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Endophytes and soil microorganisms participate in the germination of Sinojackia xylocarpa Hu by degrading the pericarp

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Abstract

Sinojackia xylocarpa is a rare endemic genus in China, and is endangered in their wild habitats. For endangered rare species, sexual propagation is significant for their protection of genetic diversity and restoration of wild population. Especially for *S. xylocarpa*, the artificially cultivated population is expanded by vegetative propagation, which could lead to a lack of diversity. The fruits of *S. xylocarpa* require stratification to germinate, during which the hard pericarp becomes soft, and endophytes and soil microorganisms are considered to be involved in the process. Amplicon sequencing was used to analyze the changes in microbial community structure and the role of microorganisms in pericarp degradation. Analyses of pericarp from fresh and stratified fruits of different stages revealed that the abundance of endophytic bacteria in pericarp presented an overall increasing trend as the stratification processing; endophytic bacteria in stratified pericarp can produce beta-glucosidase to participate in the degradation of the pericarp. Soil fungal diversity analyses of different stages showed that the highest levels of the two samples of fungi function clustering is saprophytic in nutritional type; guild model also indicated that wooden saprophytic fungi abundance increased obviously; further the effect of degrading pericarp of four strains isolated from stratified pericarp are confirmed. Results above indicate that soil fungi are involved in the degradation. Breaking seed dormancy with microorganisms has the potential to be applied to endangered plants that face difficulties in germination, especially in their natural habitats.

Introduction

Sinojackia, a genus of eudicot in the *Styracaceae*, is a rare genus endemic to China. There are seven species in the *Sinojackia* genus, including *Sinojackia xylocarpa* Hu, *Sinojackia rehderiana* Hu, *Sinojackia sarcocarpa* L. Q. Luo, *Sinojackia microcarpa* T. Chen□G.Y.Li, *Sinojackia oblongicarpa* T. Chen & T. R. Cao, *Sinojackia huangmeiensis* J. W. Ge□X. H. Yao and *Sinojackia dolichocarpa* C. J. Qi, among which *Sinojackia xylocarpa* Hu (*S. xylocarpa*, Hu 1928) was published first and is the model species of the genus. At present, the original habitats of several species of *Sinojackia* have been destroyed, and the wild populations are small and distributed in fragmented habitats.

The wild *S. xylocarpa* is extinct in its native range in Jiangsu Province, China. It is currently known to exist in only one or two individuals in Henan and Hubei Provinces, China, and two wild populations in Zhejiang Province, China (Yao et al. 2005; Xu et al. 2022). *S. xylocarpa* has been assessed as a second-class national protected plant and is rated endangered by the IUCN (International Union for Conservation of Nature). Research of the wild populations of other species of the genus *Sinojacka* showed that the number of individuals of *Sinojackia oblongicarpa* was less than 30, and less than 10 for *Sinojackia sarcocarpa* (Yao et al. 2005).

In addition to human activities and habitat destruction, low germination rate due to the recalcitrance and the deep dormancy of the seeds is another major reason for the extinction of the wild populations of *Sinojackia*. On account of the characteristics of deep dormancy, under natural conditions, seeds need stratification in wet sands of two winters and one summer to germinate. It is generally accepted that *S.*

xylocarpa exhibits integrated dormancy, meaning that the observed physical dormancy is attributed to the hard and lignified pericarp. (Huang et al. 1998; Shi et al. 1999; Xu et al. 1999; Luo 2002; Jia 2007), while the physiological dormancy caused by the germination inhibitors (Huang et al. 1998; Yao et al. 2008) also plays a role.

In wild condition, natural germination is difficult and the germination rate is low (Ye et al. 1996; Huang et al. 1998), which leads to the gradual reduction of the population of wild populations. The air permeability and water permeability of the pericarp were increased after acid etching (Xu et al. 1999), and the essence of this method resembles the degradation of microorganisms. With the period of stratification prolonged, the texture of the pericarp becomes softer, and the germination rate increases to some extent. The germination rate of *S. xylocarpa* seeds after stratification for one winter is zero, while the rate after stratification for two winters reaches about 80%. Thus, we hypothesize that endophytic and soil microorganisms are involved in the pericarp degradation process. The involvement of microorganism accelerates the process by degrading lignin and cellulose in the pericarp, promoting appropriate condition for germination. Microorganisms thus improve the germination rate of *S. xylocarpa*.

In the field of botany, current research on microorganisms can be mainly divided into two categories: one is the interaction between soil microorganisms and plants; the other is the interaction between endophyte and plants. The concept of endophytes and their beneficial relationship with plants is widely recognized as an important step in plant co-evolution and diversity (Ghasemnezhad et al. 2021). Fungi and bacteria are the most common microorganisms that exist as endophytes, and they usually maintain a symbiotic or reciprocal relationship with their hosts. It has been found that endophytes exist in various organs of plants, including vegetative organs and reproductive organs. Endophyte plays an important role in breaking seed dormancy and increasing stress resistance (Chen et al. 2021). Meanwhile, it has been reported that bacteria present in seeds and preserved from generation to generation have functional traits adapted to restrictive environments, such as metal-contaminated slag, which can bring beneficial results for host plants (Truyens et al. 2016).

16S and ITS (Internal Transcribed Spacer, a nucleic acid sequence located in the rRNA gene transcription region, widely used for fungal identification) amplicons sequencing are widely used to compare and analyze the differences and changes in microbial community structure among different samples through the process of stratification, especially for endophytes in plant tissues (Klindworth et al. 2013; Brown et al. 2018; Bziuk et al. 2021; Tarquinio et al. 2021, Wijekoon et al. 2021). To reveal the effects of microorganisms in *S. xylocarpa* seed germination, the community composition of endophytes and soil microorganisms and their functions were investigated by amplicon sequencing. Further, four strains of fungi with strong cellulose degradation ability were isolated and their capabilities of degradation were tested on fresh pericarp to further verify the assumption. We would like to reveal the effect of endophytes and soil microorganisms in promoting the germination of *S. xylocarpa*, and make microorganism-involved stratification a general measure to deal with endangered plants facing difficulties in germination, especially for seeds with a hard pericarp and seeds needing long term of stratification.

Materials and methods

Plant material selection and pretreatment

The plant material used in this experiment was obtained from Longganhu National Nature Reserve in Huangmei County, Hubei Province (115°56' E, 29°48' N). The flowers of *S. xylocarpa* are white and its fruits are shaped like a steelyard, giving the plant a high ornamental value, as shown in Figure S1.

The amplicon sequencing was performed twice in this project with different methods of selection and pretreatment to better illustrate the effects of endophytic bacteria and soil microorganisms, respectively. It is worth noting that considering no obvious mycelium has been found in the stratified pericarp, the detection of endophytic microorganisms mainly focused on endophytic bacteria. Also, the stronger infectivity of fungi indicates that the community of endophytic fungi can be infected, or merge together with soil fungi community, and even strict sterilization cannot separate endophytic fungi from the mixed fungal community. Corresponding to this consideration, *S. xylocarpa* fruits used for endophytic bacteria amplicon sequencing have been strictly sterilized, and only 16S amplicons were sequenced and analyzed. Considering that the soil environment influences the structure of endophytes in seeds, we believe that the soil microorganisms are also involved in the process of pericarp degradation (Chandel et al. 2022). However, this experiment alone cannot fully reveal microbial influence; therefore, a second amplicon sequencing was performed. To expound the co-effect of soil microorganisms and endophytes, especially for the merged and complicated changing fungi community, fruits used for soil bacterial and fungal amplicon sequencing were not sterilized, and surface soil was also collected for sequencing. Additionally, both ITS and 16S amplicons were sequenced and analyzed. The detailed method for sequencing the two amplified fragments is as follows:

Group for endophytic bacteria sequencing: Fresh fruits of *S. xylocarpa* and fruits after different period of stratification were used in the experiment. Fresh fruits were collected from Longganhu National Nature Reserve in October 2021. Stratified fruits were collected from Longganhu National Nature Reserve in January 2022, when the fruits had been stratified for 3 months and 14 months, respectively. The ratio of fruits to soil was one to three, and the humidity is 80–90%. The group of fresh fruits was marked as FR, the group of 3-months-stratified fruits was marked as S1, and the group of 14-months-stratified fruits is marked as S2, with each group containing 3 fruits as biological replicates. Surface sterilization of the fruits was performed (Liu et al. 2013): the surface was washed with sterile water, immersed in 70% alcohol for 3 min, immersed in fresh sodium hypochlorite solution with 2.5% effective chloride ions for 5 min, washed with 70% alcohol for 30 s, rinsed with sterile water 7 times, and the sample was drained with sterile filter paper. The sterile water of the last rinse was spread on LB medium and cultivated to detect the effect of sterilization. The pericarp was collected with sterilized instruments and 16S amplicon was sequenced.

