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Targeted Manipulation of Vertically Transmitted Endophytes to Confer Beneficial Traits in Grapevines

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Abstract: Vertically transmitted endophytes (VTEs) with multi-host-supporting functions are considered plant-acquired heritable traits, which can be manipulated to develop plants with the stable inheritance of these VTEs, defined here as 'plant endophytic modification (PEM)'. To translate this hypothetical strategy into agricultural and horticultural practice, a PEM was carried out by introducing an anti-fungal pathogenic bacterium, *Bacillus cereus* (strain ID: ZX-2), into grapevine cuttings and growing the cuttings into vine plants. Bacterial strain XZ-2 is highly efficient in infecting grapevine cuttings and colonizing the interior of the infected cuttings, various parts of the subsequently established vine plants, and next year's emerging vine shoots and leaves. Profiling the endophytic microbiota by high-throughput sequencing to the grapevines revealed that the colonization with exogenous ZX-2 slightly affected endophytic diversity, while significantly altering the composition and the predicted phenotypes of endophytic microbiota in ZX-2-modified grapevines (ZX-2mg). Most importantly, leaves (from both first- and second-year grapevines) of ZX-2mg conferred significantly higher ($p < 0.001$) anti-fungal pathogen abilities and a reduction in naturally occurring lesion area than leaves compared to the control grapevines. For all detected vines, a significant correlation (N = 37, r = 0.418; $p < 0.01$) between fungal pathogen inhibition rates and *B. cereus* (ZX-2) isolation rates was observed. In addition, ZX-2mg showed some growth promotion and a delay (15–20 days) in leaf abscission. The work established an alternative strategy to create plant lines with functions of specific VTEs via PEM, confirming the practical value of PEM in future organic farming systems.

Keywords: vertically transmitted endophytes (VTEs); cutting treatment; plant endophytic modification (PEM); acquired heritable trait; disease resistance; organic farming

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1. Introduction

The colonization of plants by endophytes greatly enhances the genomic and metabolic characteristics of plants, providing the host plants with a range of essential life-supporting functions or improving their survival strategies [1]. The plants, in turn, provide nutrients and habitats that support the survival of the host endophytes [2]. Because of the multiple benefits of plant–endophyte interactions, studies of endophytes are extensive and some of these endophytes have been used in agricultural and horticultural practices as microbial agents for biological control and growth promotion [3]. However, the efficacy and persistence of most of the used endophytic agents in the agricultural system are very limited due to the stability of the effects.

The endophytes within certain plants can be horizontally transmitted endophytes (HTEs) or vertically transmitted endophytes (VTEs). On the other hand, the symbiosis of endophytes with their host plants could be facultative or obligate [4]. Obligate endophytes require plant tissues to complete their life cycle, whereas facultative endophytes periodically colonize internal plant tissues [5]. To some extent, plant preference for VTEs with obligate traits may imply that these endophytes have exceptional functions from which plants could derive long-term benefits. The biotechnologies developed to purposefully manipulate and manage these valuable endophytic resources in sustainable agriculture could facilitate a second green revolution [6].

Seed-borne endophytes are an important concern for VTEs due to their potential presence in emerging progeny plants and play an important role in these plants during seed germination and early seedling establishment [7]. Core seed endophytes have been shown to be vertically transmitted in rice and several other crops, opening up new opportunities to improve plant fitness by manipulating the seed-associated microbiome [8–10]. Similarly, in vegetative propagated crops, vegetative propagules (e.g., shoot tips or cuttings) carrying endophytes can also undergo vertical transmission and confer functions equal to or greater than those conferred by seeds on host plants [11–13]. Compared to the seeds of most plants, vegetative propagules have the advantage of larger volumes and a lower threshold for accommodating larger quantities and more diverse endophytes. Our previous study showed that plants dominantly inherit functionally important endophytes from stock plants via in vitro-cultured plantlets [13]. In addition, most plant–bacteria interactions are revealed in the long-term, resulting in a fairly stable microbiome composition throughout the vegetative phase [14]. As a result, some of the stably symbiotic seed-borne or vegetative propagule-borne VTEs could be considered a type of plant "acquired heritable trait" [15,16] and can be manipulated to "breed" plants with the traits of specific functional VTEs, which we refer to as "plant endophytic modification (PEM)" [13]. Instead of modifying genes through plant genetic modification (PGM), PEM involves modifying the VTEs of a plant to achieve the long-term or even heritable effects of these VTEs.

To date, PEM has been proposed to have great prospects for application in agriculture and horticulture, while practical cases demonstrating this are lacking. In the present work, a successful PEM is carried out using grapevine as an example.

2. Material and Methods

2.1. Candidate VTE Strains and Grapevine Cuttings

Three candidate endophytic microorganisms *Bacillus cereus* (strain ZX-2, Genebank accession No. OQ216591), *Pantoea ananas* (strain P1, Genebank accession No. OQ216615), and *Didymella* sp. (strain YA, Genebank accession No. OQ195745), were previously isolated and identified by comparing their ITS or 16S rRNA sequences in the NCBI database, and these endophytic operational taxonomic units (OTUs) were confirmed as VTEs in our previous study. These microbial strains were preserved as the collection of our lab (laboratory of plant–microbe interactions, Yunnan university). The VTE strains were subcultured on plates using appropriate culture media [ZX-2 and P1 in peptone beef agar (PBA) medium (1000 mL medium containing beef extract 10 g, peptone 3 g, Nacl 5 g, and agar 10 g) for 3 days, and YA in potato dextrose agar (PDA) medium for 7 days]. Then, candidate strains were suspension-cultured using the corresponding liquid culture media. Cultures were harvested from the suspensions by centrifugation at 800 rpm and then diluted (bacteria: ~10^6 cells/mL; fungi: mycelium fresh weight 4 mg/mL) for further applications.

Dormant cuttings of a locally grown grapevine cultivar, Rose Honey (*Vitis vinifera* L. × *V. labrusca* L.), were harvested from a vineyard planted in 2017 as a target plant for carrying out the PEM. The vineyard was located in the campus of Yunnan university, Kunming, China. The grapevines in the vineyard were cordon-pruned. In addition, cuttings of several grapevine cultivars, such as Shuijing (*Vitis vinifera* L. × *V. labrusca* L.), Faguoye (*V. vinifera* L.) and Qiuzi (*V. davidii*), were also harvested from the same vineyard and used in the experiments. All grapevine cuttings were harvested in February 2022. Cuttings with one

nodal segment were preserved in a freezer at 4 °C and used within one week. About 200 cuttings from the grapevine cultivar Rose Honey and 50 cuttings from other cultivars were used in the experiments.

2.2. Anti-Pathogenic Fungi Assay of the Candidate VTE Strain ZX-2 In Vitro

Double-culture experiments were carried out to determine the antagonism of the candidate endophytic bacterial strain ZX-2 to the pathogenic fungal strains VN8 (*Fusarium graminearum*), VN4 (*Verticillium* sp.), VN6 (*Botryosphaeria dothidea*), VN13 (*Alternaria alternata*), and VN14 (*Pestalotiopsis trachycarpicola*). All these fungal strains were previously isolated from grapevines, molecularly identified, and preserved in our lab collection. A 5 mm diameter mycelial disc from a 7-day-old PDA culture of the pathogenic fungus was inoculated into the center of a PDA plate. Four ZX-2 discs (prepared from a 5-day-old PBA plate) were then placed around the fungal disc at 8 mm intervals. Individually grown pathogenic fungus was used as a control. The colony diameters of the double- and single-cultured pathogenic fungi were measured after 7 days of incubation at 28 °C and the antifungal activity was estimated via the inhibition of mycelial growth of the fungi in the direction of actively growing bacteria. The percentage inhibition was calculated using the following formula:

$$\text{Inhibition rate (IR\%)} = R_{single} - R_{dual}/R_{single} \times 100$$

In the formula, R_{single} is the radial growth of the single-cultured fungal colony; R_{dual} is the radial growth of the fungal colony opposite the bacterial colony.

2.3. Testing the Ability of Candidate VTE Strains to Infect and Colonize Vine Cuttings

To test the infection and colonization abilities of the candidate VTE strains, the bottom ends (cut in an inclined plane immediately before use) of vine cuttings were immersed in VTE strain suspensions for 48 h at room temperature. Cuttings immersed in sterilized water were used as a control group. The VTE-infected cuttings, as well as the control cuttings, were then incubated in 100 mm diameter pots with sterilized soil (soil: humus = 3:1), with one cutting per pot. Cuttings were incubated in an aseptic culture room with 12/12 light/dark periods at 25 °C, 60–75% relative humidity. At different days after treatment (DAT), cuttings were harvested and divided into upper, middle, and lower sections to determine the isolation rates of the candidate endophytes and other endophytic taxa using a tissue patch method (isolation rate (%) = number of isolates/tissue patches × 100) [17]. The candidate VTE strain isolates and the other co-emerging endophytic isolates were morphologically categorized and then identified by comparing the certain DNA sequences (16S rRNA for bacterium and ITS V3–V4 for fungus) in the National Center for Biotechnology Information (NCBI) database.

To study the infection pathway of the candidate bacterium *B. cereus* in vine cuttings, 1 DAT and 30 DAT cuttings were first divided into upper, middle, and lower segments; then, the segments were further divided into pith, xylem, and phloem tissues. The isolation rates of pith, xylem, and phloem tissues were determined separately using the same method as above.

