Changes in endophytic microbial diversity and function in the root system affect the growth of continuously planted *Casuarina equisetifolia*

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ABSTRACT: Continuous planting barrier is an important limiting factor for the growth of *Casuarina equisetifolia* (*C. equisetifolia*), an ecological and economic tree varieties, but its underlying microbial mechanism has not been fully elucidated. This study revealed microbial-driven barriers by examining the impact of continuous planting on root endophyte communities in *C. equisetifolia*. The results showed that continuous planting significantly reduced the antioxidant enzyme activities, root activity, and nutrient accumulation in the root system of *C. equisetifolia*, and inhibited root length and plant height growth. Microbial community analysis revealed that continuous planting led to a significant decrease in the abundance of Nitrobacter, a key endophytic bacterium in the root system, which weakened the nitrogen metabolism functions mediated by nitrate reduction, nitrogen respiration, and nitrate respiration, and undermined the nitrogen conversion efficiency of the root system. At the same time, continuous planting promoted the enrichment of pathogenic endophytic fungi (*Phomopsis*, *Pseudocercospora* and *Diaporthe*) in the root system and enhanced their plant pathogen functions, which further reduced the antioxidant and nutrient uptake capacities of the *C. equisetifolia* root system. It was shown that continuous planting inhibited *C. equisetifolia* growth through a dual microbial mechanism: on the one hand, it reduced functional flora, weakening nitrogen metabolism and stress tolerance; on the other hand, it increased pathogenic fungi and intensified disease impact. This study provides a new perspective for analyzing the endogenous microecological mechanism of the continuous planting disorder, and provides a theoretical basis for alleviating the disorder by regulating the root microbial community.

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IMPORTANCE: As an important ecological and economic tree species, the sustainable management of *Casuarina equisetifolia* plantations is severely challenged by the continuous planting barrier. While prior studies focused on soil changes, the role of root endophytic microbes remains unclear. This study reveals coordinated changes in root endophytic microbial structure and function under continuous planting and their effects on root physiology. Continuous planting disrupts microbial stability and inhibits growth by weakening nitrogen metabolism and promoting disease. These findings deepen understanding of plant–microbe interactions and offer theoretical and practical guidance for alleviating plantation degradation via microbial regulation, aiding the development of healthy, stable, and efficient forest ecosystems.

KEYWORD: *Casuarina equisetifolia*; key endophyte; nitrogen metabolism; pathogen; growth

INTRODUCTION

Casuarina equisetifolia (*C. equisetifolia*), as an important ecological and economic tree variety in tropical and subtropical regions, plays a key role in the construction of coastal protection forests and the restoration of degraded ecosystems (1). However, with the continuous planting of plantation forests, the problem of continuous planting barriers has become more and more prominent, which is manifested in the phenomena of forest growth decline, biomass decline, weakening of stress resistance, and intensification of pests and diseases, which seriously restricts the sustainable management of plantation forests (2). Recent studies have shown that the disorganization of microbial communities is one of the core drivers of plantation barriers, with endophytic microorganisms playing a crucial role in plant-soil system interactions (3, 4). As a special group of microorganisms colonizing the interior of plant tissues, endophytes are not only involved in nutrient uptake and stress tolerance regulation of the host, but also play a key role in maintaining rhizosphere microecological balance (5, 6). However, the structural and functional changes of endophyte communities in the root system of *C. equisetifolia* under continuous planting conditions and their relationship with the formation of continuous planting barriers are still unclear, and in-depth analysis will help to unravel the mechanism of the role of endophytes in the formation of continuous planting barriers in *C. equisetifolia*.

Plant endophytes are plant parasitic bacteria that enhance the immune response of plants and establish symbiotic relationship with plants, and they have plant growth promoting activity while inhibiting phytopathogenic fungi (7). Andrade et al. (8) isolated 67 strains of endophytic bacteria from *Cattleya walkeriana*

and found that most of the endophytic bacteria were favorable to increase the antioxidant enzyme activities of *Cattleya walkeriana*, enhance the nutrient uptake and accumulation capacity, and promote plant growth. In recent years, the role of root endophytes in mitigating plant succession disorder has received much attention and achieved many results (9). For example, Guo et al. (10) found that continuous planting significantly altered the community structure of endophytic bacteria in the root system of *Salvia miltiorrhiza* and reduced its yield quality, whereas inoculation of *Pseudomonas* in the root system significantly improved the growth of continuously planted *Salvia miltiorrhiza* seedlings and increased their yield and quality. Soybean root endophytes are beneficial in increasing the nitrogen pool in the soil to provide nutrients to the plant, and also maintain plant growth and health by producing antibiotics against plant pathogens, stimulating phytohormone biosynthesis, to decrease the effect of continuous planting on the growth of soybeans (11). Hwang et al. (12) found that inoculation of the endophytic bacterium *Priestia megaterium*, isolated from Bolboschoenus planiculmis, could effectively improve the antioxidant capacity of the plant root system, enhance the plant's ability to resist salt stress and drought stress, and promote plant growth. It can be seen that endophytic bacteria play an important role in maintaining plant health and promoting plant growth, and that the obstacles to continuous planting of plants may be closely related to changes in the community structure and function of their endophytic bacteria. It has been reported that continuous planting of *C. equisetifolia* easily leads to a decrease in the diversity and function of bacteria in the rhizosphere soil and an increase in the number of fungi, especially pathogenic fungi, which reduces the nutrient cycling capacity of the soil, decreases the ability of the root system to absorb and accumulate nutrients, and hinders the growth of *C. equisetifolia* (13). The endophytic bacteria that exist in symbiosis within the root system for a long period of time play an important role in maintaining plant growth (14). Whether the reduced nutrient accumulation capacity of the root system and stunted growth of *C. equisetifolia* are related to the changes in the structure and function of its endophyte community is still unreported. And in-depth understanding of the effects of continuous planting on the structure and function of the endophyte community in the root system of *C. equisetifolia* is of great significance in alleviating the obstacles of continuous planting in *C. equisetifolia*.

Accordingly, the present study took *C. equisetifolia* with different generations of continuous planting as the research object, and integrated multidisciplinary methods such as physiological ecology, microbial ecology and bioinformatics to systematically investigate the interactions between the growth performance of *C. equisetifolia*, the physiological characteristics of the root system, and the endogenous microbial community under continuous planting. By comparing and analyzing the growth indices, root physiological and biochemical characteristics,

nutrient content, and the structure of endophytic bacterial and fungal communities between the first planting and the third plantings of *C. equisetifolia*, combining with the methods of covariance network analysis and functional prediction, the effects of continuous planting on the growth of *C. equisetifolia* and root physiology were explored, and the response of the endophytic bacterial communities in terms of their diversity, structure, and function to continuous planting was analyzed, the interaction mechanism of key endophytes and their functional properties and the growth inhibition of *C. equisetifolia* was revealed. The present study not only provides new insights into the microbiological mechanism of the obstacles to continuous planting of *C. equisetifolia*, but also provides theoretical support for the realization of sustainable management of plantation forests through microbial regulation, which is of great significance to ensure the health and stability of plantation forest ecosystems.

RESULTS

Analysis of growth and root physiological and biochemical indices of *C. equisetifolia* in continuous planting

In this study, growth and root physiological and biochemical indices of *C. equisetifolia* were determined after continuous planting. Among them, the results of *C. equisetifolia* growth indices were determined (Fig. 1A), which showed that the root length and plant height of ML were significantly inhibited, with inhibition rates of 30.64% and 49.34%, respectively, when compared with those of MC. Secondly, it was found (Fig. 1B) that superoxide dismutase, peroxidase, catalase and root activity were reduced by 36.35%, 22.18%, 31.92% and 22.19%, respectively, in the ML root system as compared to MC, whereas malondialdehyde increased by 167.39%. In addition, the results of total nitrogen, phosphorus and potassium measurements in the root system (Fig. 1C) showed that the total nitrogen, phosphorus and potassium contents in the ML root system decreased by 6.15%, 24.70% and 10.86%, respectively, compared with those in the MC. It can be seen that continuous planting led to the reduction of antioxidant capacity of *C. equisetifolia* root system, and the ability of root system to absorb and accumulate nutrients such as nitrogen, phosphorus and potassium, which led to the growth inhibition of *C. equisetifolia* root system.

