Analysis of the Structure of Phyllosphere Epiphytic Microorganisms in Tea Plants Under the Influence of ZnO NPs

Screening and Random Forest Analysis of Differential Epiphytic Microorganisms

To clarify which microbial groups cause the differences in the phyllosphere epiphytic microorganisms of tea plants caused by ZnO NPs, we used LefSe (LDA Effect Size) analysis to screen for differentially abundant microorganisms, i.e., to find statistically significant biomarkers. There were 14 important biomarkers affecting the bacterial community structure differences between CKEP and T1EP, with CKEP dominated by *Marinococcus sp JL786*, *Marinococcus*, *Bacilli*, unidentified *Bacillales*, *Bacillales*, unidentified *Myxococcota*, and *metagenome*, while T1EP was dominated by *Halomonadaceae*, unidentified *Actinobacteria*, *Actinobacteria*, *Micrococcales*, *Microbacterium*, *Microbacteriaceae*, and *Micrococcaceae* (Fig.25A). There were 18 important biomarkers affecting the bacterial community structure differences between CKEP and T2EP, with CKEP dominated by *Marinococcus sp JL786*, *Marinococcus*, *Bacilli*, unidentified *Bacillales*, *Bacillales*, *Firmicutes*, *Xanthobacteraceae*, unidentified *Myxococcota*, and unidentified *Myxococcota*, while T2EP was dominated by *Microbacterium*, unidentified *Actinobacteria*, *Micrococcales*, *Microbacteriaceae*, *Actinobacteria*, *Candidatus Portiera aleyrodidarum*, *Patulibacter minatonensis*, *Micrococcaceae*, and unidentified *Halomonadaceae* (Fig.25B). There were 29 important biomarkers affecting the fungal community structure differences between CKEP and T1EP, with CKEP dominated by *Hypocreales*, *Acremonium alternatum*, *Sterigmatomyces halophilus*, *Glomerellaceae*, *Acremonium*, *Sterigmatomyces*, *Agaricostilbaceae*, *Sordariomycetes order Incertae sedis*, *Hypocreales family Incertae sedis*, *Agaricostilbales*, *Agaricostilbomycetes*,

Colletotrichum, *Trichocomaceae*, *Penicillium*, *Eurotiales*, and *Eurotiomycetes*, while T1EP was dominated by *Pleosporales*, *Schizophyllum commune*, *Agaricales*, *Schizophyllum*, *Lophiostoma*, *Pleosporales family Incertae sedis*, *Schizophyllaceae*, *Lophiostomataceae*, *Filobasidiales*, *Monographella cucumerina*, *Zygomycota*, *Polyporales*, and *Monographella* (Fig.25C). There were 17 important biomarkers affecting the fungal community structure differences between CKEP and T2EP, with CKEP dominated by *Sterigmatomyces halophilus*, *Acremonium alternatum*, *Hypocreales*, *Sterigmatomyces*, *Agaricostilbaceae*, *Acremonium*, *Agaricostilbales*, *Hypocreales family Incertae sedis*, *Agaricostilbomycetes*, *Trichocomaceae*, *Penicillium*, *Eurotiomycetes*, *Eurotiales*, *Hansfordia pulvinata*, and *Pleosporaceae*, while T2EP was dominated by *Cystofilobasidiales* and *Cystofilobasidiaceae* (Fig.25D). The results indicate that ZnO NPs have a significant impact on both phyllosphere epiphytic bacteria and fungi in tea plants.