2.4. Establishment of ZX-2-Modified Grapevines (ZX-2mg)

Grapevine cuttings of Rose Honey (RH) cultivar with or without ZX-2 infection were all grown in 100 mm diameter pots (24 February 2022) until the survey of first-year (90–340 days after treatment (DAT)) and second-year (520 DAT) vine plantlets. During the growth of the cutting seedlings, the bud rates, number of leaves, leaf areas, and height of new shoots were measured on 20 randomly selected vines from each of the ZX-modified and control vines. In addition, root number, root length, and fresh weight were determined for five randomly selected vine plants at 90 DAT. *B. cereus* (ZX-2) in vine cuttings and the established vine plants were tracked to determine the isolation rates at different DAT using the patch culture method, followed by a molecular identification.

2.5. Proportional Assay for Culturable Endophytic Fungi and Bacteria in Vine Cuttings

Tissue patches of grapevines incubated on PBA plates can be used to observe the emergence of both the endophytic bacterial and fungal colonies. The isolation rates of all the bacteria and fungi growing on PBA media were calculated and isolated in different DAT cuttings. The pure cultured isolates were morphologically classified as fungi or bacteria and then identified via comparison of the ITS or 16S rRNA sequences in the NCBI database. Endophytic strains with different ITS or 16S rRNA sequences were conserved as different OTUs.

2.6. Profiling of the Endophytic Microbiota in Different Parts of ZX-2mg and Control Grapevines

Genomic DNA extraction, subsequent profiling of the endophytic microbiota in ZX-2mg and control vines at 90 DAT, and subsequent computational analyses were performed according to the procedures of Xiang et al. [13]. The raw fungal and bacterial sequence data were deposited in the NCBI under accession numbers PRJNA977117 and PRJNA977126, respectively.

2.7. Determining the Disease Resistances of Grapevine Leaves

An antagonistic assay of plant leaves against pathogenic fungi was proposed to evaluate the disease resistance of plants to certain pathogenic microbes. Briefly, leaf discs (5 mm diameter) were harvested from fully developed grapevine leaves to test their antipathogenic ability against fungi. An antipathogenic fungal test of grapevine leaves was carried out according to the procedures described above (Section 2.2) for the antipathogenic fungal test of ZX-2. Instead of the ZX-2 bacterium, vine leaf discs were placed around the pathogenic fungi to test the anti-fungal effects of the vine leaves. Leaves from all surviving vines (25 ZX-2mg, 12 control vines) were tested for their antifungal activity and three fully expanded leaves from each vine were tested as replicates.

To further evaluate the disease resistance of the vines, the percentage of necrotic lesion area caused by naturally occurring diseases (mainly downy mildew and powdery mildew) in each vine leaf was estimated for both the second-year ZX-2mg and control vines in later autumn (18 October 2023). The percentage of necrotic lesion area was also measured on the leaves of all surviving vines.

2.8. Statistical Analyses and Graphical Works

All statistical analyses of data of endophytic microbiota in ZX-2mg and control vines were performed using dedicated packages in R, version 3.5.1 (The R Foundation for Statistical Computing, Vienna, Austria), unless otherwise stated. Alpha diversities between sample groups were compared using box plots. A principal coordinate analysis (PCoA) of endophytes in ZX-2mg and control vines was performed to determine beta diversity at the ASV level. The relative abundance (RA) of a given endophyte genus (or other taxonomic levels) was calculated using the following formula:

$$RA\% = N_i/N_{all} \times 100$$

In the formula, N_i represents the obtained clean reads of the genus in the sample; N_{all} represents the total obtained clean reads of all genera in the sample. Linear discriminant analysis effect size (LEfSe) and a Bugbase phenotype prediction analysis were performed to determine the composition and phenotype changes in the endophytic microbiota in ZX-2mg compared to the control vines using the tools provided by LC-BioTechnologies company, Wuhan, China (https://www.omicstudio.cn/home (accessed on 29 May 2023)).

Data on plant growth parameters, pathogen inhibition rate, lesion area percentage of vine leaves, and the isolation rates of ZX-2 and other endophytes are presented as the mean ± standard variation of multiple replicates and were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) for Windows. One-way ANOVA followed by Tukey's multiple comparison test was used to determine significance at $p < 0.05$. The various packages in R, Sigma Plot 12.5 (Systat Software Inc., San Jose, CA, USA) and the tools in Excel and PowerPoint were used to perform most of the statistical analyses and to graph the results.

3. Results

3.1. ZX-2, an Anti-Fungal Pathogen VTE with Higher Colonization Ability in Vine Cuttings

The bacterium *B. cereus* strain ZX-2 was tested to inhibit the pathogenic fungal strains VN4, VN6, VN8, VN13, and VN14. In addition to VN4, the growth of all other fungi was significantly inhibited, with the inhibition rates ranging from 35% to 66%. The growth of VN6, a pathogen that causes grapevine canker and fruit rot, was largely suppressed by 66%, followed by VN8 (49%), VN14 (38%) and VN13 (35%) (Figure 1A,B).

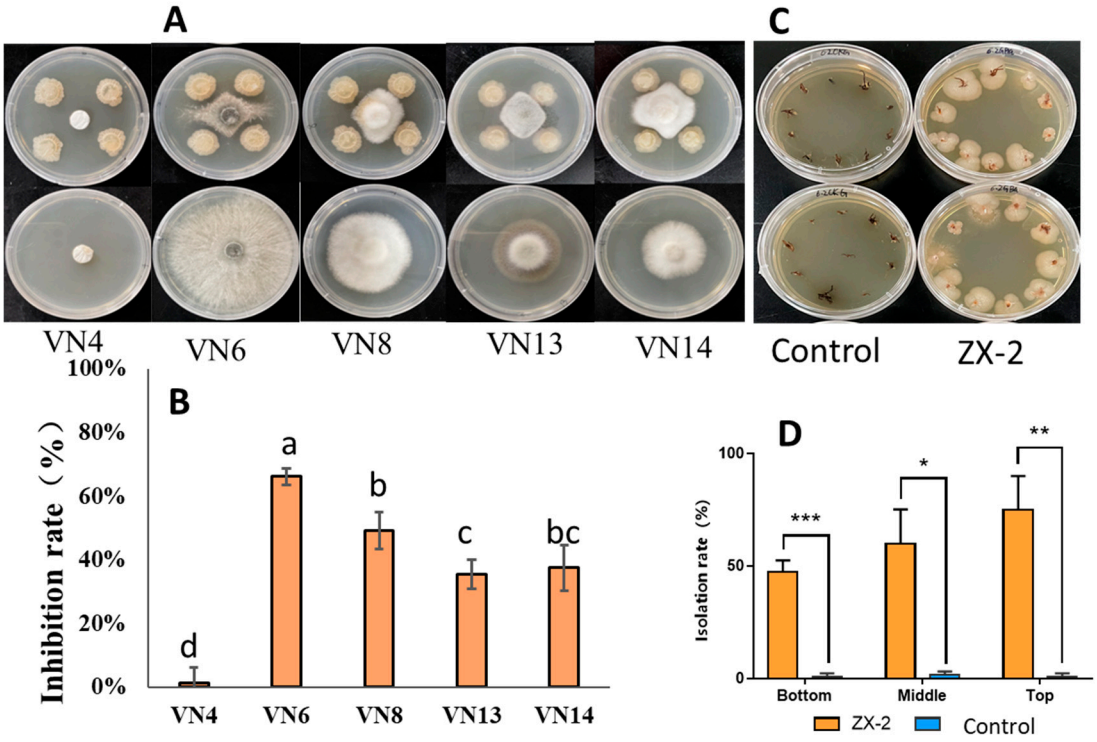

Figure 1. VTE strain ZX-2 conferred antifungal pathogen functions and a high ability to colonize vine cuttings. (**A**,**B**) The antagonistic assays of ZX-2 against pathogenic fungi. The pathogenic fungal strains used were VN4 (*Verticillium* sp.), VN6 (*Botryosphaeria dothidea*), VN8 (*Fusarium graminearum*), VN13 (*Alternaria alternata*), and VN14 (*Pestalotiopsis trachycarpicola*); (**C**,**D**) Detection of the endophytic colonization of VTE strains ZX-2 on grapevine cuttings of cultivar Rose Honey. Different lowercase letters represent the inhibition rate of ZX-2 significant differences among pathogenic fungi; * significance at 0.05 ($p < 0.05$); ** significance at 0.01 ($p < 0.01$); and *** significance at 0.001 ($p < 0.001$).

In addition to its anti-pathogen functions, ZX-2 could efficiently infect and colonize the interior of 30 DAT RH vine cuttings at a relatively higher frequency. The isolation rates of the bacterium *B. cereus* (ZX-2) in different sections of the infected 30 DAT vine cuttings were significantly higher than those of the control cuttings (Figure 1C,D), confirming ZX-2's ability to colonize the RH vine cultivar. In comparison, RH vine cuttings with or without infection by another VTE bacterial strain P1 (*Panteoa* sp.) all showed high isolation rates of the genus *Panteoa* (Supplementary Figure S1) due to the increased native colonization of this bacterium in RH vines. However, in RH vine cuttings with or without infection by the VTE fungal strain YA (*Pestalotiopsis* sp.), no colonization of YA was detected at 30 DAT (Supplementary Figure S1). In addition to the vine cultivar RH, the candidate bacterium ZX-2 was highly efficient in infecting the cuttings of the vine cultivars Faguoye, Shuijing, and Qiuzi (Supplementary Figure S2).