Group for soil microorganism sequencing: Fruits of *S. xylocarpa* after different period of stratification were used in the experiment. The stratified fruits used for soil microorganism sequencing were collected

from Longganhu National Nature Reserve in March 2022, when the fruits had been stratified respectively for 5 months and 16 months. The 16S amplicon sequencing group for soil bacteria was marked as BS1(stratified for 5 months) and BS2(stratified for 16 months), and the ITS amplicon sequencing group for soil fungi and endophytic fungi was marked as FS1(stratified for 5 months) and FS2(stratified for 16 months), with each group containing 3 fruits as biological replicates. The surface soil and pericarp were collected and 16S and ITS amplicon was sequenced.

Extraction of genomic DNA and PCR amplification

16S rDNA and ITS amplicon sequencing has become an important tool in the study of microbial community composition in environmental samples (Youssef et al. 2009; Caporaso et al. 2011; Hess et al. 2011). The genomic DNA of the samples was extracted by CTAB method, and the purity and concentration of the extracted DNA were detected by agarose gel electrophoresis. An appropriate amount of DNA was then placed in a centrifuge tube and diluted to 1 ng/μL with sterile water. Using diluted genomic DNA as a template, PCR was performed using specific primers with barcode, Phusion® High-Fidelity PCR Master Mix with GC Buffer from New England Biolabs, and high-efficiency and high-fidelity enzymes were selected according to the amplification region to ensure the efficiency and accuracy of amplification. 16SV5-7 sequences of endophytic bacteria samples were chosen for analysis because they avoid chloroplast cross-amplification (Bodenhausen et al. 2013). For soil microbial samples, 16SV5-7 and ITS1-1F sequences were amplified using their respective primers.

Mixing and purification of PCR products

PCR products were detected by 2% agarose gel electrophoresis, and the same number of samples were mixed according to the concentration of PCR products. After mixing, the samples were detected again by 2% agarose gel electrophoresis, and the target bands were recovered by using the gel recovery kit provided by Qiagen.

Library construction and sequencing

NEBNext® Ultra™ IIDNA Library Prep Kit was used for Library construction, and the constructed libraries were quantified by Qubit and q-PCR. After the library was qualified, NovaSeq6000 was used for sequencing.

Sequencing data processing

Data of each sample was separated from the off-machine data according to barcode sequence and PCR amplification primer sequence. After the barcode and primer sequences were cut off, FLASH was used to splice the reads to obtain Raw Tags. Then fastp was used to control the quality of Raw Tags, and obtain high quality Clean Tags. Finally, Usearch was used to compare Clean Tags with the database to detect and remove chimeras, so as to obtain the final Effective data, also known as Effective Tags.

ASVs denoise and species annotation

For the Effective Tags obtained above, the DADA2 module in QIIME2 software was used to denoise and filter out the sequences with abundance less than 5, to obtain the final ASVs (Amplicon Sequence Variants) and feature sheets (Fung et al. 2021). Then, the classify-Sklearn module in QIIME2 software was used to compare the obtained ASVs with the database to get species information for each ASV.

Sample complexity analysis

The observed_otus, shannon, simpson, chao1, goods_coverage, dominance and pielou_e indices were calculated using QIIME2. The Rarefaction Curve and Species Accumulation Boxplot were analyzed.

Comparative analysis of beta diversity

QIIME2 software was used to calculate the Unifrac distance, and R software was used to draw the reduced-dimension maps of PCA, PCoA and NMDS. Adonis and Anosim functions in QIIME2 software were used to analyze the significance of differences in community structure between groups. LEfSe (Linear discriminant analysis Effect Size, LDA Effect Size) and R software were used to analyze the significantly different species among groups. LEfSe analysis was performed with the LDA score threshold set to 4. For MetaStat analysis, R software was used to compare the two groups at six taxonomic levels: phylum, class, order, family, genus, and species, and to obtain p-values. Species with p-values less than 0.05 were identified as significantly different species among the groups. The t-test also uses R software to analyze the significant differences of species at each taxonomic level.

Functional annotation

PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved Stats 2) is an analysis method that infers the gene function spectrum of common ancestors based on the ASV tree and ASV gene information in the Greengene database. The gene function spectrum of other unknown species in the Greengene database was also inferred, and the gene function prediction spectrum of the whole lineage of the archaea and bacteria domains was constructed. Finally, the microbiota composition obtained by sequencing is "mapped" to the database to predict the metabolic function of the microbiota.

FunGuild is an environmental function database of fungi. Based on the support of literature, the ecological functions of fungi were classified and the FunGuild database was constructed (Douglas et al. 2020; Chen et al. 2020). Based on the species information obtained by amplicon analysis, the ecological functions of species described in the literature can be queried.

Isolation and functional analysis of fungi capable of degrading pericarp

Four strains of fungi capable of degrading carboxymethyl cellulose were isolated from pericarp stratified for two winters by culturing them on selective media in which the only carbon source was carboxymethyl cellulose (2%). The DNA of these strains was extracted by the paramagnetic particle method. ITS fragments were amplified with their corresponding primers (ITS1: 5’-TCCGTAGGTGAACCTGCGG-3’; ITS4:

5′-TCCTCCGCTTATTGATATGC-3′) and sequenced by the Sanger dideoxy sequencing method. The sequencing results were joined with DNAMAN software and compared using NCBI.

Detection of lignin and cellulose degradation ability of the strain

Four strains of fungi were cultured on PDA plate adding aniline blue (0.04%), guaiacol (0.1%) and Mn^{2+} (0.025% $MnCl_2$) respectively to detect their ability of producing lignin degrading enzymes; these fungi were also cultured on selective media, in which the only carbon source was carboxymethyl cellulose (2%), to detect their ability of using cellulose. These four strains were then cultured with sterilized fresh fruits of *S. xylocarpa* to further demonstrate their roles in pericarp degradation. Each strain was cultured with two halves of fruit, in 100 mL of selective media containing carboxymethyl cellulose (2%) as the sole carbon source. The cultures were incubated for 14 days at 26 ℃ to observe the effects on sections. The positive control group was *S. xylocarpa* fruits treated with *Humicola fuscoatra* (CCTCC AF 2018019, from China Center for Type Culture Collection), which was capable of degrade lignin, with same method like strains under test mentioned above; another positive control group is *S. xylocarpa* fruits degraded by cellulase for 24 h at 50 ℃. The effects of degradation were recorded by Stereo Fluorescence Microscope (SMZ25).

Results

Diversity of endophytic bacteria in pericarp of S. xylocarpa

We evaluated the quality of sequencing data (Table S1) and found that three groups of samples in this experiment have all reached the saturation stage in dilution curve (Figure S2A), indicating that the sequencing captured almost all bacterial species in the pericarp of *S. xylocarpa*, proving that the amount of data is sufficient for the subsequent function prediction.