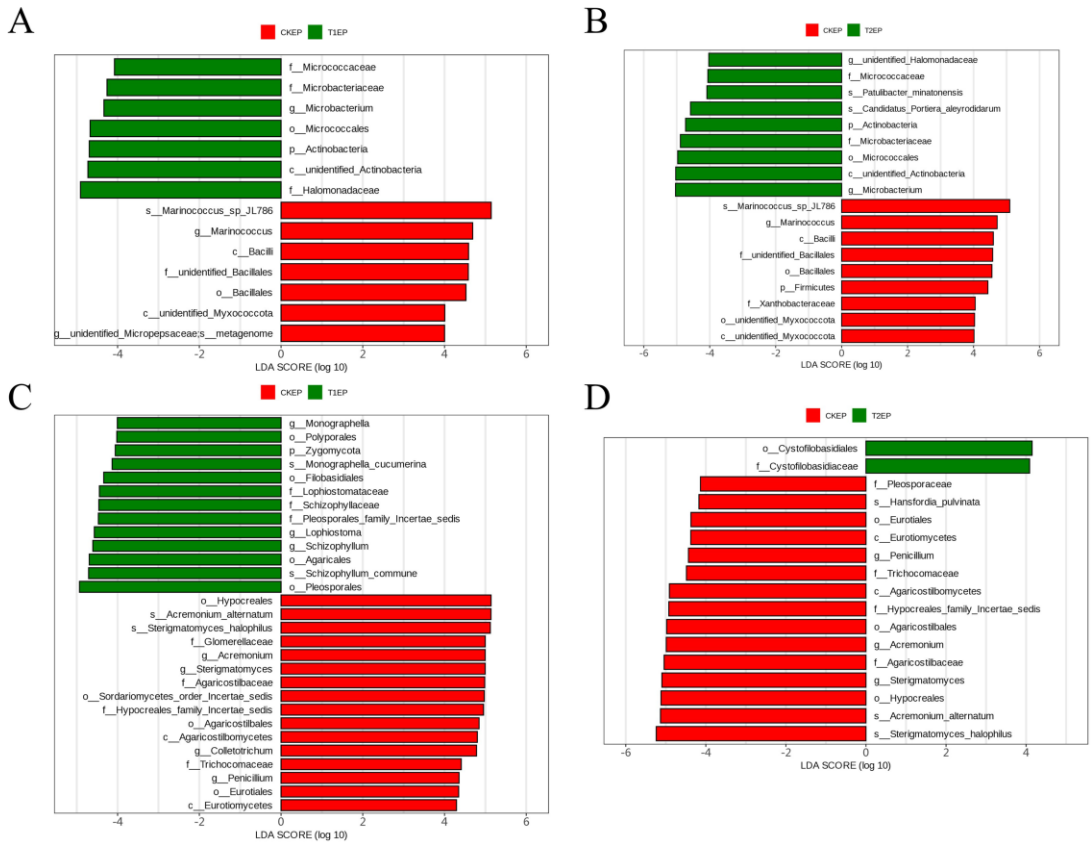

Fig.25 Significant species with differential bacterial abundance between CKEP and T1EP

under the influence of ZnO NPs (A); significant species with differential bacterial abundance between CKEP and T2EP (B); significant species with differential fungal abundance between CKEP and T1EP (C); significant species with differential fungal abundance between CKEP and T2EP (D). The LDA value distribution bar chart shows species with an LDA Score greater than the set value (default is 4), i.e., biomarkers with statistical differences between groups, and the length of the bar chart represents the impact size of the differential species.

To identify key species of phyllosphere epiphytic microorganisms in tea plants under the influence of ZnO NPs, and thus understand the diversity and function of the microbial community, we conducted a random forest analysis (Fig.26). MeanDecreaseGini calculates the impact of each variable on the heterogeneity of observations at each node of the classification tree through the Gini index, thereby comparing the importance of variables. The larger the value, the more important the variable is. *Marmoricola* and *Penicillium* were able to explain the largest changes in the epiphytic bacterial and fungal communities, respectively, at 50mg L^{-1} ZnO NPs; *Microbacterium* and *Pseudeurotium* were able to explain the largest changes in the epiphytic bacterial and fungal communities, respectively, at 100mg L^{-1} ZnO NPs. The Area Under Curve (AUC) was 1 for both, indicating good accuracy and discrimination ability.