As a result, the bacterial VTE strain ZX-2 could be an ideal candidate PEM strain with the functions of a broad spectrum of antifungal pathogens and a high colonization ability in different grapevine cultivars.

3.2. The Infection of ZX-2 in Vine Cuttings Initially Occurred in the Xylem and Then Moved Horizontally to the Pith and Phloem

The isolation rates of ZX-2 in different sections of ZX-2-infected vine cuttings at 1 and 3 DAT were determined. In 1 DAT cuttings, the introduced ZX-2 was mainly distributed in the xylem and showed an obvious gradual decrease in isolation rates from the morphological bottom (82 ± 7%) to the top (18 ± 15%) of the cuttings (Figure 2A–C). In contrast, in cuttings at 3 DAT, higher abundances (with average isolation rates of more than 40%) of *B. cereus* (ZX-2) were detected in all cutting sections (pith, xylem, and phloem) (Figure 2D,E). These results suggested that the infection of the vine cuttings with ZX-2 occurred first in the xylem, from the morphological bottom to the top, and then moved horizontally to the pith and the phloem.

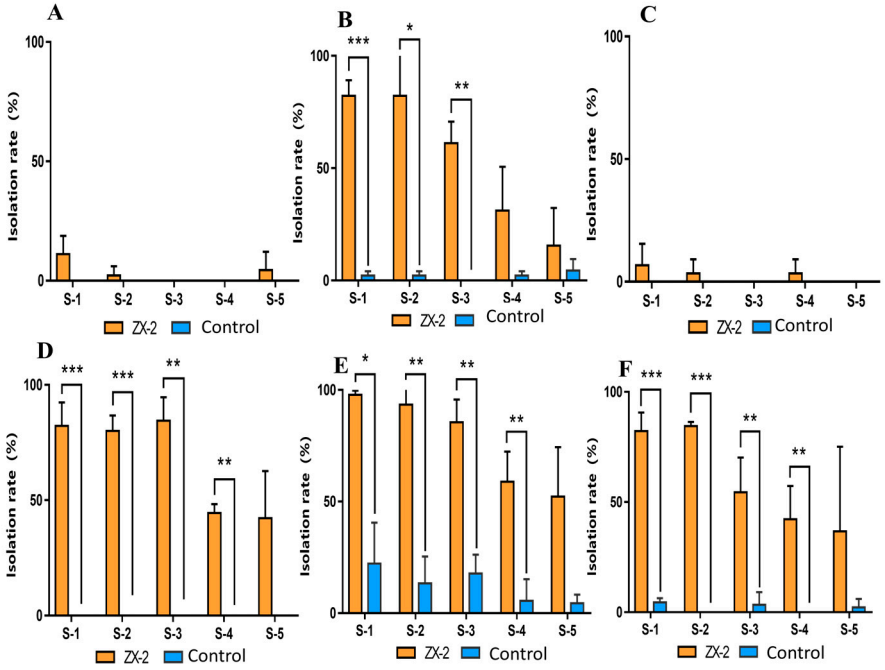

Figure 2. ZX-2 infects vine cuttings initially in the xylem and then moves horizontally to the pith and phloem sections. (**A**–**C**) Isolation rates of *B. cereus* (ZX-2) in the pith (**A**), xylem (**B**), and phloem (**C**) sections of 1 day after treatment (DAT) cuttings; (**D**,**E**) isolation rates of *B. cereus* (ZX-2) in the pith (**D**), xylem (**E**), and phloem (**F**) sections of 3 DAT cuttings. * significance at 0.05 ($p < 0.05$); ** significance at 0.01 ($p < 0.01$); and *** significance at 0.001 ($p < 0.001$).

3.3. ZX-2-Modified Cuttings and the Established Vine Plants Maintained Higher Abundances of ZX-2

The bacterial species *B. cereus* was monitored for abundance in ZX-2-infected and control vine cuttings at 1, 10, and 30 DAT and in the later established vine plantlets at 90, 230, 280, and 340 DAT, as well as in the next year's sprouted shoots at 520 DAT. Compared to the controls, significantly higher isolation rates of *B. cereus* were detected in all tissue sections of the ZX-2-modified vine cuttings and the established vine plants (Figure 3). In ZX-2-infected 1 DAT cuttings, the isolation rate of *B. cereus* peaked in the lower section (95%), followed by the middle (80%) and upper sections (75%) (Figure 3A). In contrast to 1 DAT cuttings, the *B. cereus* in ZX-2-infected cuttings at 10 DAT maintained an isolation rates from 25% to 58%, but peaked in the upper section of the cuttings, followed by the middle and lower sections (Figure 3B). ZX-2-infected vine cuttings at 30 DAT maintained a similar isolation rate trend to cuttings at 10 DAT, with higher isolation rates (68%) of *B. cereus* occurring in both the upper and lower sections (Figure 3C). By 90 DAT, the cuttings had developed into vines with roots and shoots. Compared to the control vines, the cutting part of the ZX-2-modified grapevines (ZX-2mg) maintained a significantly higher level of colonization (average isolation rates from 25% to 78%) of *B. cereus* and the isolation rates of

bacteria *B. cereus* increased along the lower, middle, and upper sections. Most importantly, the newly formed shoots (including stems and leaves) and roots of the ZX-2mg also showed higher isolation rates of *B. cereus* than the control vines, especially in the leaves (the average isolation rate in leaves of ZX-2mg was 78%, while in leaves of control grapevines, the average isolation was 4.8%) (Figure 3D). Notably, ZX-2mg maintained significantly higher isolation rates of *B. cereus* in first-year vine leaves at 230 (89%), 280 (97%), and 340 (82%) DAT, and in the next-year sprouted leaves, at 520 (50%) DAT, compared to the control vines (Figure 3E–H). However, lower isolation rates (from 1% to 57%) of *B. cereus* were also detected in some of the control vine cuttings and vine plants (Figure 3).

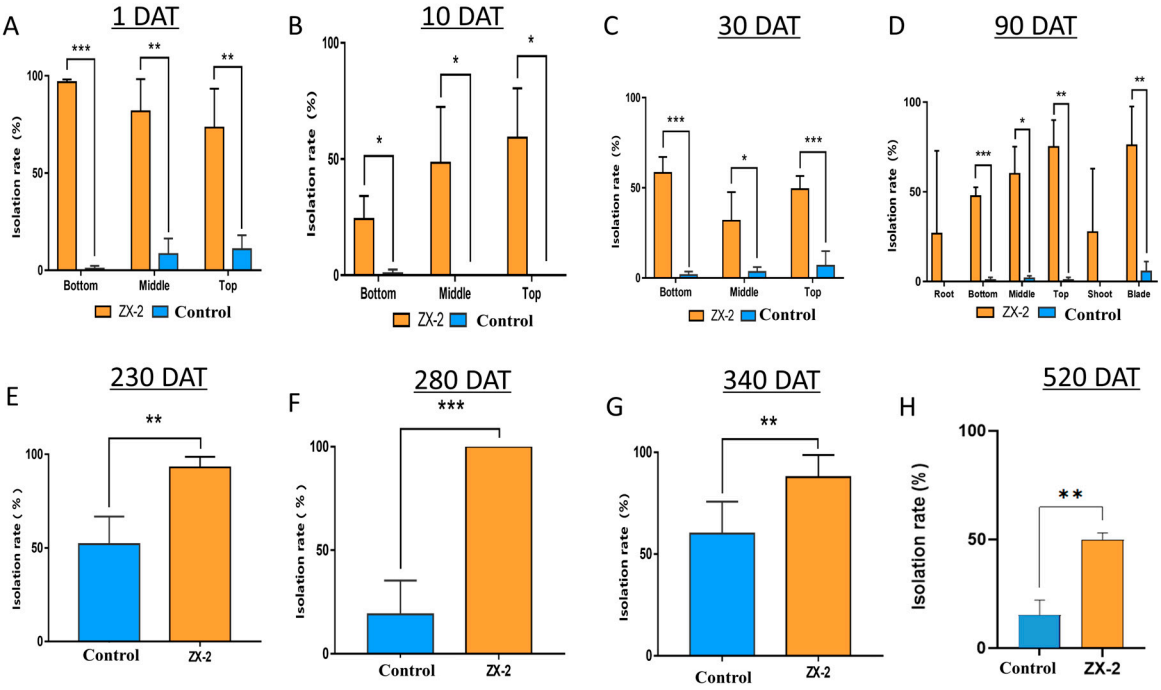

Figure 3. Dynamic distribution of *B. cereus* (ZX-2) in cuttings and the established grapevines at different days after treatment (DAT). (**A**–**C**): dynamic colonization of *B. cereus* in cuttings at 1 (**A**), 10 (**B**) and 30 (**C**) DATs; (**D**) the compartmental colonization of *B. cereus* in the established first-year grapevines at 90 DAT; (**E**–**G**) the distribution of *B. cereus* in leaves of the first-year grapevines at 230 (**E**), 280 (**F**) and 340 (**G**) DATs; (**H**) the distribution of *B. cereus* in leaves of the next-year grapevine leaves at 520 DAT. * significance at 0.05 ($p < 0.05$); ** significance at 0.01 ($p < 0.01$); and *** significance at 0.001 ($p < 0.001$).