Compared with FR, species were more evenly distributed and more diverse in S2 group (Table S2). Therefore, the stratification helped the endophytic bacteria to shift towards a richer and more uniform community. According to the ASVs annotation results and the relative abundance table of each sample (Fig. 1A), the dominant genera were changed from *Pseudomonas*, *Ralstonia* and *Sphingobacterium*, with relative abundance of 40.2%, 37.5% and 6.5% in FR group, respectively, to *Streptomyces, Lysinibacillus, Bacillus, Staphylococcus* and *Pseudorhodoplanes* with relative abundance of 17.8%, 13.2%, 11.3%, 6.4% and 3.0% in S2 group, respectively. Other unidentified bacteria accounted for 46.6% in S2, whereas they accounted for 13.7% in FR. Venn diagram (Fig. 1B) revealed a total of 729 ASVs in S2 group, with 495 ASVs in FR group and 316 ASVs in S1 group.

FR group and S1 group share more similarities than FR group and S2 group, which can be explained by the longer time of stratification. It can be observed that 41% of the ASVs in the S1 group were shared with the FR group (Fig. 1B), decreasing to 26% of the ASVs in the S2 group between the FR group and S2 group. PCA analysis (Figure S2B) showed that the differences between S1 and FR groups were

significantly smaller than those between the FR and S2 groups. As shown in Fig. 1C and 1D, taxonomic units with statistical differences in each group were demonstrated in LEfSe analysis. The red-highlighted taxonomic units, including *f_Hyphomicrobiaceae*, *o_Dongiales*, *f_Dongiaceae*, *g_Dongia* and *g_ Hyphomicro- bium*, were considered to contribute to the main differences in the S2 group (Fig. 1C), and they were also marked red in the phylogenetic tree (Fig. 1D).

Analysis of bacterial diversity of S. xylocarpa in pericarp and soil

After splitting the original data of off-machine and removing barcode and primers, splicing and filtering were performed, and effective data for subsequent analysis was obtained (Table S3). As can be seen from Table S4, compared with BS1, BS2 had more uniform species distribution and more intuitive species and low abundance species. It can be seen from the dilution curve (Fig. 2A) that all of the six groups of samples in this experiment reached the saturation stage, indicating that the sequencing has captured almost all the bacterial species in pericarp and outer soil, proving that the amount of data is sufficient for the subsequent function prediction.

The species in BS2 group were more evenly distributed and had a greater diversity than BS1 group. According to the ASVs analysis of relative abundance (Fig. 3A), *Bradyrhizobium*, *Pseudorhodoplanes* and *Acidibacter* have increased significantly and *Pseudomonas, Flavobacterium* have remarkably decreased and the sum of the relative abundance of all the genera, excluding thetop 30 genera, increased from 29.6–52%. As shown in the Venn diagram (Fig. 3B), BS1 group and BS2 group shared 747 ASVs, with BS2 having more ASVs. PCA analysis between the groups (Fig. 2B) showed a significant difference between BS1 and BS2 groups, as well as individual differences within BS2 group.

When taking soil into consideration, more species with significant differences between BS2 and BS1 were observed than merely pericarp. And the evolution connection was better revealed. It was found that when BS2 group evolve their own community structure during stratification, the previous bacterial species were selectively preserved and some bacterial species became dominant for potential environmental reasons. According to the histogram of LDA value distribution (Fig. 3C), *f_ Xanthobacteraceae*, *o_Rhizobiales* and *c_Alphaproteobacteria* showed the greatest difference in BS2 group while *f_Pseudomonadaceae*, *c_Gammaproteobacteria* and *f_Oxalobacteraceae* had the greatest differences in BS1 group. From the analysis of the evolutionary cladistic diagram (Fig. 3D), a close relationship within BS1 group and BS2 group can be seen, and the two groups were partially related. For instance, there were many subspecies of *p_Proteobacteria* found in BS1 and BS2, such as *o_Gammaproteobacteria_Incertae_Sedis* in BS2 and *f_Pseudomonadaceae* and *o_Pseudomonadales* in BS1. Venn diagram (Fig. 2C) illustrated this point more intuitively. Figure 2D showed that the relatively high abundance of *Pseudomonas* and *Flavobacterium* in BS1 decreased in BS2, while the abundance of other species increased in BS2.

Fungal diversity analysis of pericarp and soil

After splitting the original data of off-machine and removing barcode and primers, splicing and filtering were performed, and effective data for subsequent analysis was obtained (Table S5). The template similarity during PCR was high, resulting in many chimeras. Different Alpha Diversity analysis indexes were used to conduct statistics on different samples, and the results were shown in Table S6. Compared with FS1, FS2 had more low-abundance species, more abundant species and a more uniform species distribution. The results indicated that stratification had a great influence on the species of fungi in the outer pericarp and surrounding soil. It can be seen from the dilution curve (Figure S3A) that all the six groups of samples in this experiment have reached the saturation stage, indicating that the sequencing has captured almost all the fungal species in pericarp and surrounding soil, proving that the amount of data was sufficient for the subsequent function prediction.

Similar to the results of bacteria, species were more evenly distributed and more diverse in FS2 group than FS1 group. The relatively abundant species in FS1 group, *Coprinopsis*(2.3%), *Trichothecium*(27.7%), *Sarocladium*(28.3%) and *Diaporthe*(32.2%) respectively, were replaced in FS2 group by *Humicola*(1.1%), *Tausonia*(1.3%), *Exophiala*(3.1%), *Idriella*(3.7%) and *Nothophoma*(4.3%), respectively (Fig. 4A). The total relative abundance of all other genera except these 30 genera was also increased from 4.5–77.0%. The relative abundance of *Diaporthe*, *Sarocladium* and *Trichothecium*, the dominant species in FS1, have decreased sharply in FS2, while the relative abundance of *Idriella*, *Exophiala* and *Nothophoma* have increased significantly in FS2. FS1 group and FS2 group had 120 ASVs that overlapped, while FS2 had more ASVs (Fig. 4B). Figure S3C showed the top 10 FS1 and FS2 species and their abundance at the genus level. The overall content of all other genera except these 9 genera was high in FS2 group.

The result of soil fungi also revealed more species with significant differences and a clearer evolution relationship than merely taking pericarp into consideration. Differences between groups can be shown in Fig. 4C as well, where the species with statistical difference between groups were obtained by histogram of LDA value distribution. The abundance differences of *p_Basidiomycota*, *g_Striatibotrys* and *s_Striatibotrys_eucylindrospora* in FS2 group were the greatest. The abundance differences of *c_Sordariomycetes*, *p_Ascomycota* and *g_Diaporthe* are the greatest in FS1 group. The nodes in the evolutionary diagram (Fig. 4D) showed that FS1 and FS2 groups were closely related, and there were species in FS2 that were closely related to FS1. The *f_Trichomeriaceae* and *o_Chaetothyriales* contained in FS2 were closely related, and *f_Plectosphaerellaceae* and *f_Stachybotryaceae* had corresponding species in families, genera and species. *f_Diaporthaceae* and *o_Diaporthales* were closely related in FS1. PCA analysis showed partial overlap (Figure S3B), along with larger individual differences within FS2 group.

Microorganisms participate in the degradation of S. xylocarpa pericarp

From the first time of sequencing, we found that during the two years of stratification, the main function of enzymes produced by endophytic bacteria remained stable and there were some differences in details. From the Venn diagram of bacterial function prediction (Figure S2C), 1945 genes were shared among all three tested groups. After one-year stratification, the seed was prepared to germinate and

more substances need to be synthesized. So enzymes which were closely related to the energy conversion process have increased, such as long-chain-fatty-acid—CoA ligase (6.2.1.3), succinate-semialdehyde dehydrogenase [NAD(P)+] (1.2.1.79), glutarate-semialdehyde dehydrogenase (1.2.1.20) (Figure 5A). The same is true when soil microorganisms were taken into account, where acetyl-CoA carboxylase(6.4.1.2), DNA Topoisomerase(5.99.1.2), Ribonucleoside-diphosphate reductase(1.17.4.1), beta-glucosidase(3.2.1.21) and RNA Helicase(3.6.4.13), Beta-ketoacyl -[Acyl-carrier-protein] synthase III(3.6.4.13) were dominant in BS1 and glutathione transferase(2.5.1.18), non-specific Serine /threonine protein kinase(2.7.11.1), 4–hydroxy– tetrahydrodipicolinate synthase(4.3.3.7), 3 -- oxoacyl - [ACyl -- carrier-protein] reductase(1.1.1.100), acetyl-CoA C-acetyl transferase(2.3.1.9) were dominant in BS2.