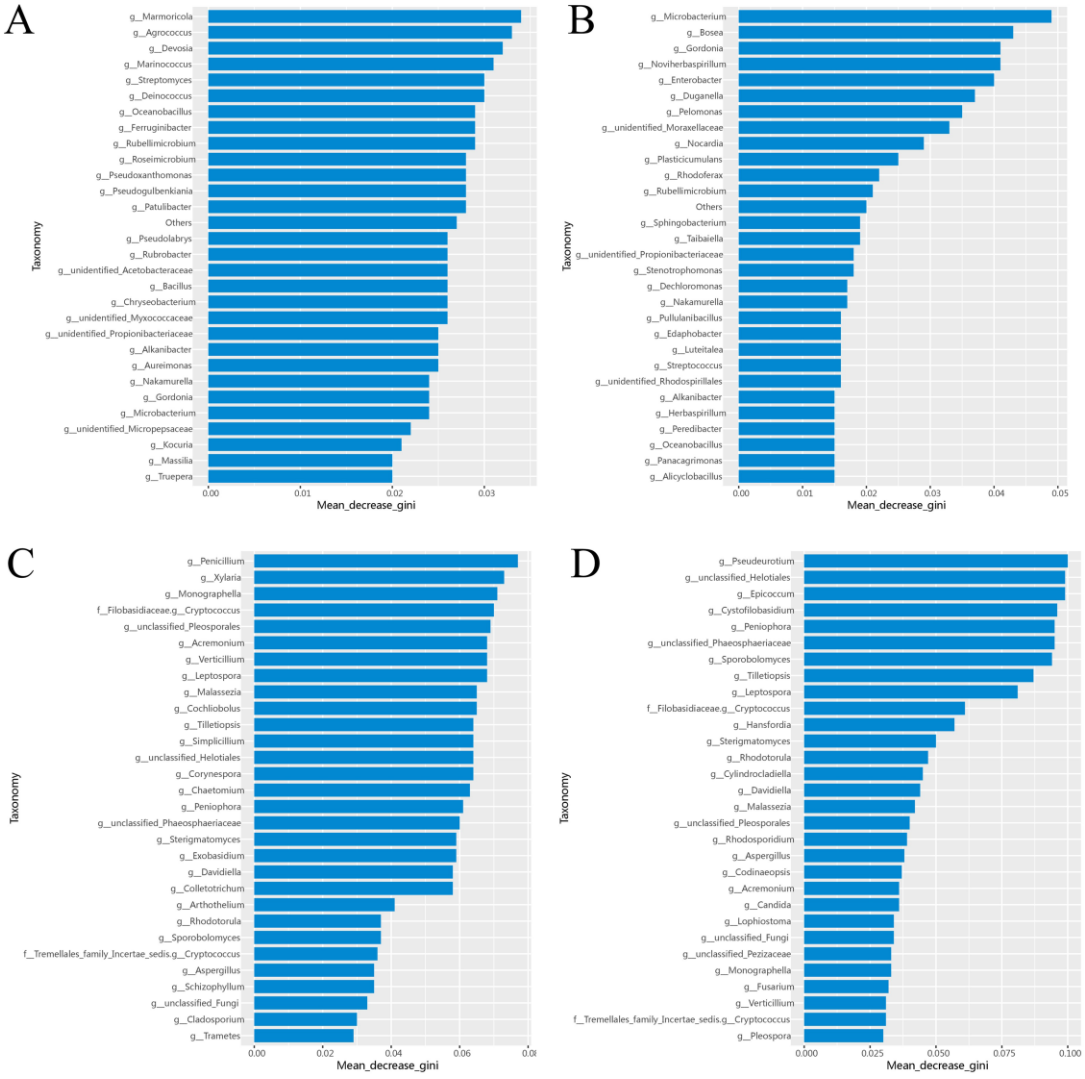

Fig.26 Key species of phyllosphere epiphytic microorganisms (at the genus level) under the influence of ZnO NPs. Key epiphytic bacteria in tea plants under the influence of T1 (A); key epiphytic fungi under the influence of T2 (B); key epiphytic bacteria under the influence of T1 (C); key epiphytic bacteria under the influence of T2 (D).

Functional Clustering Analysis of Phyllosphere Epiphytic Bacteria and Fungi

To analyze the impact of ZnO NPs on the functions of phyllosphere epiphytic bacteria in tea plants, we used Tax4Fun2 for functional annotation and clustering. As shown in Fig.27A, the bacterial

functions in CKEP were mainly enriched in Microbial metabolism in diverse environments, Carbon fixation pathways in prokaryotes, Valine, leucine and isoleucine degradation, Propanoate metabolism, Benzoate degradation, Butanoate metabolism, Pyruvate metabolism, Degradation of aromatic compounds, Fatty acid degradation, Glyoxylate and dicarboxylate metabolism, Fatty acid biosynthesis, Phenylalanine metabolism, Fatty acid metabolism, Sulfur metabolism, and Biotin metabolism. The bacterial functions in T1EP were primarily clustered in Quorum sensing. The bacterial functions in T2EP were primarily clustered in Carbon metabolism, Amino sugar and nucleotide sugar metabolism, Methane metabolism, Pyrimidine metabolism, Ribosome, Biosynthesis of amino acids, Glycolysis/Gluconeogenesis, Biosynthesis of antibiotics, Metabolic pathways, Cysteine and methionine metabolism, and Biosynthesis of secondary metabolites.