3.4. The Colonization of ZX-2 Promoted the Abundance of Bacterial Endophytes While Suppressing Fungal Endophytes in Vine Cuttings

All isolates from the tissue patches of vine cuttings and the established vines were identified by a comparison of their ITS/16S rRNA sequences in the NCBI database. Vine cuttings of cultivar RH were mainly colonized by several culturable bacterial and fungal endophytic genera, including *Bacillus*, *Pestalotiopsis*, *Pantoea*, and others (Figure 4A,B). In cuttings 90 DAT, 95% of the *B. cereus* isolates in ZX-2mg were identical to ZX-2 in 16S rRNA sequences, whereas *B. cereus* isolates from control cuttings and established vines differed from the ZX-2 in 16S rRNA sequences (Figure 4B). Interestingly, the isolation rate of fungal and bacterial endophytes in vine cuttings between ZX-2-infected and control cuttings at 1 and 10 DAT were not obviously different. However, the isolation rate of fungal endophytes in ZX-2-infected cuttings significantly decreased at 30 (4% in ZX-2-infected cuttings and 23% in control cuttings) and 90 (4% in ZX-2-infected cuttings and 58% in control cuttings) DAT, whereas the isolation rate of bacterial endophytes increased (Figure 4C,D). In this experiment, the most suppressed culturable endophytic fungi belong to the genus *Pestalotiopsis* (Figure 4E).

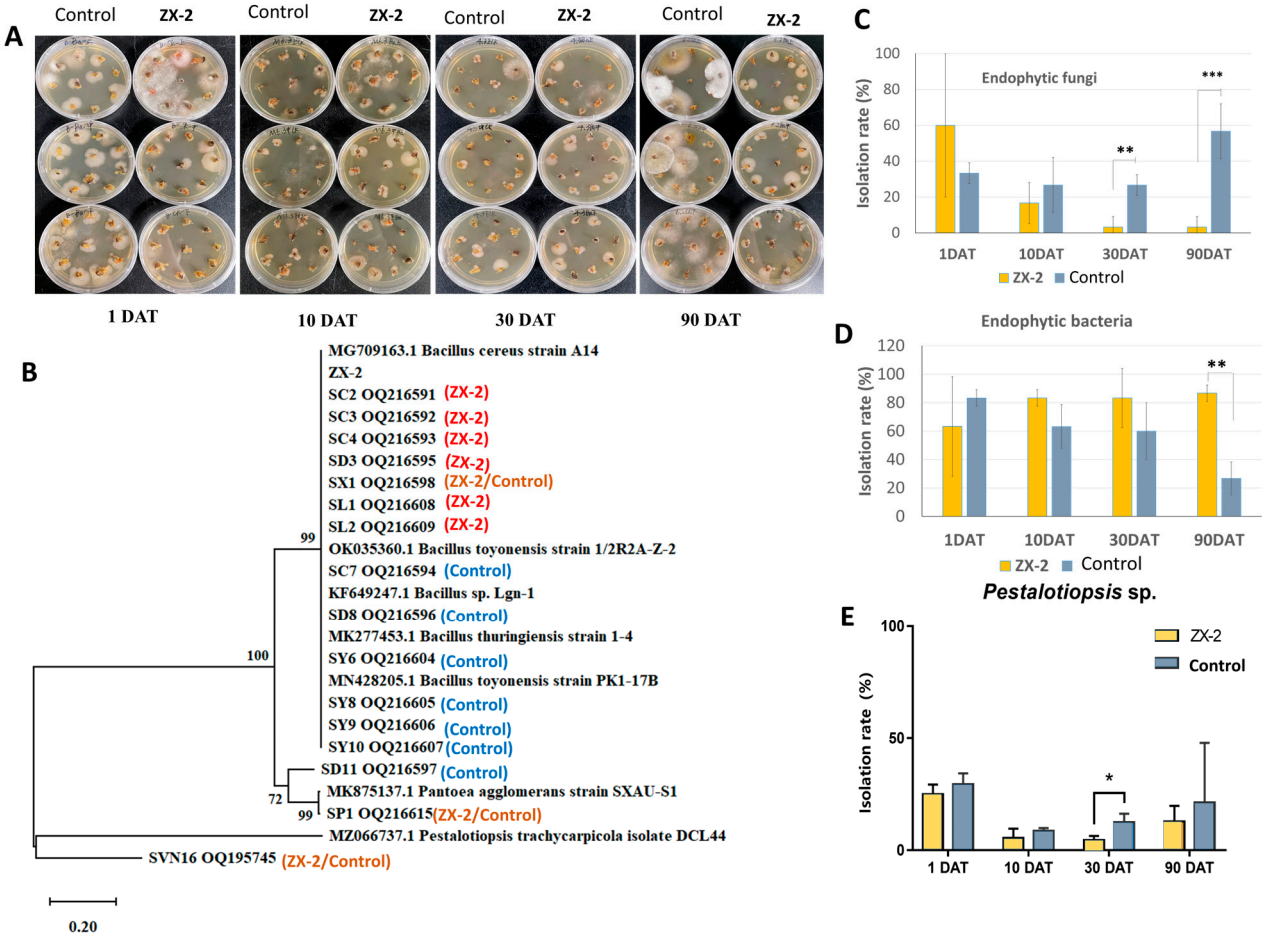

Figure 4. The introduction of ZX-2 suppressed the abundances of fungal endophytes while promoting the bacterial endophytes in grapevine cuttings. (**A**) Emergence of culturable endophytic isolates in grapevine cuttings at different days after treatment (DAT); (**B**) identification and phylogenetic analysis of the endophytic isolates from grapevine cuttings and the established grapevines based on 16S rRNA and ITS sequences; (**C**,**D**) comparison of the isolation rates of culturable endophytic fungi (**C**) and bacteria (**D**) between ZX-2-modified and control grapevine cuttings at different DAT; (**E**) comparison of isolation rates of the fungus *Pestalotiopsis* sp. in ZX-2-modified and control grapevine cuttings at different DAT. * significance at 0.05 ($p < 0.05$); ** significance at 0.01 ($p < 0.01$); and *** significance at 0.001 ($p < 0.001$).

3.5. The Endophytes in ZX-2mg Were Compositionally and Functionally Altered

Profiling the endophytic microbiota in grapevines using high-throughput sequencing technology provides detailed information to assess the impact of ZX-2 colonization on grapevine endophytes. The introduction and colonization of ZX-2 had no significant impact on the overall endophyte diversity in grapevines (Figure 5). The PCoA results indicated that the bacterial endophytic microbiota of grapevines had a compartmental specificity. The bacterial endophytic microbiota in roots, cuttings, and shoots are separately clustered in the PCoA plot (Figure 5E). However, the beta diversity of both the bacterial and fungal endophytes was not significantly affected by the ZX-2 colonization (Figure 5E,F).