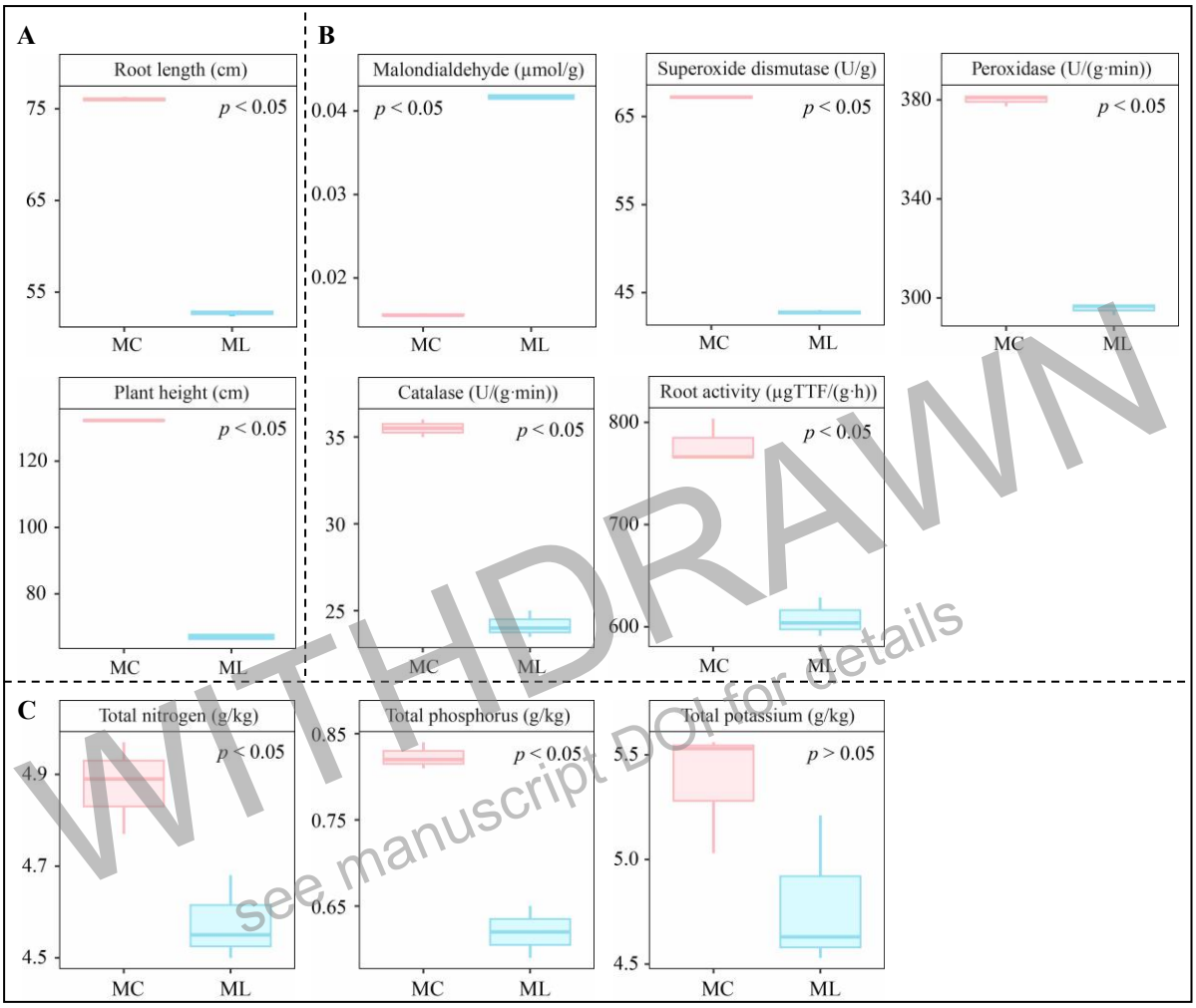

FIG 1 Analysis of growth and root physiological and biochemical indices of *C. equisetifolia* in continuous planting. MC and ML denote the first planting and third continuous plantings of *C. equisetifolia*, respectively; (A) Plant height and root length of *C. equisetifolia*; (B) Physiological indices of the root system of *C. equisetifolia*; (C) Total nitrogen, phosphorus, and potassium content of the root system of *C. equisetifolia*.

Diversity analysis of endophytic bacteria and fungi in the root system of *C. equisetifolia*

High-throughput sequencing results of endophytic bacteria in the root system of *C. equisetifolia* showed (Fig. 2A) that a total of 666 bacterial OTUs were detected, of which 485 were common to MC and ML, 85 were unique to MC, and 96 were unique to ML. The analysis of α-diversity indices of endophytic bacterial communities showed (Fig. 2B) that the ACE and Chao1 of ML were not significantly different from MC ($p > 0.05$), while Shannon and Simpson were significantly larger than MC ($p < 0.05$). The results of PCoA analysis of β-diversity of endophytic bacterial communities showed (Fig. 2C) that the two principal components could

effectively differentiate between MC and ML with an overall contribution of 77.04%. It can be seen that there was a significant difference in community diversity between root endophytes of MC and ML, and the diversity of endophytic bacteria in the root system of *C. equisetifolia* was enhanced in the continuous planting.

Further analysis using the covariance network revealed (Fig. 2D) that the covariance network of endophytic bacteria in the MC root system existed 570 nodes, 8911 edges, and an average connectivity of 31.27, while the covariance network of endophytic bacteria in the ML root system existed 581 nodes, 9346 edges, and an average connectivity of 32.17. All the indices of ML were larger than that of MC, this can be seen that the endophytic bacteria of ML root system were more complex than MC. Secondly, the neutral community model analysis (Figure 2E) showed that the R^2 value of MC was 0.765, which was higher than that of ML (0.732), and the mobility m was 0.116, which was lower than that of ML (0.119), which indicated that the endophytic bacteria of the root system of *C. equisetifolia* were more diffuse, more diversified, and the bacterial community structure was more complex after continuous planting of *C. equisetifolia*.

Further analysis of *C. equisetifolia* root endophytic bacterial community structure revealed (Fig. 2F) that at the gate level, among the top ten root endophytic bacteria in MC and ML in terms of their respective abundance, ML roots showed a decrease in the abundance of Proteobacteria, Acidobacteriota, Bacteroidetes, Chloroflexi, and Armatimonadota compared to MC, while the abundance of Verrucomicrobiota, Actinobacteriota, Planctomycetota, Myxococcota, Patescibacteria and Desulfobacterota increased in abundance. At the genus level, the abundance of *Bradyrhizobium*、*Ktedonobacter*、*Niastella*、*Pseudolabrys*、*Terracidiphilus* and *Opitutus*、*Rhodoplanes* decreased in the ML root system as compared to MC, while *Pedosphaera*、*Candidatus Koribacter*、*Gemmata*、*Actinocorallia*、*Candidatus Saccharimonas* increased in abundance. It can be seen that the abundance of endophytic bacterial communities in the root system of *C. equisetifolia* changed significantly after continuous planting.