Beta-glucosidase (3.2.1.21), which is an indispensable enzyme in conventional cellulose degradation system was found in both two sequencing analyses of *S. xylocarpa* in pericarp and soil (Fig. 5). The enzyme is a microorganism cellulose polyenzyme, which plays an important role in the whole process of cellulose hydrolysis. Most of the bacteria secreting this enzyme belong to *Firmicutes* and *Proteobacteria* (Su et al. 2021), which is consistent with our previous detection results. From the traditional perspective, cellulose degradation is mainly caused by the synergistic action of several cellulose hydrolases, like endoglucanase, cellulose biohydrolase and beta-glucosidase (Martínez et al. 2005). But in this experiment, we only successfully identified beta-glucosidase. In addition, the discovery of lytic polysaccharide monooxygenase (LPMOs) has broken through the classic model. LPMOs is a kind of enzymes that helps to degrade crystalline cellulose through REDOX reaction, providing a new pathway for beta-glucosidase to degrade fibrinosaccharides (Martínez et al. 2016). However, as LPMOs is a new discovery in recent years, the sequence of its synthesis is still unclear, which means that if LPMOs enzyme is involved in cellulose decomposition in the fruits of *S. xylocarpa*, there is a large possibility that it cannot be detected by endophytic bacteria diversity analysis. In addition, in the function prediction, we also find ethanol dehydrogenase in the pericarp during the first winter of stratification. Ethanol is the main component of the metabolites of decomposing cellulose (Zhao et al. 2019), which further confirms the important role of microorganisms in cellulose degradation.

In terms of fungi functional annotation, the relative abundance of saprophytic and wood saprophytic fungi in the two groups is higher no matter whether Trophic Mode (Figure S3D) or Guild (Fig. 6A) is based on. On FunGuild annotation of fungi, we successfully locate two fungi with demonstrated lignin decomposition ability (Table S7), namely two white rot fungi (ASV410 and 484) with remarkable extracellular lignin decomposition ability (Cerro et al. 2021). In addition, a species of *Aspergillus flavus* (ASV53), which may have lignin decomposition ability, is located. Current studies have confirmed that *A.flatus* F-1 can cut lignin into fragments (Li et al. 2020). In conclusion, soil colonies and endophytic bacteria interact together to decompose lignin and cellulose in pericarp and promote seed germination. Except for the indispensable Saprotroph Mode in both group FS1 and FS2, FS2 has less abundance and more diversity. According to the analysis of Guild, even though the relative abundance of Undefined_Saprotroph type has decreased, it has become the group of the highest abundance except for the unassigned species in FS2 group. The abundance of Plant_Pathogen–Soil_Saprotroph–Wood_Saprotroph, Endophyte-Undefined_Saprotroph are higher in FS2 group, while the abundance of

Endophyte-Plant_Pathogen, Undefined_Saprotroph, Soil_Saprotroph are higher in FS1 group. Even when taking individual differences into consideration, the analysis of Trophic Mode reveals that Saprotroph, Saprotroph-Symbiotroph, Pathotroph-Symbiotroph, Pathotroph have the highest abundance in FS1 group and Pathotroph-Saprotroph, Pathogen-Saprotroph-Symbiotroph have the highest abundance in FS2 group.

It is noteworthy that the content of beta-glucosidase of one-year stratification is higher in abundance than that of two-year stratification in both the endophytic and soil bacteria analysis, while fungal analysis had the opposite result, whre the content of Plant_Pathogen-Soil_Saprotroph-Wood_Saprotroph and Undefined_Saprotroph-Wood_Saprotroph increase greatly in FS2. Since endophyte is a whole, the roles of fungi and bacteria cannot be viewed separately, and so this phenomenon can be explained that bacteria and fungi play the leading role respectively at different periods of stratification in the degradation of cellulose and lignin.

Based on the overall structure of the community, there are also microorganisms that can decompose cellulose, but the specific species could not be accurately located due to the incomplete understanding of the bacteria that can produce cellulase and ligninase so far. At the genus level, the bacteria mainly consist of *Pseudorhodoplanes, Flavobacterium, Bacillus* and so on. After stratification, their content has increased. The relative abundance of *Flavobacterium* is up to 31% in the one-year stratification group, but decreases to about 2.2% in the two-year stratification group, showing a “down after rising first” trend. The reports by the authors (Abd et al. 2020; Jiang et al. 2022) suggest that *Bacillus* is the dominant bacterium in soil cellulase generation, which increases the possibility of microorganisms in fruits participating in decomposition of pericarps. Studies have shown that the GS13T strain of *Flavobacterium* has the ability to decompose cellulose as well (Kim et al. 2019). It is reasonable to speculate that there may be other strains that can decompose cellulose from *Flavobacterium* and *Bacillus* genus. In addition, according to the heat map of fungal species cluster (Fig. 6B), the amount of saprophytic and wood saprophytic fungi are high in both FS1 and FS2. Among the advantageous fungal genus, we found *Trichothecium* and *Humicola*. *Humicola* fungi can produce a variety of heat-resisting cellulose enzymes, such as endoglucanase (endo-1,4-beta-D-glucanase, EC 3.2.1.4), fiber biohydrolase (cellulose 1,4-beta-cellobiosidase, EC 3.2.1.91) and beta- glucosidase (1,4-beta-d-glucosidase, EC 3.2.1.21) (Takashima et al. 2007). *Trichothecium roseum* also has the ability to produce beta-glucosidase and carboxymethyl cellulase (Zheng et al. 2020). The other dominant fungal genus like *Trichothecium*, *Sarocladium* and *Diaporthe* also have strains which can produce cellulase (Li et al. 2021; Zhou et al. 2016).

At the level of phylum, *Proteobacteria, Actinobacteriota, Bacteroidota* and *Firmicutes* are the dominant bacteria in pericarp. After one-year storage, the relative abundance of *Actinobacteriota* have increased significantly. The relative abundance of *Firmicutes* have increased significantly after two years of stratification. It is found that *Firmicutes* and *Bacteroidota* are similar to the dominant microbial communities discovered (Zhao et al. 2015; Qiao et al. 2013; Du et al. 2010; Jiang et al. 2022; Müller et al.

2006). It further confirms that soil microorganisms and endophytes have participated in the degradation of *S. xylocarpa* pericarp.

Isolation of potential fungal strains and their effects on the pericarp

We use several media to isolate and identify the fungal strains. The results of cultivation are enumerated in Fig. 7. Four morphologically different strains are selected according to their outstanding abilities of degrading cellulose or lignin and sequenced for further verification. All four strains cultivated on CMC selective media show decolorizing circle after being dyed with Lugol's iodine, suggesting that these strains are able to degrade cellulose in pericarp. Strain 1, 2 and 3 show decolorizing circle on PDA plate with aniline blue, indicating that they can producing laccase, which is capable of degrading lignin; strain 4 is not capable of producing laccase. All four strains cannot produce lignin degrading lignin peroxidase or Mn-dependent peroxidase. Sequence analyses through blast reveal the taxonomic status and relatedness of four isolated strains (Table S8). Strain 1 belongs to *Penicillium*; Strain 2 belongs to *Fusarium*; Strain 3 belongs to *Aspergillus*; Strain 4 belongs to *Aspergillus*.