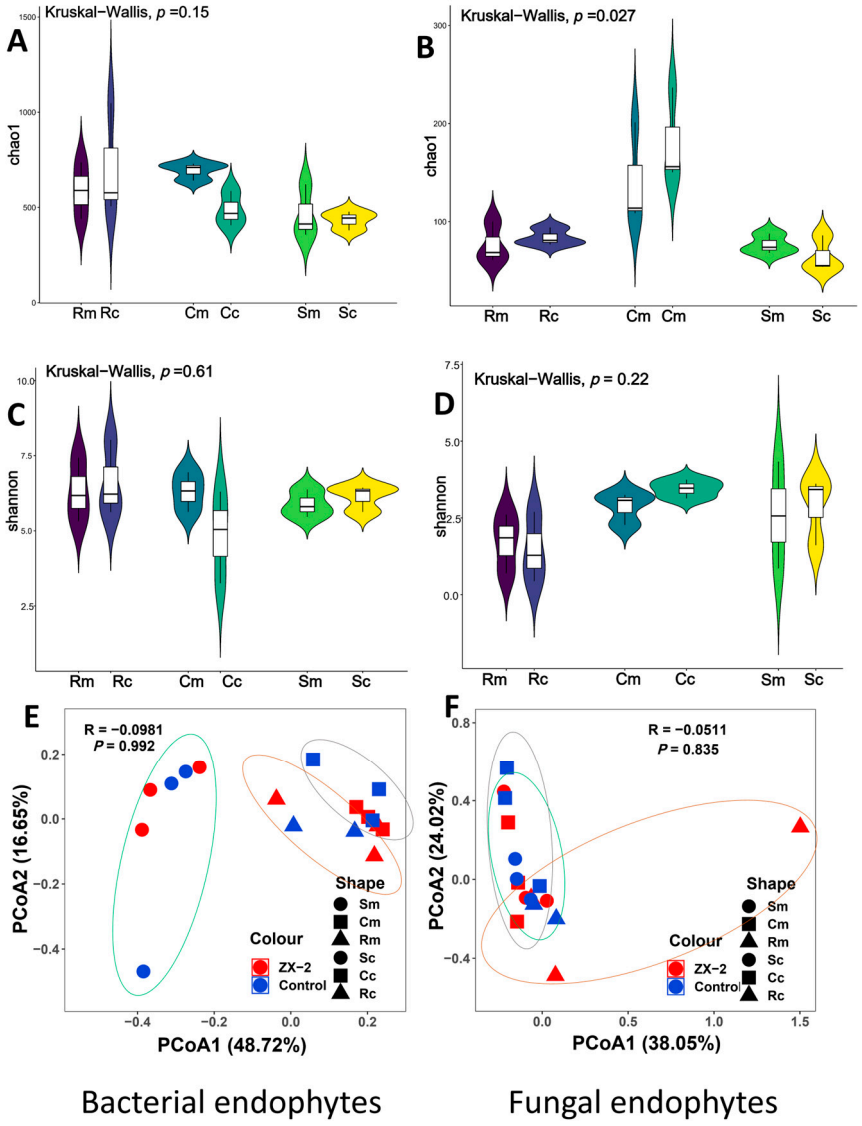

Figure 5. ZX-2-based PEM did not significantly affect the global diversities of endophytic microbiota in grapevines. (**A**,**B**) Comparison of the alpha diversities (Chao1 index) of endophytic bacteria (**A**) and fungi (**B**) between ZX-2-modified and control grapevines; (**C**,**D**) comparison of the alpha diversities (Shannon index) of endophytic bacteria (**C**) and fungi (**D**) between ZX-2mg and control grapevines; (**E**,**F**) comparison of the beta diversity (PCoA) of endophytic bacteria (**E**) and fungi (**F**) between ZX-2mg and control grapevines. Sample groups: Rm, roots of ZX-2mg; Cm, cutting part of ZX-2mg; Sm, shoots of ZX-2mg; Rc, roots of control grapevines; Cc, cuttings of control grapevines; Sc, shoots of control grapevines.

In contrast to the diversity, the composition of the endophytes in ZX-2mg was greatly altered by the introduction and colonization of ZX-2 (Figure 6). Compared to the controls, in ZX-2mg, higher relative abundances (RA) of *B. cereus* were detected in shoots, cuttings, and roots. New shoots of the ZX-2mg had the highest RA of ZX-2 (RA = 3.3%), followed by the cuttings (0.27%) and roots (0.09%) (Figure 6A). The composition of other bacterial endophytes in ZX-2mg changed accordingly (Figure 6B–E). Among the most abundant (RA > 3%) endophytic bacterial genera, the RAs of *Ralstoea*, *Bacillus*, an unclassified genus in Fimicutes, *Lysinibacillus*, *Aneurinibacillus*, an unclassified genus in Muribaculaceae, *Bradyrhizobium*, and *Halomonas* were strongly increased in new shoots of ZX-2mg (Figure 6B), and the RA differences of the genera *Bacillus*, *Aneurinibacillus*, and *Bradyrhizobium* between the shoots of ZX-2mg and controls reached statistical significance (Figure 6C). According to the

LEfSe analysis, ZX-2-based PEM significantly increased (LDA > 3; $p < 0.05$) the abundance of 6 families (Dysgonomonadaceae, Aneurinibacillaceae, Bacillaceae, Desulfotomaculaceae, Bradyrhizobiaceae, and Shewanellaceae), 9 genera (*Proteiniphilum*, *Aneurinibacillus*, *Anoxybacillus*, *Bacillus*, *Lysinibacillus*, *Clostridium*, *Bradyrhizobium*, *Comamonas*, and *Shewanella*) and 12 species (including the *B. cereus* and 3 other species of the genus *Bacillus*) in shoots of ZX-2mg (Figure 6C). On the other hand, ZX-2 colonization clearly suppressed the RA of the endophytic bacterial genera from the family Lachnospiraceae (LDA > 3) in vine shoots (Figure 6C). One phylum (Dependentiae), five families (an unclassified family in Flavobacteriales, Ermiphilaceae, Bacillaceae, Lachnospiraceae, Enterobacteriaceae), thirteen genera (*Vicinamibacter*, *Chitinophaga*, unclassified genus in Flavobacteriales, an unclassified genus in *Vermiphilaceae*, *Bacillus*, *Anaerocolumna*, *Bosea*, *Shinella*, *Novosphingobium*, *Variovorax*, *Methylobacillus*, an unclassified genus in Nitrosomonadaceae, *Klebsiella*), and fourteen species (including the *B. cereus*) were significantly promoted (LDA > 3; $p < 0.05$) as distinct bacterial endophytes in the cutting part of the ZX-2mg (Figure 6D).

In addition to the shoot parts, the roots of ZX-2mg significantly promoted only one genus (*Methyloversatills*) and three species of bacterial endophytes, but significantly suppressed one phylum (Actinobacteriota), two families (*Micrococcaceae* and *Sphingobacteriaceae*), three genera (*Rothia*, *Pedobacter* and *Xanthobacter*), and five species of bacterial endophytes (Figure 6E). Among the most abundant endophytic bacterial genera (RA > 3%), *Achromobacter*, *Curvibacter*, *Neorhizobium*, and *Ralstonia* were strongly promoted, whereas the genera *Acidovorax*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, and *Flavobacterium* were decreased in the roots of ZX-2mg (Figure 6B,E). In addition, the genus *Bacillus* in grapevine showed a strong positive relationship with the genera *Ralstonia*, *Aneurinibacillus*, *Lysinibacillus*, and an unclassified genus in Firmicutes (Figure 6F).

Fungal endophytes were also compositionally altered in ZX-2mg (Figure 7A,B). Among the most dominant (RA > 3%) fungal endophytic genera, *Pestalotiopsis*, *Davidiella*, *Alternaria*, *Kabatiella*, and *Trametes* were strongly suppressed in the cutting part of ZX-2mg, whereas *Phialophora*, *Didymella*, a genus in Halosphaeriaceae, *Valsa*, and *Cercophora* were significantly ($p < 0.05$) promoted in the cutting part of ZX-2mg (Figure 7B,D). In the shoots, the endophytic fungal composition between ZX-2mg and controls was not obviously different at the phylum level, while it was different at the class and genus levels (Figure 7A–C). ZX-2mg had a significantly higher RA of the fungal class Pezizomycetes, but a significantly lower RA of endophytic fungal genus *Cladosporium* in the shoots (Figure 7C). The fungal endophytes in the roots of ZX-2mg were also profoundly altered by the ZX-2-based PEM (Figure 7B,E). ZX-2mg had significantly higher RAs of two classes (Dothihideomycetes and Tremellomycetes), one genus (*Didymella*) and one species (*Peyronellaea curtisii*), but significantly lower RAs of one order (Eurotiales), two families (Trichocomaceae, Pyronemataceae), three genera (*Petromyces*, an unclassified genus in Pyronemataceae, and *Podospora*) and four species of fungal endophytes in the roots (Figure 7E).