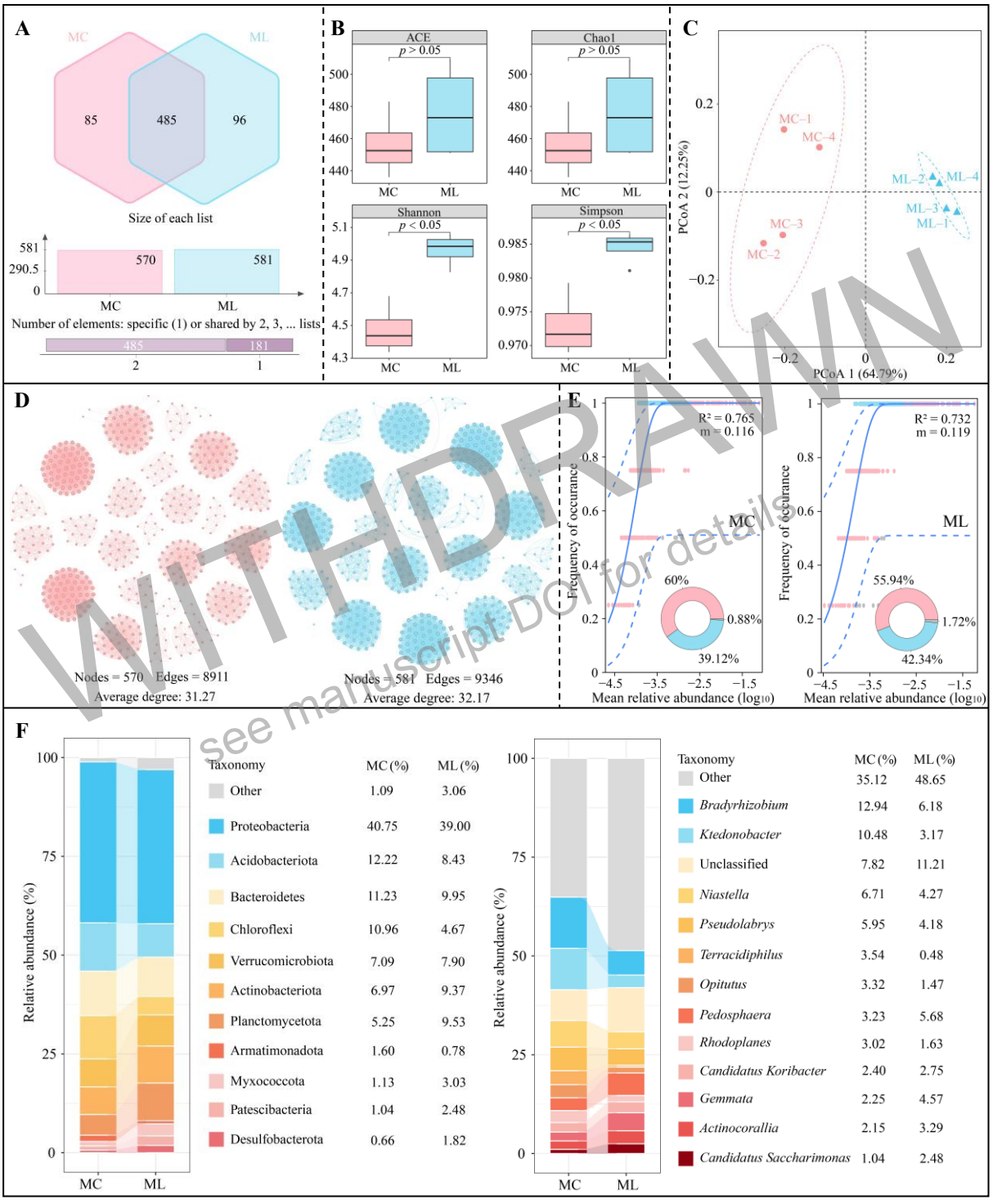

FIG 2 Endophytic bacterial diversity analysis of *C. equisetifolia* root system. MC and ML denote the first planting and third continuous plantings of *C. equisetifolia*, respectively; (A) Venn diagram analysis of endophytic bacterial community; (B) α-diversity analysis of endophytic bacterial community; (C) PCoA analysis of β-diversity of endophytic bacterial community; (D) Covariance network diagram analysis of endophytic bacterial community; (E) Neutral community model analysis of endophytic bacterial community; (F) Abundance analysis of endophytic bacteria at phylum and genus levels.

High-throughput sequencing results of endophytic fungi in the root system of *C. equisetifolia* showed (Fig. 3A) that a total of 165 fungal OTUs were detected, of which 92 were common to MC and ML, 27 were unique to MC, and 46 were unique to ML. The α-diversity analysis of the endophytic fungal community showed (Fig. 3B) that ACE, Chao1, Shannon, and Simpson of ML were significantly different from MC ($p > 0.05$). The results of PCoA analysis of β-diversity of endophytic fungal communities showed (Fig. 3C) that the two principal components could effectively differentiate between MC and ML with an overall contribution of 82.12%. It can be seen that the root endophytic fungi of MC and ML did not differ significantly in community diversity, but there may be significant differences in community composition and abundance.

Further analysis using the covariance network revealed (Fig. 3D) that the covariance network of endophytic fungi in the MC root system existed 114 nodes, 385 edges, and an average connectivity of 6.47, whereas the covariance network of endophytic fungi in the ML root system existed 115 nodes, 510 edges, and an average connectivity of 8.57. All the indices of ML were larger than that of MC, this can be seen that the endophytic fungi in the ML root system were more complex than that of MC. Neutral community model analysis revealed (Fig. 3E) that MC had an R^2 value of 0.396 and a mobility m of 0.030, both of which were higher than that of ML ($R^2 = -0.008$, $m = 0.016$). It can be seen that *C. equisetifolia* reduced the diffusion of endophytic fungi in its root system after continuous planting, but its community structure was still more complex.

Further analysis of the community structure of endophytic fungi in the root system of *C. equisetifolia* revealed (Fig. 3F) that at the phylum level, among the top ten abundance of root endophytic fungi in MC and ML, the abundance of Ascomycota decreased in the ML root system as compared to MC, while Glomeromycota, Basidiomycota, Chytridiomycota, Rozellomycota, Calcarisporiellomycota and Mucoromycota increased in abundance. At the genus level, the abundance of *Venturia*、*Saitozyma*、*Oncopodiella*、*Coniochaeta*、*Robillarda* and *Pestalotiopsis* decreased in the ML root system compared to MC, while *Rhizophagus*、*Fusarium*、*Cordana*、*Arthropsis*、*Ruhlandiella*、*Cladosporium*、*Quixadomyces*、*Psathyrella* and *Diaporthe* increased in abundance. It can be seen that the abundance of endophytic fungal communities in the root system of *C. equisetifolia* changed significantly after continuous planting.

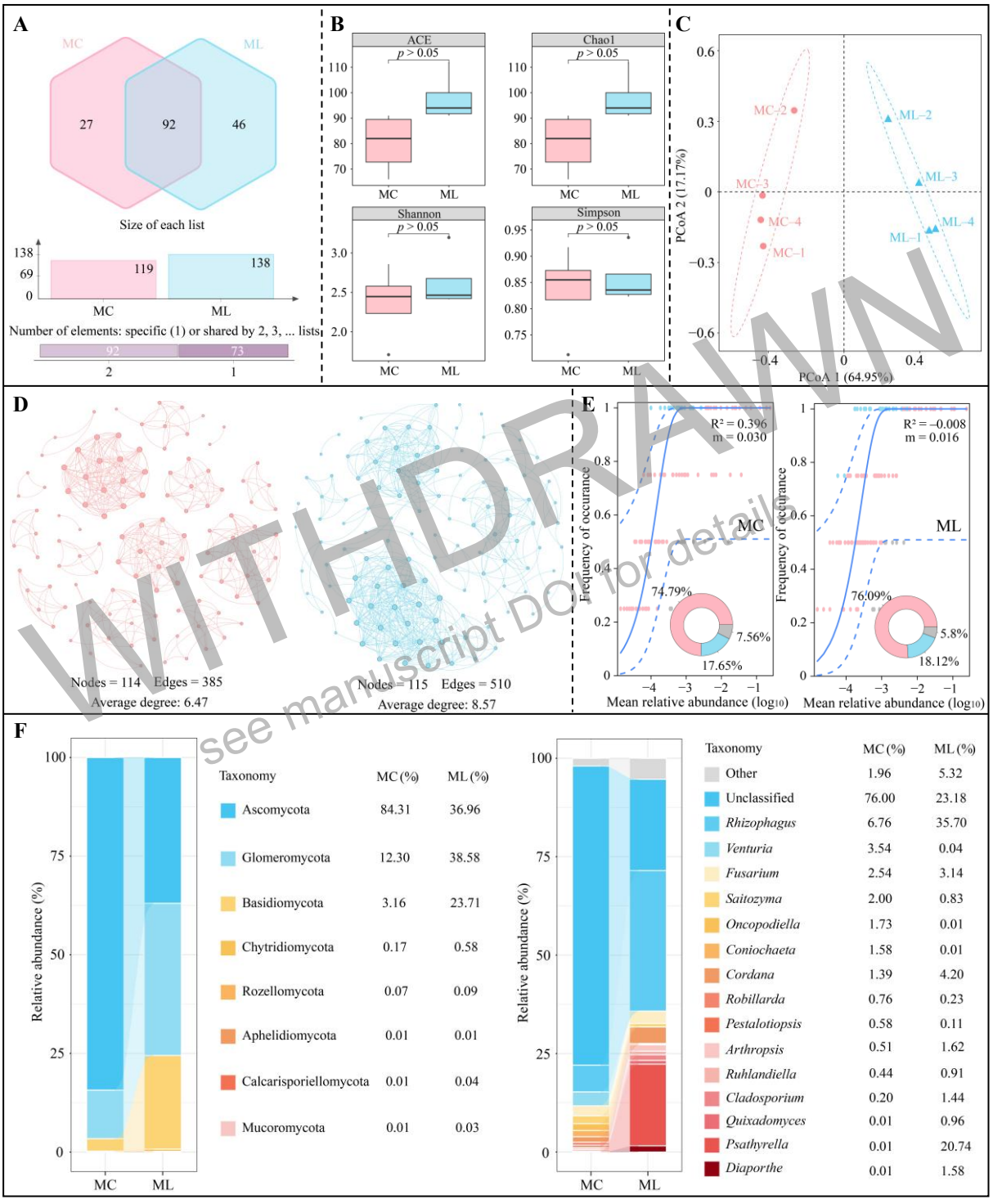

FIG 3 Diversity analysis of endophytic fungi in the root system of *C. equisetifolia*. MC and ML denote the first planting and third continuous plantings of *C. equisetifolia*, respectively; (A) Venn diagram analysis of endophytic fungal communities; (B) α-diversity analysis of endophytic fungal communities; (C) PCoA analysis of β-diversity of endophytic fungal communities; (D) Covariance network diagram analysis of endophytic fungal communities; (E) Neutral community model analysis of endophytic fungal communities; (F) Abundance analysis of endophytic fungi at phylum and genus levels.

Screening of key differential endophytic bacteria and fungi in the root system of *C. equisetifolia* in continuous plantings

Based on the above analysis, this study further screened the endophytic key differential bacteria and fungi in the root system of *C. equisetifolia* in continuous planting. The results of modular interactions network analysis of endophytic bacteria showed (Fig. 4A) that 666 bacterial OTUs from 275 genera could be classified into three modules, of which modules 1, 2, and 3 contained 86, 81, and 108 genera, respectively. The node topological role analysis of the three modules found (Fig. 4B) that a total of 32 keystone nodes were identified, and all of them showed high connectivity nodes (Connector, Zi < 2.5, Pi > 0.6), of which modules 1, 2 and 3 contained 24, 11 and 7 keystone nodes, respectively. Analysis of the endogenous bacterial abundance of keystone nodes in different modules revealed (Fig. 4C) that there was no significant difference in the endogenous bacterial abundance between MC and ML in module 1 and module 2 ($p > 0.05$), whereas the endogenous bacterial abundance of MC was significantly higher than that of ML in module 3 ($p < 0.05$). Further analysis revealed (Figure 4D) that the endophytic bacteria in the 7 keystone nodes in module 3 were mainly from 7 genera, of which 6 genera showed significant differences in abundance, namely *Chelativorans*, *Mucilaginibacter*, *Paraburkholderia*, *Rhodoblastus*, *Acidisphaera* and *Nitrobacter*. It is evident that the key differential endophytic bacteria in the root system of *C. equisetifolia* after continuous planting were mainly from 6 genera.