The comparative analysis of the four selected strains and the control groups shows that the selected strains have an effect to promote degradation on the structure and properties of the pericarp. Strain 1 demonstrates general capabilities on degrading pericarp (Fig. 8) compared with negative control and similar characteristics compared with positive control (Figure S4, Figure S3E). Strain 2, 3 and 4 also presented various degrees of abilities to degrade the structure of pericarp (Figure S4Figure S3).

Discussion

S. xylocarpa is an endangered species endemic to China. Some species in *Sinojackia* genus, like *Sinojackia huangmeiensis*, are rare and endangered, and their distribution area is extremely narrow. In recent years, artificial protection measures have been taken to increase the population of *Sinojackia* (Wang et al. 2011; Liang et al. 2019), but the low germination rate in natural condition results in the fact that it remains endangered. Studies have shown that the physical barriers to water and air around seeds can cause dormancy (Penfield 2017). The hard texture of the pericarp of *Sinojackia* isolates most of the outside air and water from the embryo, which is the main reason for the dormancy of the seeds (Luo et al. 2016). Similar to the *S. xylocarpa*, there are many endangered species with slow self-renewal due to the long seed dormancy time. For example, *Rhus Michauxii* (Pullman et al. 2021) also has hard pericarp hindering germination just like the *S. xylocarpa*; *Monimopetalum chinense Rehd* (Pan et al. 2012), of which endogenous substances interact with the pericarp to cause dormancy. The thickened and lignified pericarp of *Vanilla planifolia* formed an impermeable envelope surrounding the embryo, which may play an important role in inducing dormancy (Yeh et al. 2021). For now, artificial methods to break seed dormancy mainly include physical methods such as cutting seeds, stratification in wet sands or chemical methods like acid etching. Even though keeping the seeds in low temperature will inhibit the germination of *Ruppia sinensis* seeds, after transferring the seeds to suitable germination conditions,

the germination rate has increased compared with the seeds without being kept in low temperature (Gu 2018). This study suggests that the endophytes in seeds and soil microorganisms may provide new methods to break seed dormancy and be beneficial for long-term conservation.

As an important reproductive organ of plants, seeds are carriers of various beneficial bacteria, and endophytic bacteria also play many important roles. Seed endophytes can produce 1-aminocyclopropane-1-carboxylic acid deaminase to relieve inhibition of ethylene concentration and promote plant growth (Sperdouli et al. 2012); *Cladosporium cladosporioides* is a seed endophytic fungus of *Suaeda glauca*, which can promote seed germination and plant growth (Qin et al. 2016); *Dendrobium officinale* has a close biological relationship with different microorganisms, which can form a microbial community with *Dendrobium officinale* and affect the germination of symbiotic seeds (Teixeira et al. 2015). In addition, arbuscular mycorrhizal fungi (AMF) of which soil environment can cause functional differentiation have been shown to promote the growth of endangered plants (Bothe et al. 2010; Yang et al. 2014). In our study, amplicon sequencing of ITS fragment and 16S fragment is wildly used to expound the composition and diversity of endophytes and microorganisms and their changes during the complete process of stratification. Analysis of amplicon sequencing indicates that endophytic bacteria participate in degradation of pericarp through producing beta-glucosidase mainly in the first winter of stratification; fungi community on the pericarp contain high ratio of nutritional type of wood saprophytic or saprophytic communities; also, through FunGuild annotation, two fungi with lignin decomposition ability is located. We consider that these results, which are focusing more on the general effects of endophytes and microorganisms towards seed germination of *Sinojackia* in natural condition, will broaden up the horizon for the restoration and development of the wild population of *Sinojackia*. In regard to the criteria of sample selection, fruit samples were collected in winter because the composition of the microbial community is more stable at low temperature and reflects the condition of the seeds on the verge of germination in the second winter. Considering of the infection process during the long term of stratification process, samples were not limited to surface sterilized pericarp used to detect endophytic bacterial; we also collected and sequenced pericarp carrying surface soil to fully reveal the effect of endophytes and soil microorganisms on pericarp during stratification process.

To further elaborate on the specific role of certain species in pericarp degradation, several strains of fungi were isolated from stratified pericarp by using selective media and experiments which were performed to verify their biochemical properties. It is worth emphasizing that attempts to isolate and purify endophyte strains have also been verified in the study of *Sinojackia* (Cao 2009; Li et al. 2010), but in general many strains with possible ability of degradation are not able to grow on artificial media and are difficult to be isolated. Therefore, we choose to use amplicon sequencing results to analyze the overall changes of pericarp during stratification. The strains isolated and purified in this experiment are easy to grow on selective media and have obvious morphological differences. Their lignin degradation ability or cellulose degradation ability is used as an auxiliary basis for microbial participation in pericarp degradation. Therefore, we speculate that endophytes and soil microorganisms play a role in breaking seed dormancy by degrading pericarp of recalcitrant seeds. This finding makes it possible to break seed dormancy and increase the populations of endangered plants by modifying soil microenvironment, and it

seems a much more efficient and promising way to save these endangered species in their natural habitats.

According to Meng (Meng et al. 2015), the germination rate of 15 months stratification is 52.7% and the germination rate of 24 months stratification is 67%, which are far longer than the time needed to degrade the cellulose and lignin. Based on the community structure we observed, the community has reached a relatively stable state during the period of stratification for one year. It is possible that it will take at least one year for the endophytes to evolve a suitable community system during stratification which is capable of degrading the pericarp. Also, we observe a phenomenon that the diversity of endophytic bacteria have decreased after one-year stratification. We believe that the “down after rising first” trend could account for the less abundance in BS2 group. For example, *Bacillus amyloliquefaciens* Y19, separated from corn seeds, which have effects of promoting growth and preventing disease, present a trend of “down after rising first” in the corn of colonization in rhizosphere soil and corn tissue (Jiang et al. 2015). The trend of fruit endogenous bacteria also can appear the same downward trend after rising first. The diversity index of endophytic fungi in *Alpinia zerumbet* seeds decreased from young fruit period to middle mature period and then increased at late mature period. The reason could be the availability of a favorable environment, such as temperature, water contents, and dry matter for microorganisms’ growth at first, which were depleted later and stored at the final maturation stage (Yan et al. 2022).

Besides the evolution time of endophytes, temperature may also play a crucial part in it. The stratification temperature varying from 0–5°C to 10–20°C can account for that the two-year stratification makes highest germination rate, which go as follows. The low temperature may not only be suitable for the endophytes’ evolution, but increase germination time. For example, lowering the temperature during seed maturation progressively increased time to 50% germination and reduced the speed of seedling establishment and cotyledon opening (Chen et al. 2021). Temperature may also have an impact on the production and activity of cellulase and ligninase. The growth kinetic studies on *Aspergillus niger* MS82 producing β-glucosidase indicate that there was a maximum rate of growth at 30°C in a medium (Sohail et al. 2009). Four selected cellulolytic bacteria which was investigated at a wide range of temperature (40–80°C) showed maximum relative cellulase enzyme activities at a temperature of 60°C (Awasthi et al. 2018). During green manure composting, the results showed that efficient lignin and cellulose degradation was achieved when the temperature was between 45 and 60 ℃ (Yu et al. 2022). It indicates that the low temperature during stratification may not be suitable for either the production or the activity of cellulase and ligninase, which delays the degradation of cellulose and lignin in the pericarp as well.

Conclusions

By conducting a diversity analysis of the microorganisms in both the pericarp of *S. xylocarpa* and soil, we discovered that the diversity of microorganisms increased with prolonged time of stratification. Functional identification of the enriched genes revealed enzymes secreted by these microbial species, such as beta-glucosidase, which have a degrading effect on the pericarp. We identified endophytes and fungi that secreted enzymes capable of degrading the pericarp, and found that they played crucial roles

respectively at different stages of stratification and collectively contributed to the breakdown of the pericarp. Moreover, using various culture media, we isolated four fungal species (respectively belongs to *Penicillium*, *Fusarium*, *Aspergillus*, and *Aspergillus*) and confirmed their degradation capabilities. Our findings have potential applications for endangered plant species that experience difficulties in germination, particularly in their natural habitats.