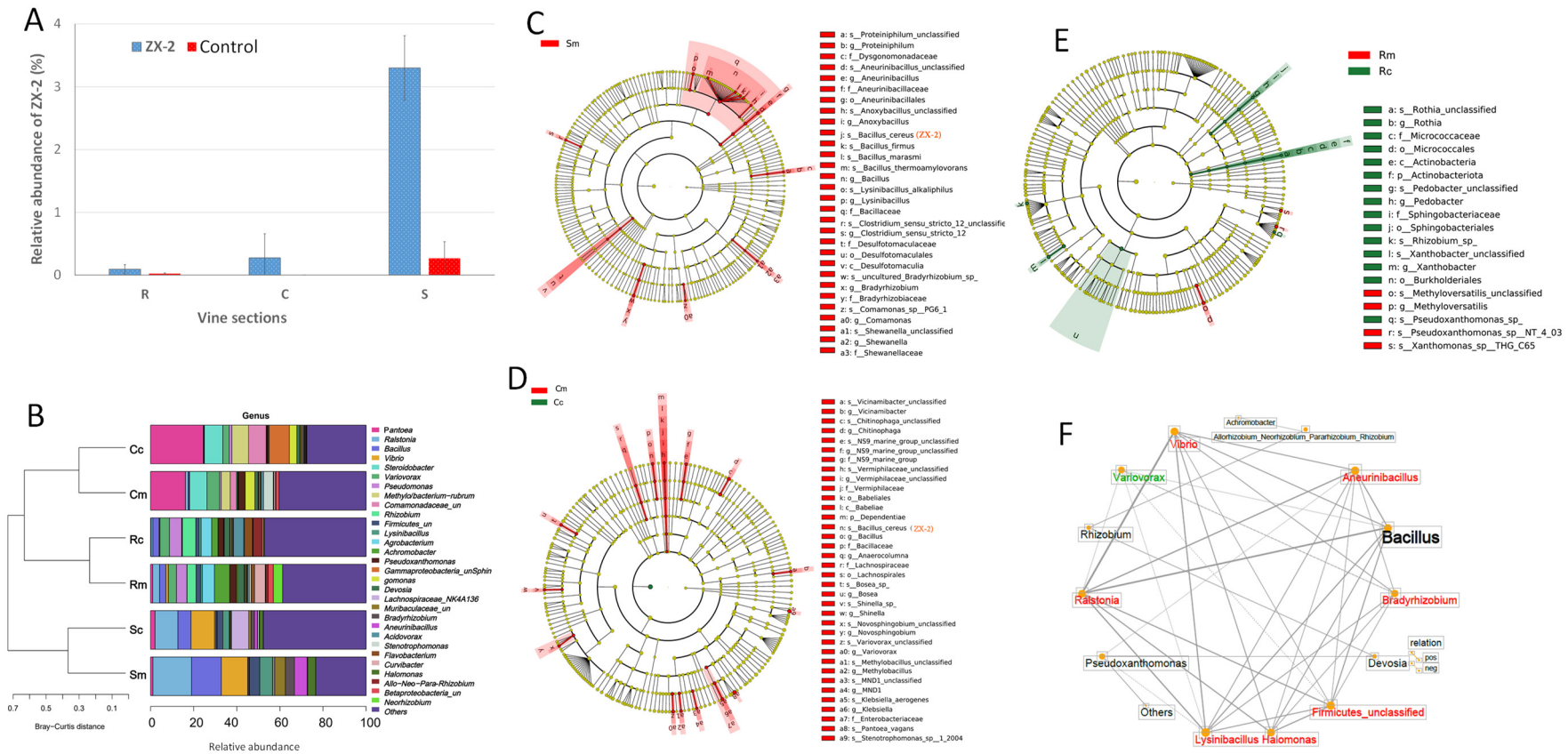

Figure 6. The endophytic bacterial microbiota in ZX-2mg was compositionally altered. (**A**) Comparison of the relative abundances of *B. cereus* between ZX-2mg and control grapevines; (**B**) comparison of the composition of bacterial endophytes (at the genus level) between ZX-2mg and control grapevines; (**C**–**E**) LEfSe assay of bacterial endophytes in grapevine shoots (**C**), cutting part (**D**) and roots (**E**) between ZX-2mg and control grapevines; (**F**) correlation analysis of bacterial genera in grapevines. Sample groups: Rm, roots of ZX-2mg; Cm, cutting part of ZX-2mg; Sm, shoots of ZX-2mg; Rc, roots of control grapevines; Cc, cuttings of control grapevines; Sc, shoots of control grapevines.

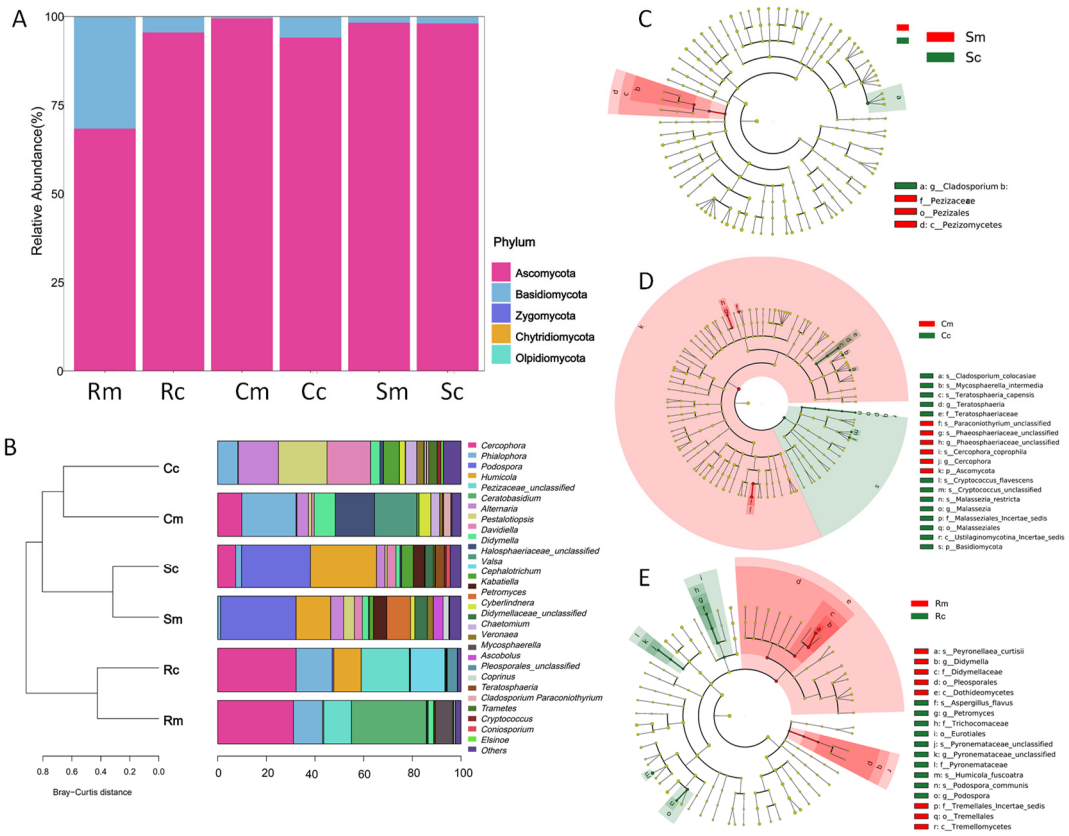

Figure 7. Compositional comparison of fungal endophytic microbiota between ZX-2mg and control grapevines. (**A**,**B**) Comparison of the composition of fungal endophytes between control and ZX-2mg at phylum (**A**) and general (**B**) levels. (**C**–**E**) LEfSe analysis of fungal endophytes in shoots (**C**), cutting part (**D**), and roots (**E**) of ZX-2mg and control grapevines. Sample groups: Rm, roots of ZX-2mg; Cm, cutting part of ZX-2mg; Sm, shoots of ZX-2mg; Rc, roots of control grapevines; Cc, cuttings of control grapevines; Sc, shoots of control grapevines.

In addition to the compositional effects, a phenotype prediction analysis revealed that the colonization of ZX-2 had an impact on the phenotypes of the endophytic microbiota in grapevines (Figure 8). ZX-2-based PEM tends to increase the endophytic bacteria with aerobic or facultatively anaerobic characteristics, while decreasing the anaerobic bacteria in grapevines, especially in new grapevine shoots (Figure 8A–C). The shoots of ZX-2mg have more mobile elements-containing endophytic bacteria, whereas the original cutting parts of ZX-2mg contained decreased RAs of this bacterial group (Figure 8D). Compared to the controls, the shoots of ZX-2mg contain more gram-negative and fewer gram-positive bacterial endophytes (Figure 8F,G). Interestingly, the bacteria with both potential pathogenicity and stress-tolerance characteristics in ZX-2mg were increased in the shoot and root parts, but decreased in the cutting parts (Figure 8H,I). In general, ZX-2-based PEM had greater effects on the phenotypes of endophytic bacteria in the shoot part than in other parts of the grapevine (Figure 8).

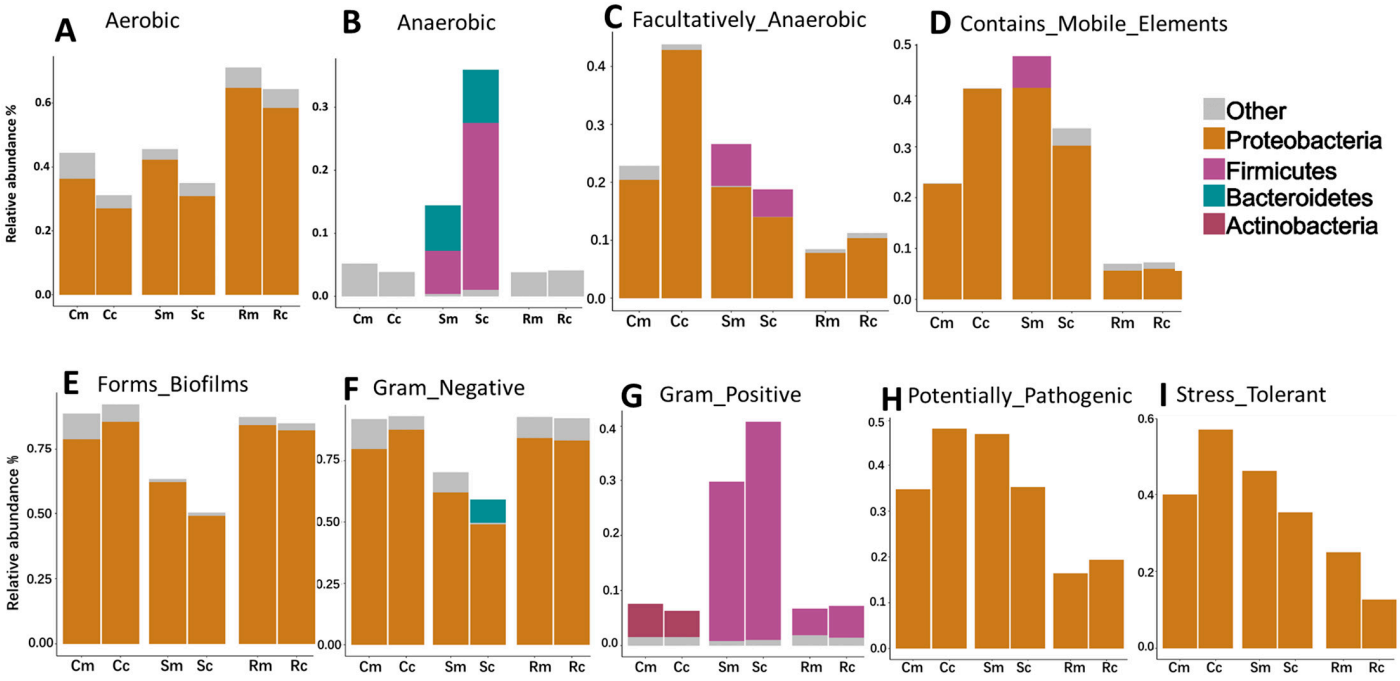

Figure 8. Predicted phenotypes of the endophytic bacteria in different parts of the ZX-2mg and control grapevines. (**A**–**I**): displayed the relative abundances of endophytic bacteria with predicted phenotypes aerobic (**A**), anaerobic (**B**), facultatively anaerobic (**C**), contains mobile elements (**D**), forms biofilms (**E**), gram native (**F**), gram positive (**G**), potentially pathogenic (**H**), and stress tolerant (**I**) in different vine samples. Sample groups: Rm, roots of ZX-2mg; Cm, cutting part of ZX-2mg; Sm, shoots of ZX-2mg; Rc, roots of control grapevines; Cc, cuttings of control grapevines; Sc, shoots of control grapevines.