In addition, the results of modular interactions network analysis of endophytic fungi showed (Fig. 5A) that 165 fungal OTUs from 65 genera could be classified into 10 modules, of which modules 1, 2, 3, 4, 5, and 6 contained 17, 15, 11, 4, 10, and 4 genera, respectively, and modules 7, 8, 9, and 10 all contained only 1 genus. The results of the node topology roles of the 10 modules showed (Fig. 5B) that a total of 14 keystone nodes were identified and all of them showed high connectivity (Connector, Zi < 2.5, Pi > 0.6), where modules 1, 2, 3, 4, 5 and 6 contained 5, 1, 2, 2, 2 and 2 keystone nodes, respectively, while the remaining four modules had no keystone nodes. Analysis of the endophytic fungal abundance of keystone nodes in different modules revealed (Fig. 5C) that there was no significant difference in endophytic fungal abundance between MC and ML in modules 2, 3, and 4 ($p > 0.05$), whereas the endophytic fungal abundance of ML was significantly higher than that of MC in modules 1, 5, and 6 ($p < 0.05$). Further analysis revealed (Fig. 5D) that the endophytic fungi of 9 keystone nodes in modules 1, 5, and 6 were mainly from 9 genera, of which 6 genera showed significant differences in abundance, namely*Rhizophagus*, *Psathyrella*, *Pseudocercospora*, *Phomopsis*, *Diaporthe*, *Neofabraea*. It is evident that the key differential endophytic fungi in the root system of *C. equisetifolia* after continuous planting were mainly from 6 genera.

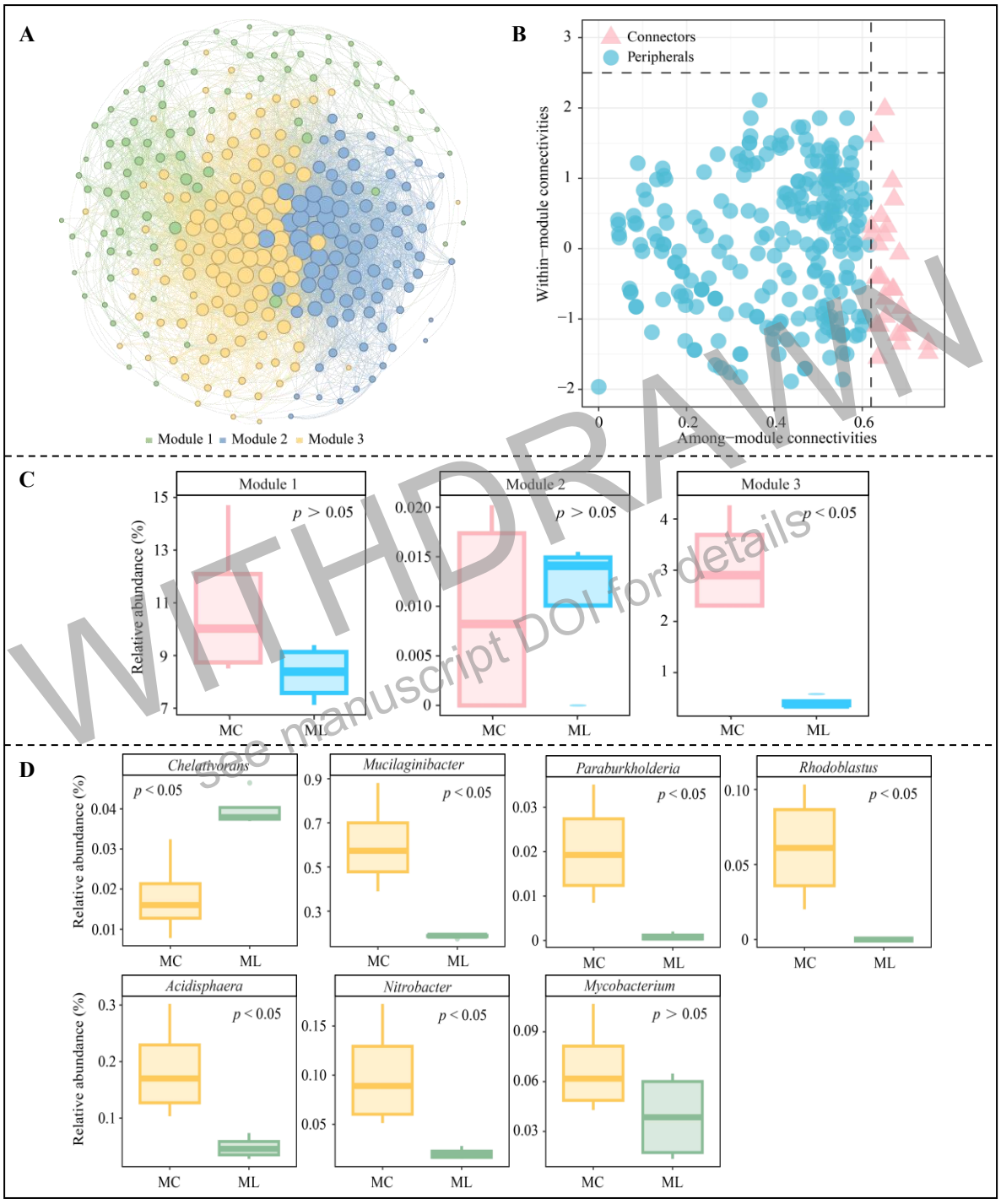

FIG 4 Modular analysis of endophytic bacteria in the root system of *C. equisetifolia* and screening of keystone differential bacteria. MC and ML denote the first planting and third continuous plantings of *C. equisetifolia*, respectively; (A) Modular interactions network analysis of endophytic bacteria; (B) Keystone node analysis of the modular network; (C) Endophytic bacterial abundance analysis of the keystone nodes of different modules; (D) Abundance analysis of different endophytic bacteria in module 3.