Declarations

Supplementary Information The online version contains supplementary material available.

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Author contributions J.L. conceived and supervised the project. J.L., Z.L. and R.Z designed this study. Z.L., X.M. and H.Y. performed the experiments and wrote the manuscript. J.L., Z.L. and R.Z were responsible for editing the manuscript. All authors read and approved the manuscript.

Data availability

All data has been uploaded to National Center for Biotechnology Information (NCBI) under the BioProject accession no. PRJNA886059, PRJNA886062 and PRJNA886063.

The authors declare no conflict of interest.

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Conflict of interest. The authors declare that no competing interests exist.

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Figures

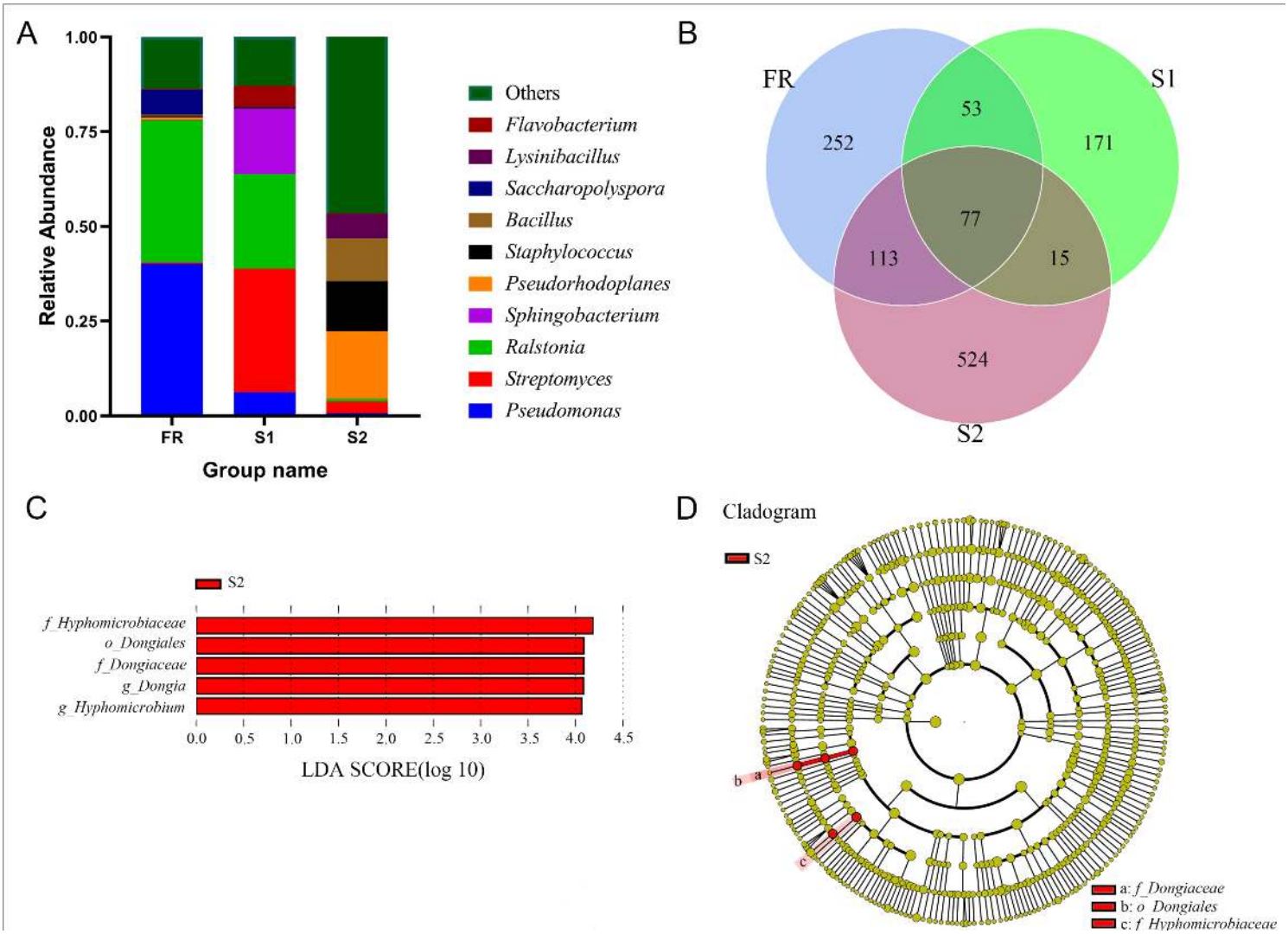


Figure 1

Analysis of endophytic bacteria in the pericarp of *S. xylocarpa*. A: Relative abundance of endophytic bacteria in pericarp of *S. xylocarpa* according to ASVs annotation. Top 10 genera with the highest relative abundance of endophytic bacteria were demonstrated. **B:** Venn graph of common and unique ASVs of each group. **C:**LEfSe analysis of endophytic bacteria in pericarp of *S. xylocarpa*. The histogram of LDA value distribution showed the taxonomic unit with LDA score greater than 4, namely the Biomarker with statistical difference between groups. The length of the bar represented the size of the impact of different species (LDA score). **D:** The phylogenetic tree of endophytic bacteria in the pericarp of *S. xylocarpa*. In the cladogram diagram, the circles radiating from the inside out represented taxonomic levels from phylum to genus. Each small circle at a different classification level represented a taxonomic unit at that level, and the diameter of the small circle was proportional to the relative abundance. Species with significant differences were colored yellow, and the red nodes represented the microbial groups that play an important role in the S2 group. The species names represented were shown around the diagram.