3.6. ZX-2mg Showed Improved Disease Resistance and Growth-Promoting Traits

The disease resistance of both ZX-2mg and control vines was assessed by counting the naturally occurring leaf lesion area and testing the antagonistic ability of vine leaves against fungal pathogens such as MH1 (*B. cinerea*), VN8 (*F. graminearum*), VN13 (*A. alternata*), and MH4 (*C. cladosporioides*). Major proportions of the leaf discs from current and next year's leaves of ZX-2mg showed stronger antagonistic abilities against the tested fungal pathogen strains (Figure 9A–D). Compared to the control vines, the inhibitory effects of ZX-2-modified vine leaves (both current and next year) on fungal strains MH1 and VN8 reached statistical significance ($p < 0.05$) and critical significance ($p < 0.01$) (Figure 9B,D). Compared to the control vines, ZX-2mg (second-year plants) showed a reduction in naturally occurring leaf lesion area (Figure 9E). When pathogen resistance (determined by the inhibition rates of vine leaves against the fungal pathogen MH1) was assessed for all second-year ZX-2mg and control vines, leaves from the ZX-2mg group (N = 25) conferred significantly higher resistance ($p < 0.001$) than those from the control vines (N = 12) (Figure 9F). In addition, leaves from the ZX-2mg group were more likely to be colonized by the culturable bacterium ZX-2 (*B. cereus*) (Figure 9G), and a significant correlation (N = 37, r = 0.418; $p < 0.01$) was observed between fungal inhibition rates and *B. cereus* (ZX-2) isolation rates in grapevine leaves (Supplementary Figure S3).

In addition to the disease resistance, the ZX-2mg showed an obvious growth promotion in leaf area, root length, and fresh weight (Figure 10A–D), as well as a delayed (15–20 days) leaf abscission in winter (Figure 10F).

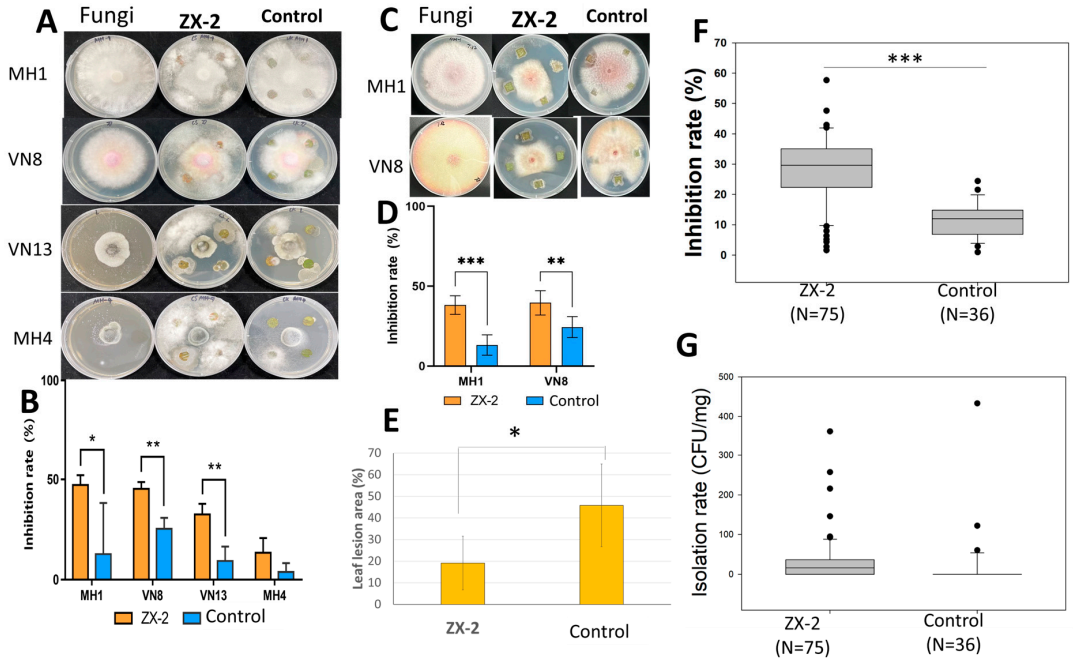

Figure 9. The ZX-2-modified grapevines showed enhanced fungal pathogen resistances. (**A**,**B**) The inhibition effects of first-year grapevine leaves to the pathogenic fungal strains, MH1(*Botrytis cinerea*), VN8 (*Fusarium graminearum*), VN13 (*Alternaria alternata*), and MH4 (*Cladosporium cladosporioides*); (**C**,**D**) The inhibition effects of second-year grapevine leaves (520 days after treatment) to the pathogenic fungal strains MH1 and VN8; (**E**) ZX-2-modified grapevines of the second year showed a reduction in the naturally occurring leaf lesion area; (**F**) the fungal pathogen inhibition rates of second-year leaves were compared between ZX-2 modified vine group and the control vine group; (**G**) the isolation rates in second-year leaves between ZX-2 modified and the control grapevines were compared. * Significance at 0.05 ($p < 0.05$); ** significance at 0.01 ($p < 0.01$) and *** significance at 0.001 ($p < 0.001$).

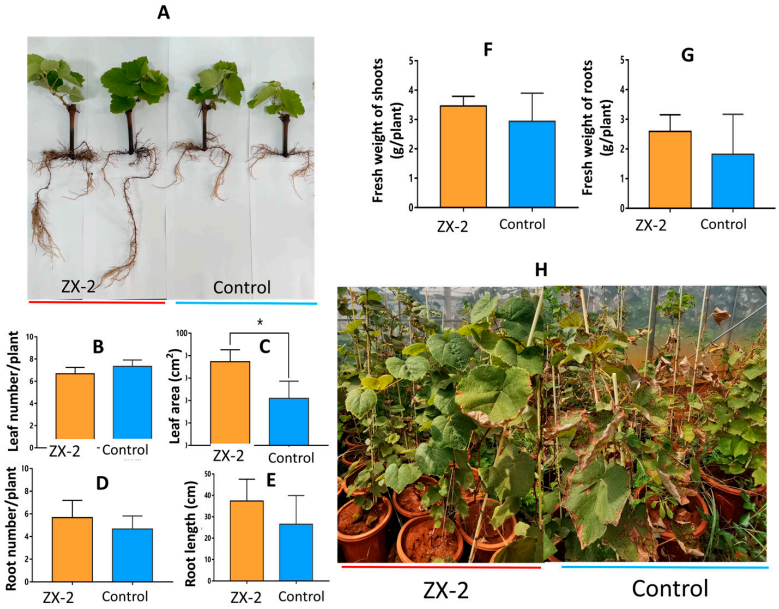

Figure 10. ZX-2-modified vines exhibited growth promotion and a delayed leaf abscission in winter. (**A**–**G**) Comparison of the growth parameters between ZX-2-modified and control grapevines at 90 DAT (**A**) regarding leaf number (**B**), leaf area (**C**), root number (**D**), root length (**E**), fresh shoot weight (**F**), and fresh root weight (**G**); (**H**) ZX-2-modified vines displayed a delayed leaf abscission in winter. * significance at 0.05 ($p < 0.05$).

4. Discussion

The vertical transmission of microbial profiles and keystone taxa such as the genera *Pantoea* and *Xanthomonas* of the rice microbiome suggest new ways to improve plant fitness by manipulating seed-associated microbiomes [8,9]. In addition, plants that can be propagated from cuttings, such as grapevines, also confirm the inheritance of beneficial endophytes along vegetative generations, leading to the birth of plant endophytic modification (PEM) [13]. PEM can be achieved technologically through the natural selection and artificial modification of the endophytic microbiome in plants. In agricultural systems, individual plants or the shoots of a plant may occasionally acquire traits associated with certain heritable endophytes so that these plants or shoots can be selected and propagated as crop lines. PEM by natural selection may have been applied in horticulture as 'bud sport selection', although we did not recognize it. We have reason to believe that certain proportional "bud mutations" are not caused by plant genetic variations (somatic cell clonal variations) but by the acquisition of certain heritable functional endophytes. PEM by artificial modification serves to directly shape the VTE of a target plant. Fortunately, this was successfully established in our current work. The addition of a disease-resistant VTE strain, ZX-2 (*B. cereus*), to freshly harvested (cut) wooden cuttings resulted in improved disease resistance of the progeny vines (Figure 9), confirming the practical value of PEM in agricultural and horticultural systems.