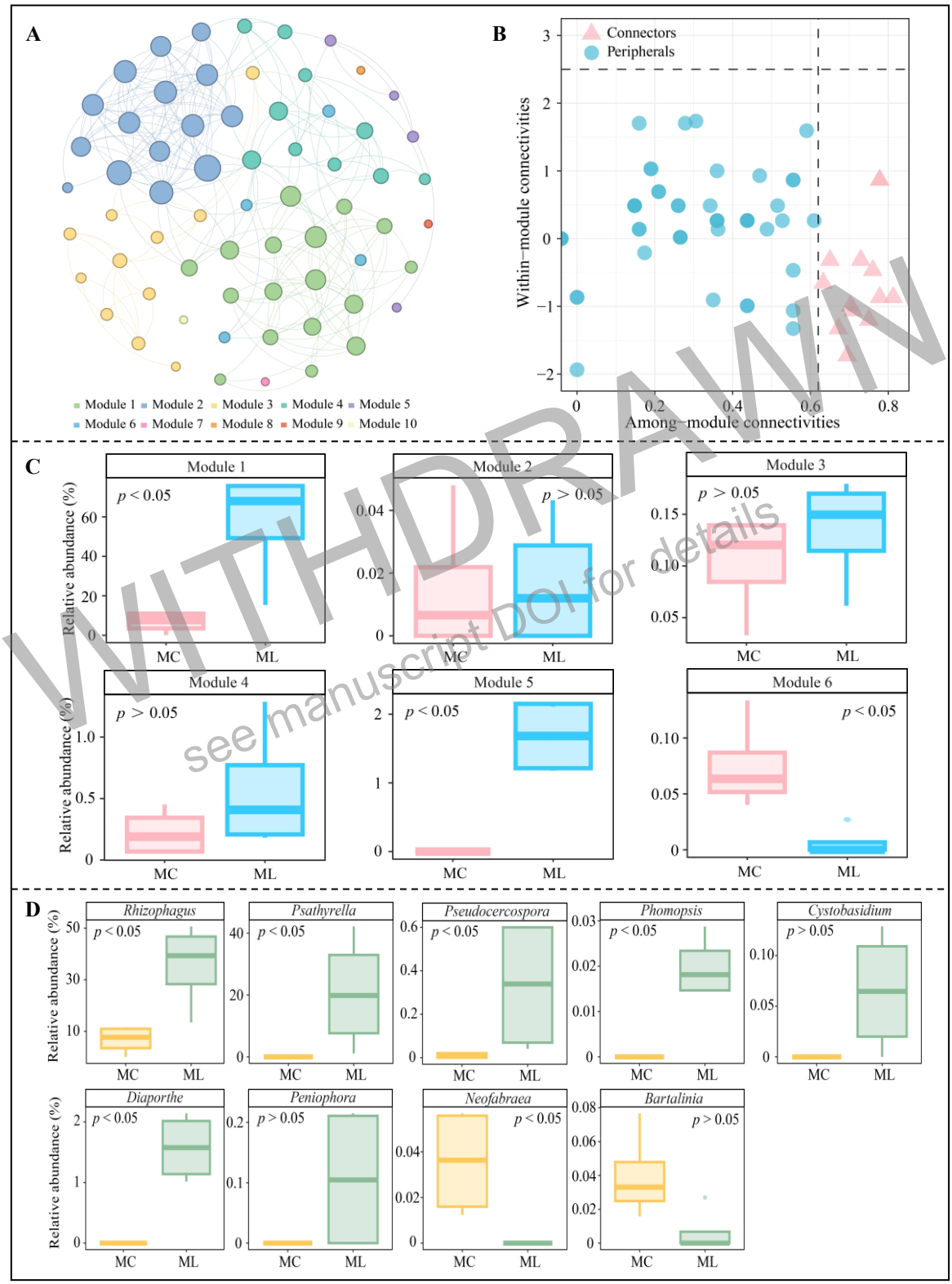

FIG 5 Modular analysis of endophytic fungi in the root system of *C. equisetifolia* and screening of keystone differential fungi. MC and ML denote the first planting and third continuous plantings of *C. equisetifolia*, respectively; (A) Modular interactions network analysis of endophytic fungi; (B) Keystone node analysis of the modular network; (C) Abundance analysis of endophytic fungi in keystone nodes of different modules; (D)

238 Abundance analysis of different endophytic fungi in modules 1, 5 and 6.

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241 **Functional analysis of key differential endophytic bacteria and fungi in the root system of *C. equisetifolia***

242 **in continuous plantings**

243 Based on the above analysis, this study found that the key differential endophytic bacteria in the root system

244 of *C. equisetifolia* were mainly from 6 genera and 33 OTUs. This was used for the functional enrichment of the

245 key endophytic bacteria and it was found (Fig. 6A) that the key endophytic bacteria were enriched to 9 functions,

246 and 3 of the functions were enriched at a significant level, namely nitrate reduction, nitrogen respiration and

247 nitrate respiration. Further analysis of the significantly enriched functions revealed that their contributions were

248 mainly from *Nitrobacter* endophytes (Fig. 6B), and the intensity of the three significantly enriched functions

249 were all significantly greater in MC than in ML (Fig. 6C). Secondly, it was found (Fig. 7A) that the key

250 differential endophytic fungi in the root system of *C. equisetifolia* in continuous planting were mainly from 6

251 genera with 20 OTUs, enriched to 4 functions, of which only one significantly enriched function was plant

252 pathogen. Further analysis of the significantly enriched functions found that their contributions were mainly

253 from *Phomopsis*, *Pseudocercospora* and *Diaporthe* endophytic fungi (Fig. 7B), and the functional intensity of

254 plant pathogen, ML was significantly greater in MLthan in MC (Fig. 7C). It can be seen that the function of

255 endophytic bacteria and fungi in the root system of *C. equisetifolia* changed significantly after continuous

256 planting, which in turn may affect the growth of *C. equisetifolia*.

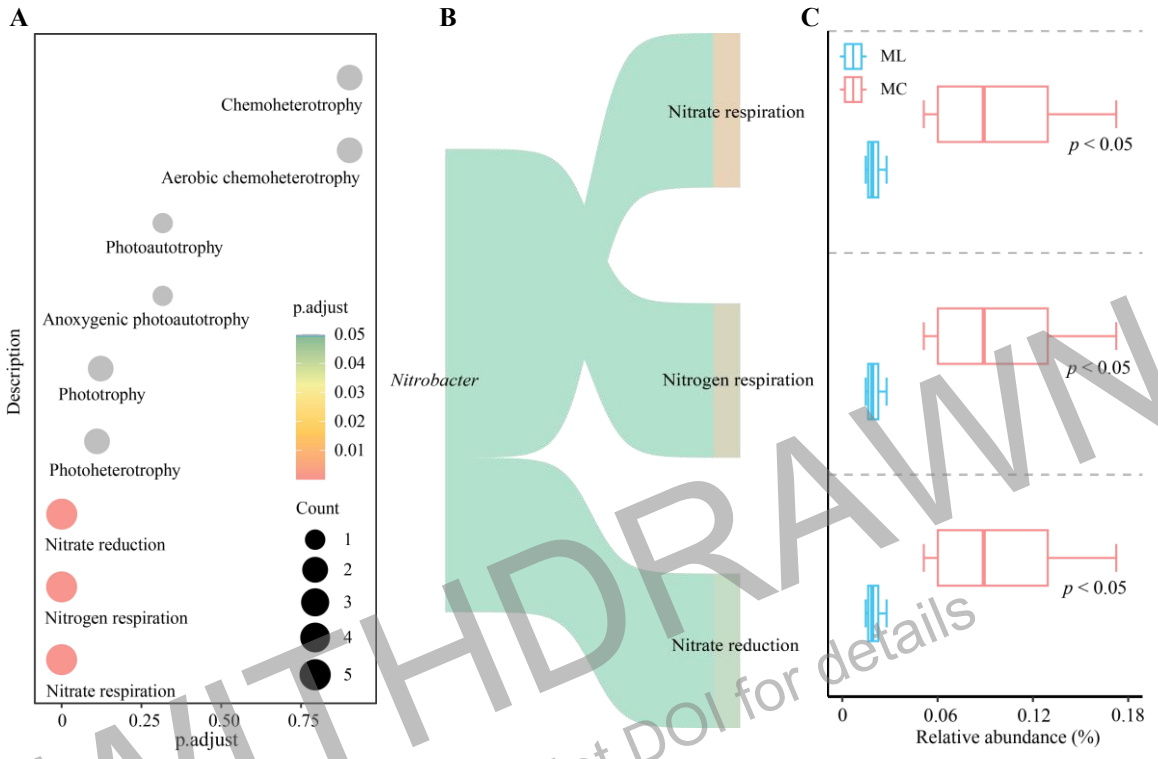


FIG 6 Functional enrichment of key differential endophytic bacteria in the root system of *C. equisetifolia* in continuous planting and their intensity analysis. MC and ML denote the first planting and third continuous plantings of *C. equisetifolia*, respectively; (A) Functional enrichment of key endophytic bacteria; (B) contribution analysis of endophytic bacteria that significantly enriched function; (C) Intensity analysis of the significantly enriched function.

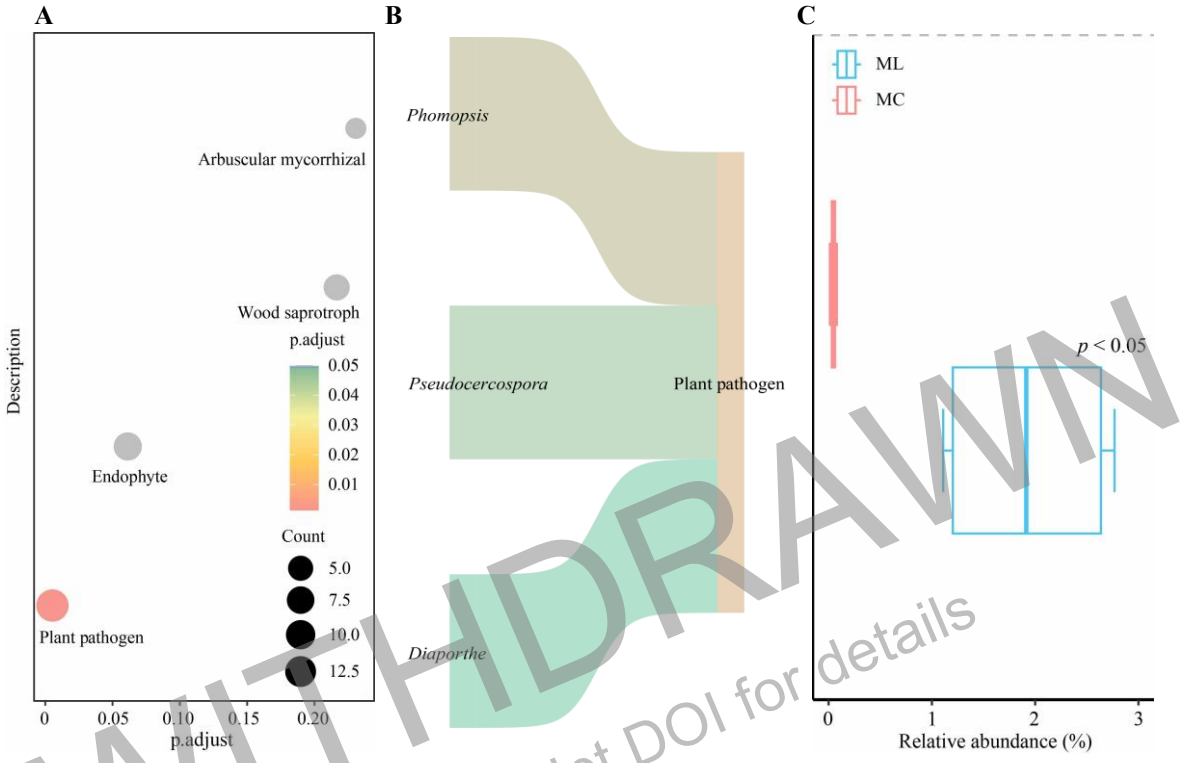


FIG 7 Functional enrichment of key differential endophytic fungi in the root system of *C. equisetifolia* in continuous plantings and their intensity analysis. MC and ML denote the first planting and third continuous plantings of *C. equisetifolia*, respectively; (A) Functional enrichment of key endophytic fungi; (B) Contribution analysis of endophytic fungi that significantly enriched function; (C) Intensit analysis of the significantly enriched function.

