathogenic bacteria (Arias and Murray, 2009). Therefore, the soil-root continuum potentially serves as a previously neglected vehicle for expanding the pool of antibiotic resistance available to human pathogens. On the other hand, we also identified a quantitative control that the soil-root interface exerted on the flux of pathogens from soil to plant, which was consistent with the previous view that soil-borne pathogens could be somehow excluded from plant root (Hardoim et al., 2008; Santhanam et al., 2015). This therefore raises an interesting question of whether the control effect could potentially reduce the plant burden of antibiotic resistance dissemination via clinical pathogens, and the clinical risks of plant resistomes to human health should be further assessed and emphasized (Chen et al., 2019).

In addition, investigating the factors affecting antibiotic resistance is crucial for better understanding the dissemination of antibiotic resistome in the soil-root continuum. For example, heavy metals are prevalent in mangrove soils and are known to play an important role in disseminating bacterial antibiotic resistance (Yan et al., 2017). In this study, we occasionally detected a cluster of heavy metal resistance genes flanked by multiple genetic contexts of mobile elements in our metagenomics datasets (Fig. S5). This co-existence of heavy metal and antibiotic resistance in the soil-root ecosystems highlights that selective



**Fig. 5.** Prevalence and flux of pathogens along the soil-root continuum. (A) Relative abundance of top 10 pathogens across four root-associated compartments. Pathogens were detected via the 16SPIP pipeline against a human pathogen 16S rRNA sequence database. (B) SourceTracker analyses illustrating the proportion of endosphere pathogens derived from exterior compartments.

effects of heavy metals or other inducers on antibiotic resistance is also a facet worth exploring in future research.

## 5. Conclusions

In this study, we described the profile and dynamics of antibiotic resistome and pathogens across four compartments of a soil-root continuum, namely: the unplanted soil; rhizosphere; episphere; and endosphere. We observed that 91.4% of total ARGs were shared across four root-associated compartments, and identified a continuous fashion by which the resistome was disseminated along the soil-root continuum. Such dissemination was independent of underlying root-associated bacterial and fungal microbiota, but might be facilitated by a multiplicity of mobile genetic elements. In addition, we observed the prevalence and flux of antibiotic pathogens along the soil-root continuum, with the dominance of multiple-drug resistant *Vibrio vulnificus*, pathogenic *Escherichia coli* and *Klebsiella pneumoniae*. Together, our findings identified the soil-root continuum as an interconnected sink through which certain ARGs and antibiotic-resistant pathogens can flow from soil into the plant.

## CRedit authorship contribution statement

**Cheng Wang:** Investigation, Data curation, Formal analysis, Writing - original draft. **Ruiwen Hu:** Methodology, Writing - review & editing. **P. J. Strong:** Methodology, Writing - review & editing. **Wei Zhuang:** Investigation, Writing - review & editing. **Weiming Huang:** Investigation, Writing - review & editing. **Zhiwen Luo:** Methodology, Writing - review & editing. **Qingyun Yan:** Methodology, Funding acquisition. **Zhili He:** Methodology, Supervision, Funding acquisition. **Longfei Shu:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2020.124985](https://doi.org/10.1016/j.jhazmat.2020.124985).

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