In general, successful PEM essentially involves (i) elite candidate VTE strains; (ii) suitable propagules of target plants; and (iii) methods for introducing the candidate VTE strain into target plants. Plant endophytes, like other plant-associated microbiota, are associated with the plant genotype in terms of host selectivity [18,19]. Studies have shown that long-term plant breeding not only shapes plant traits but also has a significant impact on the plant-associated microbiota [3]. This suggests a microbiome-integrated breeding approach [20]. Owing to the association of crop genetics with microbiota-based quantitative traits, host–microbiota interactions can be treated as an external quantitative trait, suggesting the need for strategies to integrate microbiota manipulation into crop selection programs [21]. However, it was also found that most intentionally introduced endophytic strains in the phyllosphere or rhizosphere of a plant disappear within a short time before having any detectable effect [1]. All of these studies emphasized the match between the genotype of the host plant and its associated endophytes.

As shown in the present experiment, not all VTEs are suitable for PEM (Supplementary Figure S1). Due to the complexity of a plant assembling its endophytic microbiota [4,22,23], the integration of an artificially introduced microbe into an established endophytic microbiome is difficult to predict. In general, endophytic strains for potential use in PEM should have the following characteristics: (1) a high ability to infect and colonize target plants; (2) the ability to transfer across plant compartments and generations; and (3) the ability to confer beneficial functions to host plants. Our proposal is to screen candidate PEM strains from the same plant genus, species, variety, or line for which PEM will be implemented. In this sense, the basic idea of PEM is to popularize those that incidentally acquire VTEs in most plant populations. In addition, we propose investigating candidate endophytic strains from the systematically distributed VTEs that could potentially move to different plant compartments (including propagules) and progeny plants. The currently used endophytic bacterial strain, ZX-2, was selected from a grapevine cultivar Faguoye (*V. vinifera* L.) and showed a strong ability to infect and become an endophyte within the grapevine cultivars Faguoye, Shuijing (*V. vinifera* L. × *V. labrusca* L.), Rosehoney (*V. vinifera* L. × *V. labrusca* L.), and Qiuzi (*V. davidii*) (Supplementary Figure S2), all of which taxonomically belong to the same genus, *Vitis*. Most importantly, ZX-2 could rapidly move to the newly formed shoots and leaves of grapevines with higher relative abundances (Figures 3 and 6), conferring disease resistance to the grapevines (Figure 9). The successful case of PEM using a cultivated grapevine in this work was largely attributed to the elite VTE strain, ZX-2. It is expected that more PEM strains with different functions will be developed and applied in future agricultural and horticultural practices.

The propagules that started the PEM are also important. The mechanism by which exogenous microorganisms horizontally infect intact plants and organs (e.g., seeds, bulbs, and tubers) and become endophytes is still poorly understood. Roots and leaf stomata and lenticels are considered to be plant parts and channels by which the plant can receive new endophytes. However, how these invading microbes enter plant tissues and are integrated into the existing endophytic microbiota requires further study. Our successful PEM may be partly due to the open system of freshly harvested cuttings, allowing for bacteria to easily enter the vascular bundles along the water flow (Figure 2).

However, as shown in our present research, the PEM cannot guarantee that all modified plants and their progeny will acquire the expected traits, but greatly increased the probabilities of host plants colonizing more candidate VTE strains (ZX-2) and obtaining the beneficial traits derived from the colonization of the candidate VTE strains (Figures 9 and 10; Supplementary Table S1). Therefore, a secondary or continuous selection is needed to obtain elite crop lines during a PEM. In addition, in this case of PEM, the relative abundances of the candidate bacterium ZX-2 (*Bacillus cereus*) were promoted in all parts of ZX-2mg, especially in the shoot parts (Figure 6), conferring ZX-2mg leaves with enhanced anti-pathogen abilities (Figure 9). However, some ZX-2mg leaves with relatively lower isolation rates of ZX-2 showed relatively stronger rates of fungal pathogen antagonism. On the other hand, the highest isolation rates of ZX-2 in grapevine leaves did not imply that the grapevine had the strongest pathogen antagonism (Supplementary Figure S3). The results suggest that, in addition to the ZX-2, other endophytes in ZX-2mg are involved in conferring disease resistance and other traits to the host plants.

In addition to the introduced ZX-2, the composition and abundance of other endophytes in ZX-2mg were strongly influenced (Figures 5–7). Compared to non-PEM grapevines, the introduction of ZX-2 significantly increased the relative abundance of the genera *Bacillus*, *Anoxybacillus*, and *Lysinibacillus* in grapevine shoots (Figure 6). The genus *Bacillus* is an undeniable source of biological control agents in sustainable aquaculture and agriculture [24,25]. In addition to the species *Bacillus cereus* (ZX-2), the level of three other endophytic bacterial species belonging to the genus *Bacillus* (*B. thermoamylovorans*, *B. marasmi*, and *B. firmus*) were also significantly increased in the shoots of ZX-2mg. The RA of the bacterial genus *Aneurinibacillus* was significantly increased in the shoots of ZX-2mg, and species of this genus were found to produce thermostable and organic solvent-tolerant lipase [26] and have the ability to solubilize tricalcium phosphate and fix nitrogen [27]. Other bacterial genera, such as *Proteiniphilum*, *Clostridium*, *Bradyrhizobium*, *Comamonas*, and *Shewanella*, were also significantly increased in the shoots of ZX-2mg (Figure 6). In the roots, the levels of some of the endophytic bacteria were also significantly promoted in ZX-2mg (Figure 6). Accordingly, fungal endophytes were significantly altered in the shoots and roots of ZX-2mg (Figure 7). The coordinated changes in other endophytes in ZX-2mg may confer other effects than disease resistance on host plants, such as growth promotion and delayed leaf abscission (Figure 10). The fact that one PEM had multiple effects on host plants further suggests a necessary step of continuous selections during the process of PEM. However, the alternative functions of the PEM and the modified endophytic microbiota await further investigation.

To test the disease resistance of PEM plants, we proposed a plant tissue–pathogen dual-culture system. PEM and non-PEM plant leaf discs were subjected to dual culture with different pathogenic fungal strains and, as expected, most leaf discs from PEM plants showed significant growth-suppressive effects on some pathogenic fungi (Figure 9A,C). PEM and non-PEM grapevines were of the same cultivar with similar genetic backgrounds but different endophytic microbiota. The endophytic isolates from the fungus-antagonistic grapevine leaf discs confirmed the antagonistic effects against the tested pathogenic fungi in an alternative experiment. Therefore, in addition to testing the disease resistance of plants, the method can be used to test whether the plants contain any disease-resistant endophytes and to rapidly isolate endophytes that are antagonistic to specific pathogens.

In contrast to plant genetic modification (PGM), PEM is performed by modifying the VTE of a plant to obtain stable functional endophyte traits and can be used as an alternative plant breeding strategy in agriculture and horticulture. However, there are still many problems with the successful application of this technology in the selection of candidate strains and propagules, the introduction of candidate strains into plants, the trait 'expression' of the candidate endophytic trains, and the continuous selection for obtaining stable PEM lines. Successful PEM should be carried out with a candidate VTE within the permissive range of the host genotype, where the candidate VTE has the opportunity to integrate with the endophytic microbiota of the target plants. Nevertheless, in the context of plant genetic modification, PEM is still a good compensation tool in "crop breeding" practices.

5. Conclusions

We hypothesized that the vertically transmitted endophytes (VTEs), as a kind of plant-heritable trait, and these endophytes can be directly manipulated to confer host plants with long-term beneficial functions, namely plant endophytic modification (PEM). The present work successfully carried out a PEM by introducing a disease-resistant VTE bacterium into grapevine cuttings, which resulted in improved long-term disease resistance of the progeny vines, confirming the practical value of the PEM strategy in plant improvement and in future organic farming systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10060607/s1, Figure S1: Detection of the infection colonization abilities of candidate VTE strains ZX-2 (A), P1 (B), and YA (C) on grapevine cuttings of cultivar RH. "*": significance at 0.05 ($p < 0.05$); "**": significance at 0.01 ($p < 0.01$); and "***": significance at 0.001 ($p < 0.001$); Figure S2: VTE strain ZX-2 have a broad spectrum, infecting different cultivars of grapevines. (A–D): Detecting the infection rates of ZX-2 in grapevine cuttings cultivars Rose Honey, Faguoye (B), Shuijing (C), and Qiuzi (D). "*'": significance at 0.05 ($p < 0.05$); "**": significance at 0.01 ($p < 0.01$); and "***": significance at 0.001 ($p < 0.001$); Figure S3: Correlations between fungal inhibition rates and *B. cereus* (ZX-2) isolation rates in grapevine leaves; Table S1: Values of the detected pathogen inhibition rate and osolation rate of *Bacillus cereus* in all grapevine samples.

Author Contributions: M.-Z.Y. and S.-S.Z., designed the research, wrote the main manuscript text. C.-X.C., and L.-R.G., performed the majority of the experiments and data analysis. Y.-T.W., Y.W., Y.L., C.-X.L., P.Z., S.-Y.H. and Y.-Q.L. participated in the lab works and data analysis. X.-X.P., participated in the preparation of figures and manuscript organization. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data and materials are available in the manuscript and in NCBI under the accession numbers PRJNA977117 and PRJNA977126.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

ASV	amplicon sequence variant
DAT	days after treatment
EMP	endophytically modified plant
LEfSe	Linear discriminant analysis effect size
OTUs	operational taxonomic units
PBA	peptone beef agar
PCoA	Principal co-ordinates analysis
PDA	potato dextrose agar

PEM	plant endophytic modification
ZX-2mg	ZX-2 modified grapevines
RA	relative abundance
HTEs	horizontally transmitted endophytes
VTEs	vertically transmitted endophytes

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