Association analysis of key endophytic bacteria and fungi and their functions with growth and physiological indices of *C. equisetifolia*

Based on the key endophytic bacteria and fungi obtained and their functions, the present study further analyzed their correlations with growth and physiological indices of *C. equisetifolia*. The redundancy analysis showed (Fig. 8A) that the main indices associated with ML were malondialdehyde, *Phomopsis*, *Pseudocercospora*, *Diaporthe* and plant pathogen, while the rest of the indices were associated with MC. The results of correlation network and PLS-SEM equation analysis of key endophytic bacteria and their functions with different indices showed (Fig. 8B) that *Nitrobacter* was significantly and positively correlated with nitrate reduction, nitrogen respiration, and nitrate respiration, and *C. equisetifolia* root superoxide dismutase, peroxidase, catalase and root activity, and root total nitrogen, phosphorus and potassium, and root length and plant height, while significantly and negatively correlated with and malondialdehyde. Secondly, key bacteria positively

282 regulated bacterial function (0.988**), morphological indices (0.729*) and nutrient content (0.849**) of *C.*
283 *equisetifolia* root, and growth of *C. equisetifolia* (0.878**). Interaction analysis of key endophytic fungi and
284 their functions with different indices showed (Fig. 8C) that *Phomopsis*, *Pseudocercospora* and *Diaporthe* had
285 significant positive correlations with plant pathogen, and significant negative correlations with superoxide
286 dismutase, peroxidase, catalase and root activity, and significant negative correlations with both nutrient content
287 of *C. equisetifolia* root and growth indices of *C. equisetifolia*. Secondly, key fungi positively regulated the
288 function of fungi (0.998**) and negatively regulated the morphological indices of *C. equisetifolia* root
289 (-0.886**), whereas the root morphological indices positively regulated the root nutrient content (0.849**) and
290 the growth of *C. equisetifolia* (0.878**). It can be seen that there was a close relationship between the root
291 growth and nutrient accumulation capacity of *C. equisetifolia* in continuous planting and the abundance of key
292 endophytic bacteria and fungi in the root system as well as their functional intensities, and that increasing the
293 abundance of key endophytic bacteria and decreasing the number of key endophytic fungi were conducive to the
294 growth of *C. equisetifolia*.

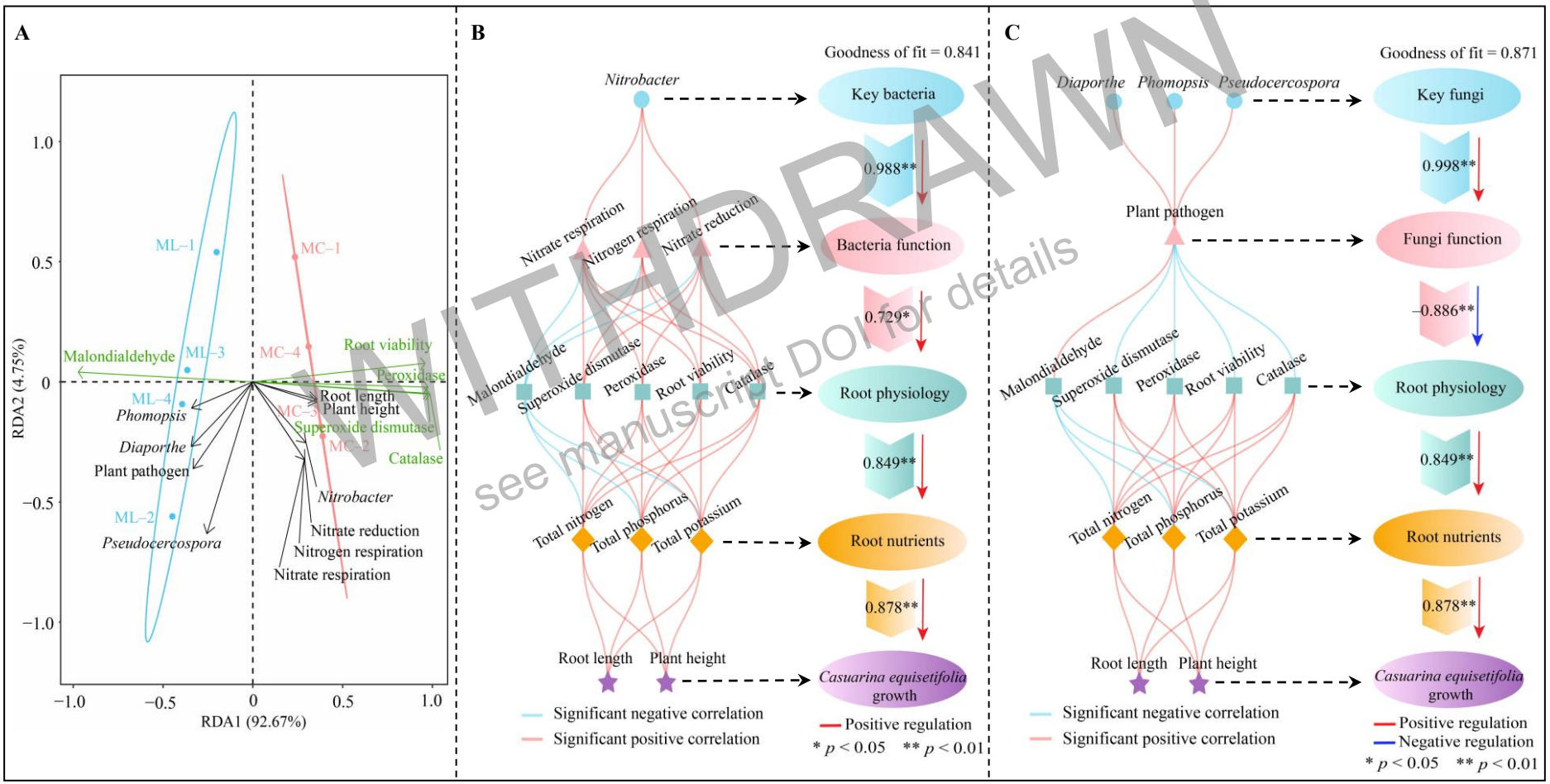


FIG 8 Correlation analysis of key endophytic bacteria, fungi and their functions with growth and physiological indices of *C. equisetifolia*. MC and ML denote the first planting and third continuous plantings of *C. equisetifolia*, respectively; (A) Redundancy analysis of different indices; (B) Correlation network and PLS-SEM equation construction of key endophytic bacteria with different indices; (C) Correlation network and PLS-SEM equation construction of key endophytic fungi with different indices.

DISCUSSION

Continuous planting obstacle is a common problem in many plants, which significantly reduces plant stress tolerance, weakens root nutrient uptake and accumulation, and inhibits plant growth, thus severely limiting the sustainable development of agriculture (15, 16). As an ecologically and economically important tree varietie, *C. equisetifolia* also faces serious continuous planting obstacle. In this study, continuous planting significantly reduced the antioxidant enzyme activities and root activity of *C. equisetifolia* roots, and led to a decrease in root nitrogen, phosphorus, and potassium accumulations, as well as inhibited root length and plant height. This result was similar to that of Wang et al. (13, 17), further confirming that the inhibitory effect of continuous planting on the growth of *C. equisetifolia* is generalized.