To analyze the impact of ZnO NPs on the functions of phyllosphere epiphytic fungi in tea plants, we used FunGuild for functional annotation and clustering. As shown in Fig.27B, the fungal functions in CKEP were mainly clustered in Endophyte-Plant Pathogen-Wood Saprotroph, Dung Saprotroph-Undefined Saprotroph-Wood Saprotroph, Animal Pathogen-Endophyte-Fungal Parasite-Plant Pathogen-Wood Saprotroph, Undefined Saprotroph, Animal Pathogen, Endophyte-Undefined Saprotroph-Wood Saprotroph, Lichenized, Endophyte-Plant Pathogen, Dung Saprotroph-Ectomycorrhizal-Litter Saprotroph-Undefined Saprotroph. The fungal functions in T1EP were mainly enriched in Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph, Plant Pathogen-Wood Saprotroph, Dung Saprotroph-Soil Saprotroph-Wood Saprotroph, Dung Saprotroph-Soil Saprotroph, Animal Pathogen-Endophyte-Fungal Parasite-Lichen Parasite-Plant Pathogen-Wood Saprotroph, Animal Endosymbiont-Animal Pathogen-Endophyte-Plant Pathogen-

Undefined Saprotroph, Animal Pathogen-Endophyte-Epiphyte-Undefined Saprotroph, Endophyte-Plant Pathogen-Undefined Saprotroph, Endophyte-Litter Saprotroph-Undefined Saprotroph, Wood Saprotroph, and Fungal Parasite-Plant Pathogen-Plant Saprotroph. The fungal functions in T2EP were mainly enriched in Endomycorrhizal-Plant Pathogen-Undefined Saprotroph, Ectomycorrhizal, Animal Pathogen-Undefined Saprotroph, Plant Pathogen-Undefined Saprotroph, Plant Pathogen, Leaf Saprotroph, Soil Saprotroph-Undefined Saprotroph, and Endophyte-Insect Pathogen.

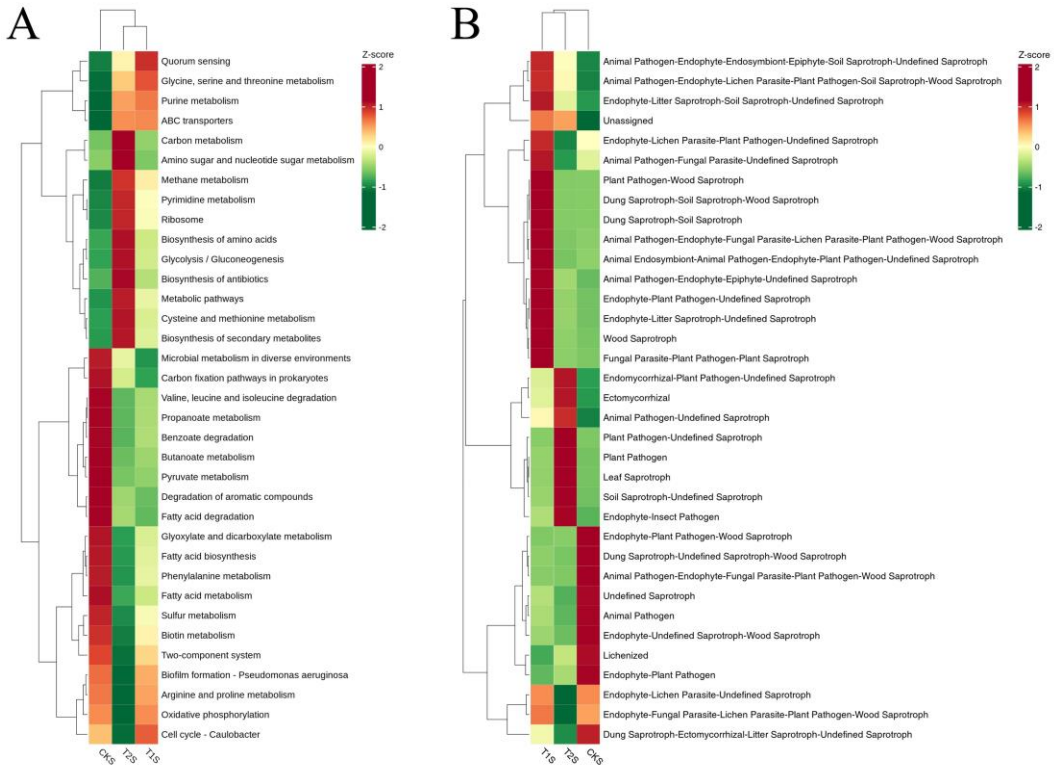

Fig.27 Heatmaps of functional annotation clustering of phyllosphere epiphytic bacteria based on Tax4Fun2 (A) and phyllosphere epiphytic fungi based on FunGuild (B) across different groups. Horizontally, the heatmaps represent functional annotation information; vertically, they represent sample information. The squares indicate relative abundance, with redder colors indicating higher relative abundance and bluer colors indicating lower relative abundance.