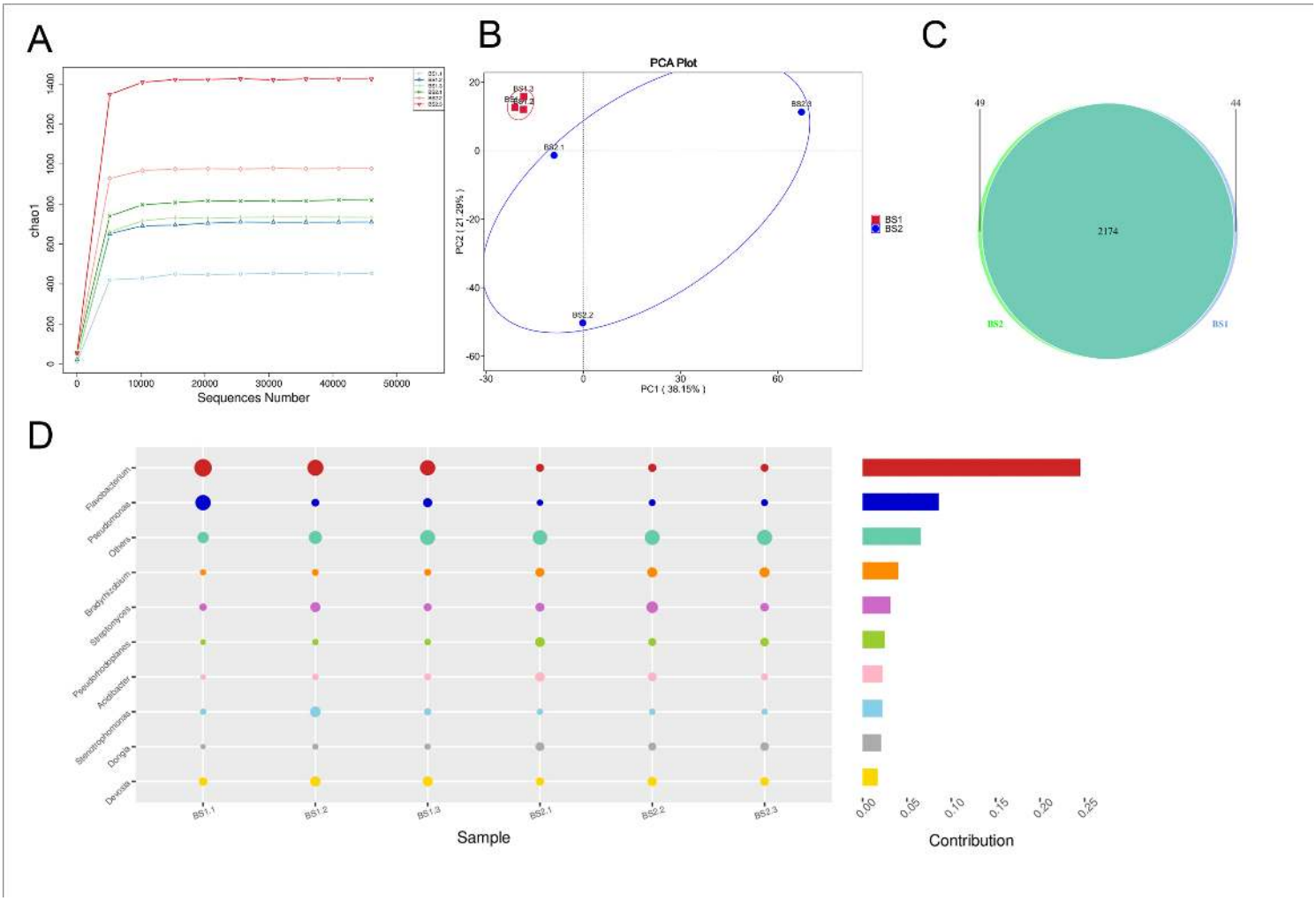


Figure 2

Supplementary data of soil bacteria. A: Dilution Curve (Chao1) of bacteria in soil. The horizontal axis represented the amount of sequencing data; the vertical axis represented the corresponding alpha diversity index, and the vertical axis in this figure was Chao1. **B:** PCA analysis results of bacterial in pericarp and soil bacteria. **C:** PICRUSt2 functional annotation Venn diagram result based on EC database. **D:** Different contributions of pericarp and soil bacteria Simper.

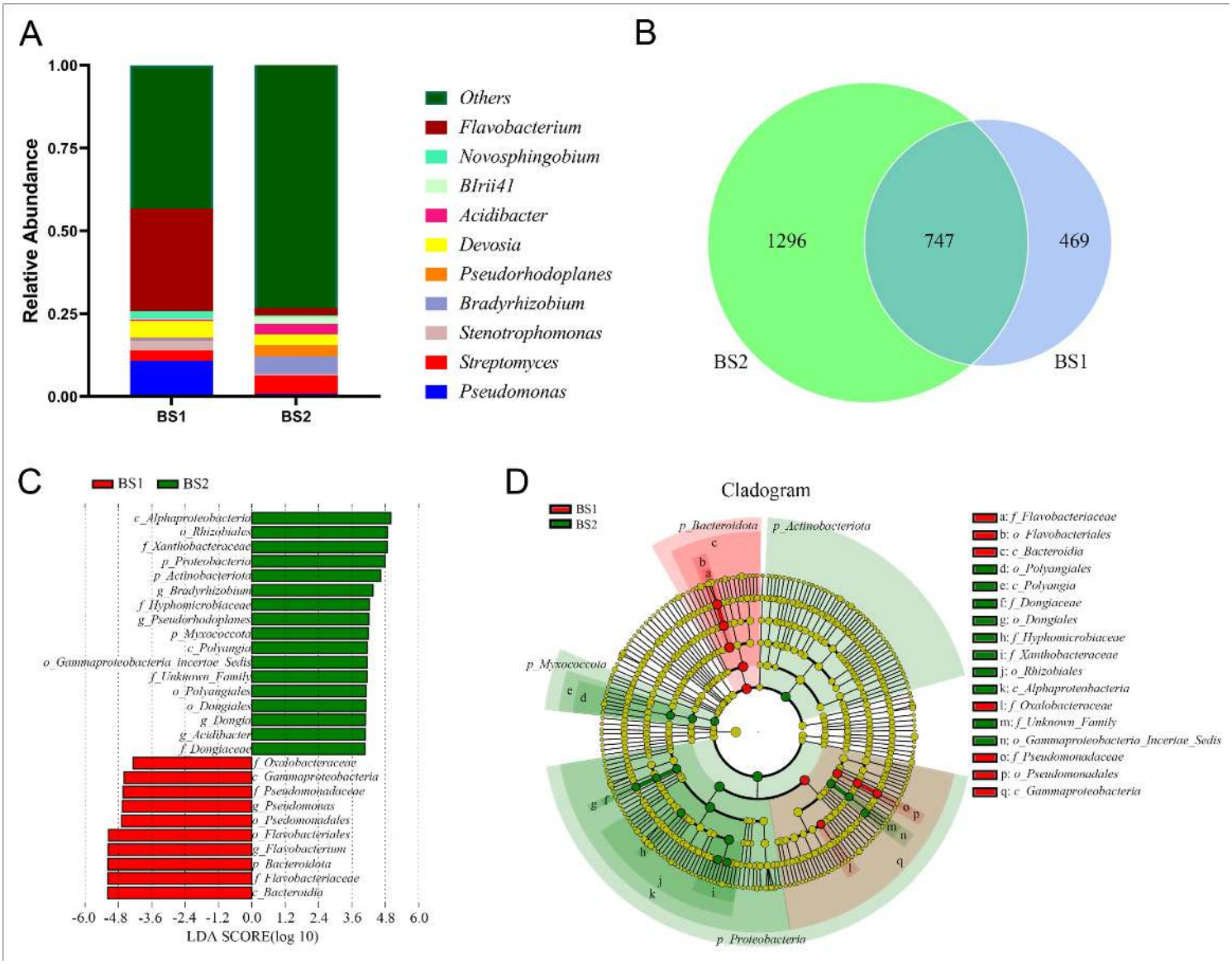


Figure 3

Analysis of soil bacteria. A: Relative abundance of soil bacteria in pericarp of *S. xylocarpa*according to ASVs annotation. Top 10 genera with the highest relative abundance are demonstrated. **B:** Venn graph of common and unique ASVs of each group. **C:** LEfSe analysis of soil bacteria. **D**: The phylogenetic tree of soil bacteria. Species with significant differences are colored yellow, and the red nodes represent the microbial groups that play an important role in the BS1 group, while green nodes represent BS2 group. The species names represented are shown around the diagram.