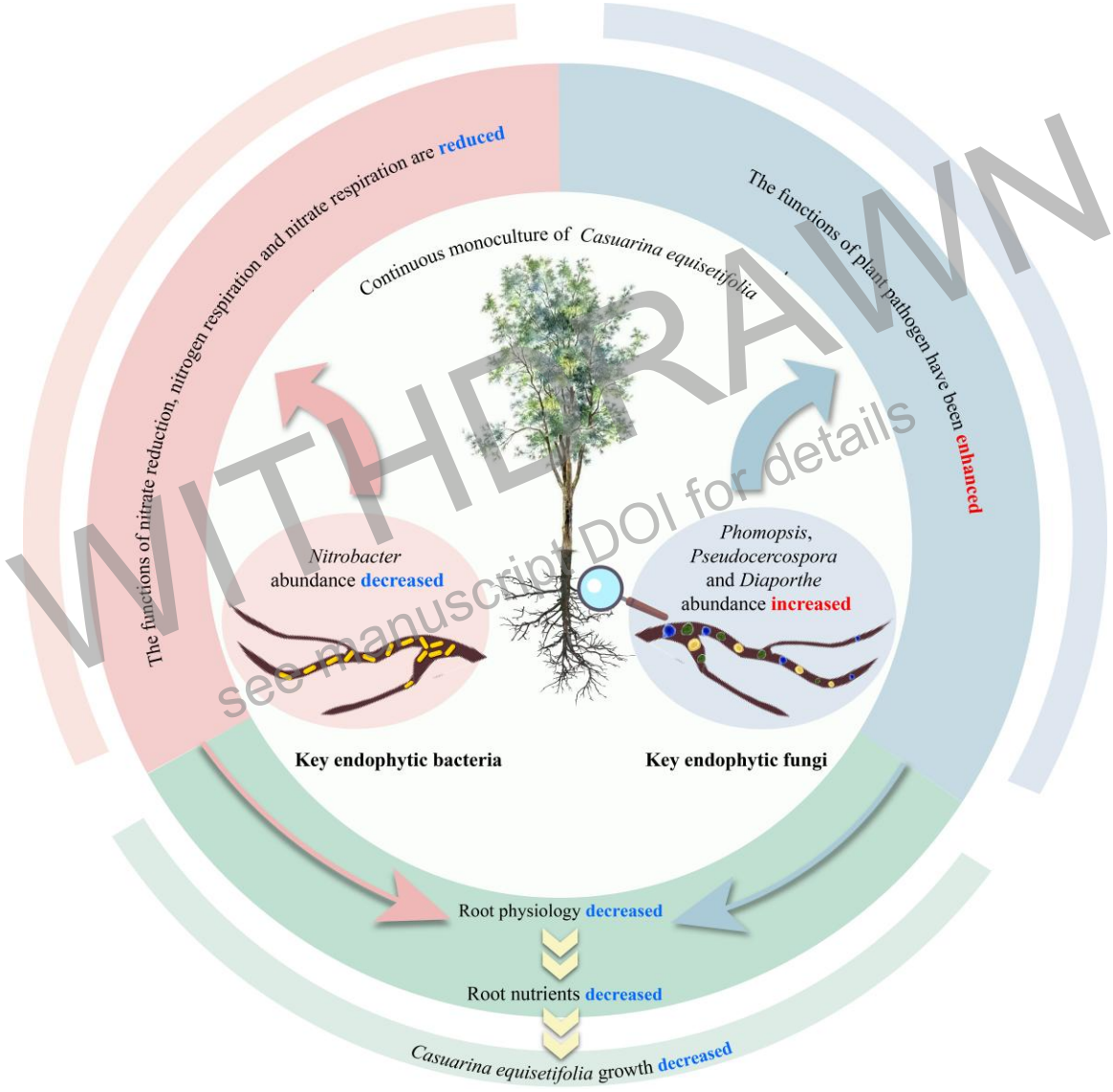
Plant endophytes play a key role in mitigating continuous planting obstacle, and their mechanisms of action include: secretion of growth hormones to promote plant growth, enhancement of plant nutrient metabolism for plant uptake, activation of antioxidant enzyme systems to increase plant resistance, and inhibition of pathogenic bacteria proliferation to maintain plant health (18, 19). However, continuous planting significantly alters the community structure and functional properties of plant endophytes, weakening their protective effects (20). In this study, *C. equisetifolia* was used as the target plant, and it was found that continuous planting led to significant changes in the structure and function of its root endophytes; the nitrogen metabolism functions of endophytic bacteria such as nitrate reduction, nitrogen respiration, and nitrate respiration were significantly reduced after continuous planting, which was mainly attributed to the reduction of *Nitrobacter* endophytes; secondly, the plant pathogen function of endophytic fungi was significantly enhanced, mainly due to the enrichment of endophytic fungi such as *Phomopsis*, *Pseudocercospora* and *Diaporthe*, etc. *Nitrobacter*, as a key chemoenergetic autotrophic nitrifying bacteria that play a central role in the nitrogen cycle by oxidizing nitrite to nitrate (21). This type of strain has been reported to have a unique metabolic flexibility that enables dynamic switching of nitrogen metabolism functions under aerobic and anaerobic conditions depending on the ambient oxygen concentration, including the key processes of nitrate reduction, nitrogen respiration, and nitrate respiration, which significantly enhances nitrogen transformation in plants (22, 23). The present study showed that *Nitrobacter* could significantly enhance the antioxidant capacity, root activity, and nutrient accumulation efficiency of *C. equisetifolia* root by positively regulating the above 3 nitrogen metabolism functions, thereby promoting plant growth. However, continuous planting of *C. equisetifolia* led to a decrease in the abundance of endophytic *Nitrobacter* in the root system, which triggered a series of chain reactions, firstly, directly weakening the nitrogen transformation function of the root microdomains, disrupting the balance of nitrogen cycling, and

leading to the limitation of nitrogen nutrient uptake by the plant; secondly, changing the rhizosphere redox environment affected the metabolic activity of other microorganisms, and ultimately changed the structure and function of endophytic communities. Therefore, continuous planting led to a decrease in the abundance of endogenous *Nitrobacter* in the root system of *C. equisetifolia*, which not only hindered the nitrogen conversion capacity of the root system, but also reduced plant resistance and ultimately inhibited the growth of *C. equisetifolia*.

Three groups of endophytic fungi, *Phomopsis*, *Pseudocercospora* and *Diaporthe*, have been reported to have significant plant pathogenicity, and their infestation of hosts easily leads to reducing host resistance, growth slowdown and disease intensification (24). Of these, *Phomopsis* primarily causes seed rot and branch blight, *Pseudocercospora* causes leaf spot, and *Diaporthe* causes stem ulcers and vascular bundle diseases (25, 26, 27). Secondly, these fungi may be latent in an asymptomatic endophytic state under non-stress conditions and rapidly turn pathogenic under environmental stress (28, 29). The rhizosphere microecological imbalance and reduced host resistance of *C. equisetifolia* caused by continuous planting are key factors promoting the colonization and spread of these pathogenic fungi. The abundance of these pathogenic fungi increased significantly after continuous planting of *C. equisetifolia*, and they positively regulated the plant pathogen function, leading to a decrease in the activity of antioxidant enzyme system, a decrease in root activity, and a weakening of the efficiency of nutrient uptake and accumulation in the root system of *C. equisetifolia*. These findings provide an important basis for analyzing the pathogenic mechanism due to continuous planting of *C. equisetifolia* and suggest that regulating the community structure of these pathogenic fungi may be an effective way to alleviate the continuous planting obstacle.

In conclusion, continuous planting obstacle significantly inhibited the growth of *C. equisetifolia*, as evidenced by reduced antioxidant enzyme activities in the root system, diminished nutrient uptake, and restricted plant growth. It was found that the underlying reason (Fig. 9) was that continuous planting disrupted the balance of endophytes in the root system, leading to a decrease in the abundance of the key endophytic bacterium *Nitrobacter*, which weakened its nitrogen metabolism function and affected nitrogen uptake and utilization efficiency, which in turn affected the growth of *C. equisetifolia*. Meanwhile, continuous planting led to the enrichment of the endophytic pathogenic fungi *Phomopsis*, *Pseudocercospora* and *Diaporthe* in the root system of *C. equisetifolia*, which reduced the root activity and resistance of *C. equisetifolia* and enhanced its pathogenicity. This study revealed that continuous planting affected *C. equisetifolia* growth through a dual microbial mechanism, i.e., the synergistic effect of attenuation of functional flora and proliferation of pathogenic

359 flora, which exacerbated the growth inhibition of *C. equisetifolia*, which provided a new perspective to analyze
360 the endogenous microbial mechanism of continuous planting obstacle, and provided a theoretical basis for
361 alleviating continuous planting obstacle of *C. equisetifolia* through the regulation of endogenous flora.



362

363 **FIG 9** Mechanisms of the effect of key endophytes of the root system on the growth of *C. equisetifolia* in
364 continuous plantings.

365

366 **MATERIALS AND METHODS**

367 **Experimental design and sample sampling**

368 The experimental site of this study was located at the *C. equisetifolia* plantation site of the national
369 protective forest in Chihu Town, Hui'an County, Fujian Province, China (118°55′E，24°35′N). Soil that had not
370 been planted with *C. equisetifolia* and root soil from two continuous plantings of *C. equisetifolia* were collected

in March 2023 from the same area within the *C. equisetifolia* planting site. Soil sampling method for unplanted *C. equisetifolia* was as follows: 20 points were randomly selected within the forest not planted with *C. equisetifolia*, with a spacing of more than 5 m. After removing the dead leaves and 10 cm of topsoil from the soil surface, soil was collected at a depth of 10-40 cm and within a radius of 10 cm, and was mixed thoroughly for one replicate, with a total of four replicates collected. The sampling method of *C. equisetifolia* root soil was as follows: 20 plants of *C. equisetifolia* were randomly selected in the forest where *C. equisetifolia* was planted two continuously, and after removing the surface layer of dead leaves and about 10 cm of topsoil, the soil was collected at a depth of 10~40 cm and within a radius of 10 cm centered on the main trunk of the plant, and was mixed thoroughly for one replicate, with a total of four replicates collected. In April 2023, soil mixed and removed its residual roots was packed into 24 cm diameter and 25 cm high pots of 9 kg soil each, and more uniformly grown *C. equisetifolia* seedlings of about 28 cm height were transplanted into pots of 1 plant each, with four replicates of each treatment and four pots per replicate, for a total of 36 pots. Of these, transplants to soil not planted with *C. equisetifolia* were the first planting of *C. equisetifolia*, labeled MC, and transplants to soil planted with two continuous plantings of *C. equisetifolia* were the third continuous planting of *C. equisetifolia*, labeled ML. After transplanting, *C. equisetifolia* was placed in a field greenhouse and watered with 200 mL of water every evening, and a compound fertilizer (N:P:K = 21:8:16) was applied in May and November 2023 at a rate of 3 g per pot per application. In April 2024, i.e., 1 year after planting, plant height and root length of *C. equisetifolia* seedlings were determined and *C. equisetifolia* root samples were collected for the determination of root physiological and biochemical indices, total nitrogen, total phosphorus, and total potassium content, and for the high-throughput sequencing analysis of the endophytic microorganisms in the root system, with four independent replicates for each treatment.

Determination of root length and plant height of *C. equisetifolia*

In order to assess the effect of different treatments on the growth of *C. equisetifolia* seedlings, plant height and root length of *C. equisetifolia* seedlings were determined under each treatment. Plant height and root length were measured by direct tape measure, where plant height was determined from the rhizome division to the highest point of the plant, and root length was determined from the rhizome division to the end of the longest primary root.

Determination of physiological and biochemical indices in the root system of *C. equisetifolia*

Physiological and biochemical indices of *C. equisetifolia* roots were determined for malondialdehyde, superoxide dismutase, peroxidase, catalase, and root activity as described in Huang et al. (30). Briefly described

as, malondialdehyde content was determined by thiobarbituric acid (TBA) method, i.e., fresh root samples ground in liquid nitrogen were extracted by adding 10% trichloroacetic acid (TCA), and the absorbance was measured at 532 nm and 600 nm, and then converted to its content. Superoxide dismutase activity was determined by nitrogen blue tetrazolium (NBT) reduction method by measuring the change in absorbance at 560 nm and calculating the enzyme activity. Peroxidase activity was determined by guaiacol method, i.e., the reaction was carried out in the presence of hydrogen peroxide to produce brownish-red color product, the change in absorbance at 470 nm was measured and the enzyme activity was calculated. Catalase activity was assessed by the rate of decomposition of hydrogen peroxide, the change in absorbance at 240 nm was measured and the enzyme activity was calculated. Root activity was performed by 2,3,5-triphenyltetrazolium chloride (TTC) reduction method and the absorbance of the extract was measured at 485 nm before calculating root activity.

Determination of total nitrogen, phosphorus and potassium content in the root system of *C. equisetifolia*

C. equisetifolia root samples were wet-cooked with concentrated sulfuric acid and hydrogen peroxide, cooled, and volume-determined to 100 mL, and the filtrate was used for the determination of total nitrogen, total phosphorus, and total potassium in the roots. Total nitrogen was determined by Kjeldahl method, total phosphorus by molybdenum-antimony resistence colorimetric method, and total potassium by flame photometric method (31).

High-throughput sequencing analysis of endophytic bacteria and fungi in the root system of *C. equisetifolia*

The collected *C. equisetifolia* roots were washed with sterile water and then sequentially soaked in 70% alcohol for 5 min, 5.25% sodium hypochlorite for 5 min, and then 70% alcohol for 30 s to remove microorganisms on the surface of the root system, and finally washed repeatedly with sterile water to avoid alcohol residue (32). The treated roots were ground using liquid nitrogen and a sterile mortar, and 0.4 g of root powder was extracted using the HiPure Soil DNA Mini Kit (Magen Biotech, Guangzhou, China). The quality and concentration of the DNA obtained were checked by Nanodrop 2000 (ThermoFisher Scientific, Inc., USA).

The primers used for amplification of the endophytic bacterial 16S rRNA gene were 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTTC-3′), and the primers used for amplification of the ITS region of endophytic fungi were ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′). The first round of PCR amplification program for both endophytic bacteria and fungi consisted of an initial denaturation at 94°C for 30 s, followed by 20 cycles (94°C for 30 s, 60°C for 20 s, 65°C for 2 min) and extension at 65°C for 10 min.

The second round of PCR amplification program for both endophytes consisted of an initial denaturation at 94°C for 30 s, followed by 10 cycles (94°C for 15 s, 60 °C for 15 s, 65°C for 2 min) and extension at 65°C for 10 min. PCR amplification products were purified using magnetic beads, and the amplification products were pooled and mixed according to the number of reads after quantification by Qubit 3.0 (Thermo Scientific, Inc., USA), then detected band length by agarose gel electrophoresis.

The method of three-generation sequencing library construction was as follows: 1.0 μg (50 μL) of the mixed amplification product was taken, and the end-repair reaction was performed, i.e., 7 μL of NEBNext End-prep buffer and 3 μL of End-prep enzyme mix were added to the amplification product, the total amount of the reaction system was 60 μL. The reaction was carried out at 20 °C for 10 min, 65°C for 10 min. At the end of the reaction, the DNA was purified using magnetic beads and eluted with 61 μL of EB buffer, and 1 μL was taken to determine the DNA concentration. The DNA after end repair was added into Ligation Sequencing Kit V14 (SQK-LSK114, Oxford Nanopore Technologies, Oxford, UK) with 25 μL of Ligation buffer, 5 μL of Adapter Mix H and 10 μL of Quick T4 DNA Ligase (New England Biolabs, Ipswich, MA, USA), totaling 100 μL of the reaction system, and the reaction was carried out at 25°C for 10 min. The product after the reaction was again purified by magnetic beads, eluted with 25 μL of Elution buffer, and 1 μL was taken to determine the DNA concentration. The 24 μL of DNA after ligating connectors was mixed with 51 μL of Loading Beads II and 75 μL of Sequencing Buffer II in a total volume of 150 μL. The constructed libraries were loaded onto the PromethION Flow Cells Sequencing Chip R10.4 (Oxford Nanopore Technologies, Oxford, UK) and sequenced on the PromethION sequencing platform (Oxford Nanopore Technologies, Oxford, UK) for 24-48 h. The sequencing data were spliced and filtered using Pear after distinguishing the samples according to barcode, removing low-quality sequences and obtaining high-quality clean tags for endophytic bacteria (Table S1) and fungi (Table S2). The splicing results were spliced using the Vsearch software (v2.7.1) to remove short sequences and chimeras, and were subsequently subjected to uparse algorithm for OTU clustering (sequence similarity threshold was set to 97%), and the samples were even drawn flat. The OTU sequences were finally aligned to the corresponding databases for species annotation using BLAST, where Silva (Release 138) was used for bacteria and Unite (Release 8.2) for fungi. Based on the endophytic bacterial and fungal OTUs obtained, the function of bacteria was predicted using FAPROTAX software and the function of fungi was predicted using FUNGuild software.

Statistical analysis

The raw data were initially organized and statistically summarized using Excel 2020 software. Differences

in indicators between groups were analyzed with $p < 0.05$ as the criterion for judging statistical significance. Data visualization and model construction were done using Rstudio (version 4.2.3), with box plots based on the ggplot2 package (v 3.5.0), Wayne diagrams using the ggVennDiagram package (v 1.5.2), microbial community α-diversity and β-diversity indices using the vegan package (v 2.6.4), and neutral community model fitting using the minpack.lm package (v 1.2.4), covariate network analysis and modular network interplots were performed using the R package igraph (v 2.0.2), partial least squares - structural equation modelling (PLS-SEM) was constructed using the plspm package (v 0.4.9), microbial functional enrichment analysis was performed using the clusterProfiler package (v 4.10.0), redundancy analysis (RDA) was performed using the vegan package (v 2.6.4), and correlation network maps between microbial communities were constructed using the linkET package (v 0.0.7.1).

Supplementary material

Table S1 Statistics of splicing results after sequencing of roots endophytic bacteria of *Casuarina equisetifolia*. Table S2 Statistics of splicing results after sequencing of roots endophytic fungi of *Casuarina equisetifolia*.

CRediT authorship contribution statement

Lei Hong and Yuhua Wang: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Mingzhe Li, Jianjuan Li, Qingxu Zhang and Miaoen Qiu:** Formal analysis, Visualization, Writing – original draft. **Xiaoli Jia:** Investigation, Writing – original draft. **Wenxiong Lin:** Writing – review & editing. **Haibin Wang and Zeyan Wu:** Project administration, Conceptualization, Resource, Supervision, Writing – original draft, 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.

Data availability

Data will be made available on request. The Miseq raw reads were deposited in the National Center for Biotechnology Information Short Read Archive database under accession number PRJNA1277710 (Endophytic

bacteria, https://www.ncbi.nlm.nih.gov/bioproject/1277710) and PRJNA1277743 (Endophytic fungi, https://www.ncbi.nlm.nih.gov/bioproject/1277743).

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