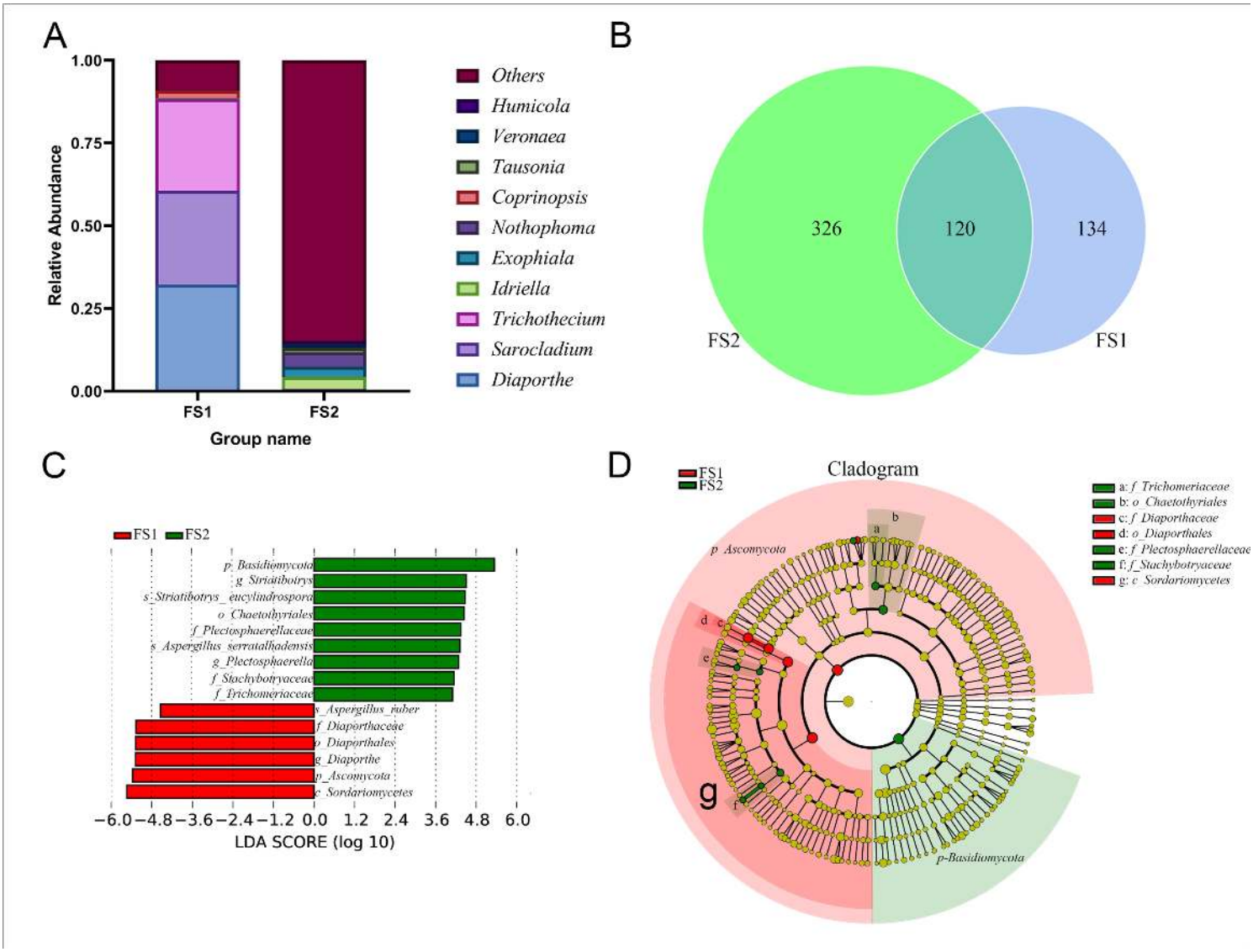


Figure 4

Analysis of ASVs and difference in soil fungi. (A) Relative abundance of soil fungi in pericarp of *S. xylocarpa* according to ASVs annotation. Top 10 genera with the highest relative abundance are demonstrated. **B:** Venn graph of common and unique ASVs of each group. **C:** LEfSe analysis of soil fungi. **D:** The phylogenetic tree of soil fungi. Species with significant differences are colored yellow, and the red nodes represent the microbial groups that play an important role in the FS1 group, while green nodes represent FS2 group. The species names represented are shown around the diagram.

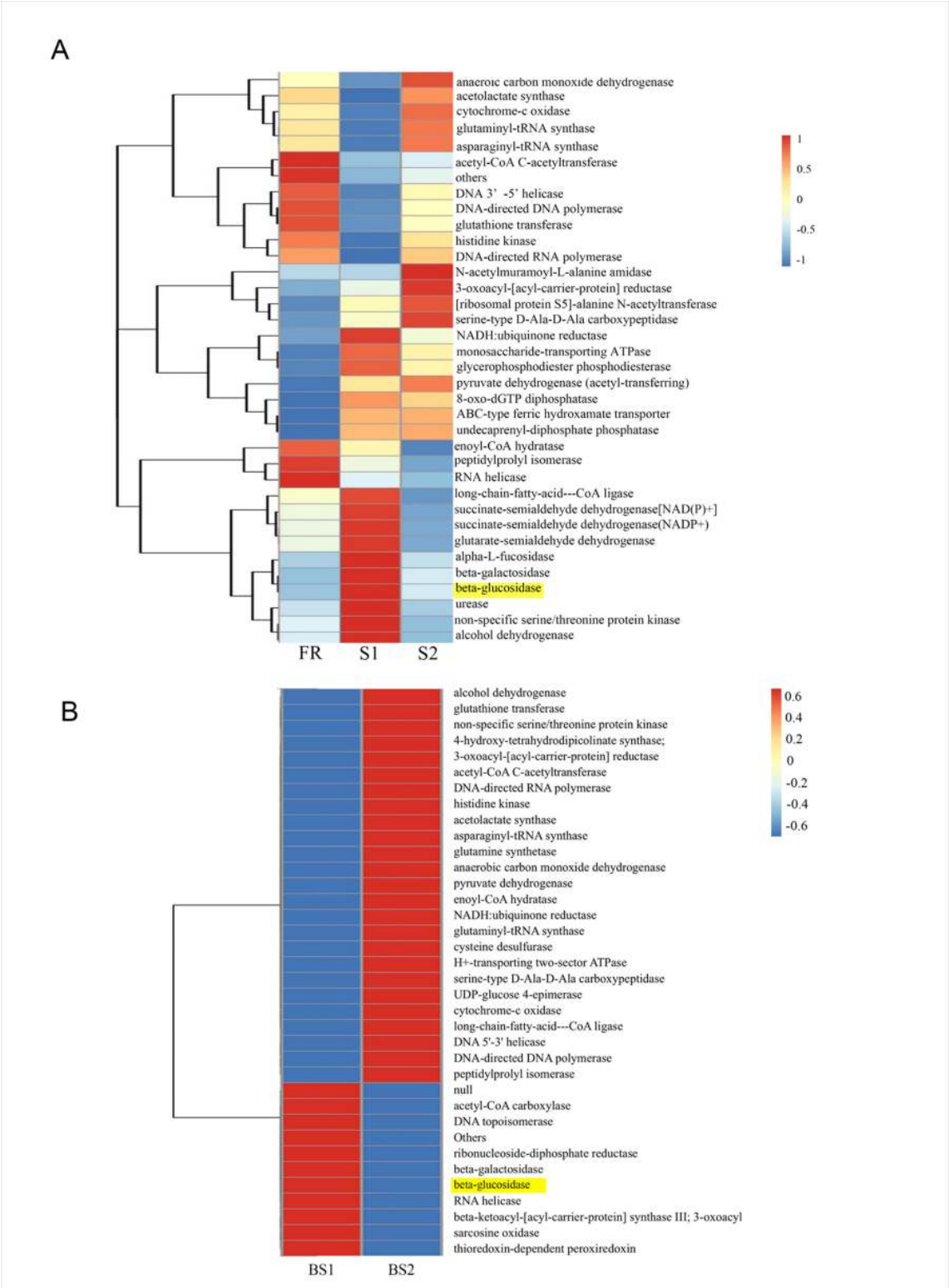


Figure 5

Function prediction of bacteria. A: Heatmap of bacterial community enzyme (EC) function annotation clustering based on EC database. The value corresponding to the heat map is the Z-value obtained from the normalized relative abundances of functions in each row. FR: fresh fruits; S1: 3-months-stratified fruits; S2: 14-months-stratified fruits. **B:** Heatmap of bacterial community enzyme (EC) function annotation clustering based on EC database. BS1: the 16S amplicon sequencing group for soil bacteria

stratified for 5 months; BS2: the 16S amplicon sequencing group for soil bacteria stratified for 16 months. The value corresponding to the heat map is the Z-value obtained from the normalized relative abundances of functions in each row.

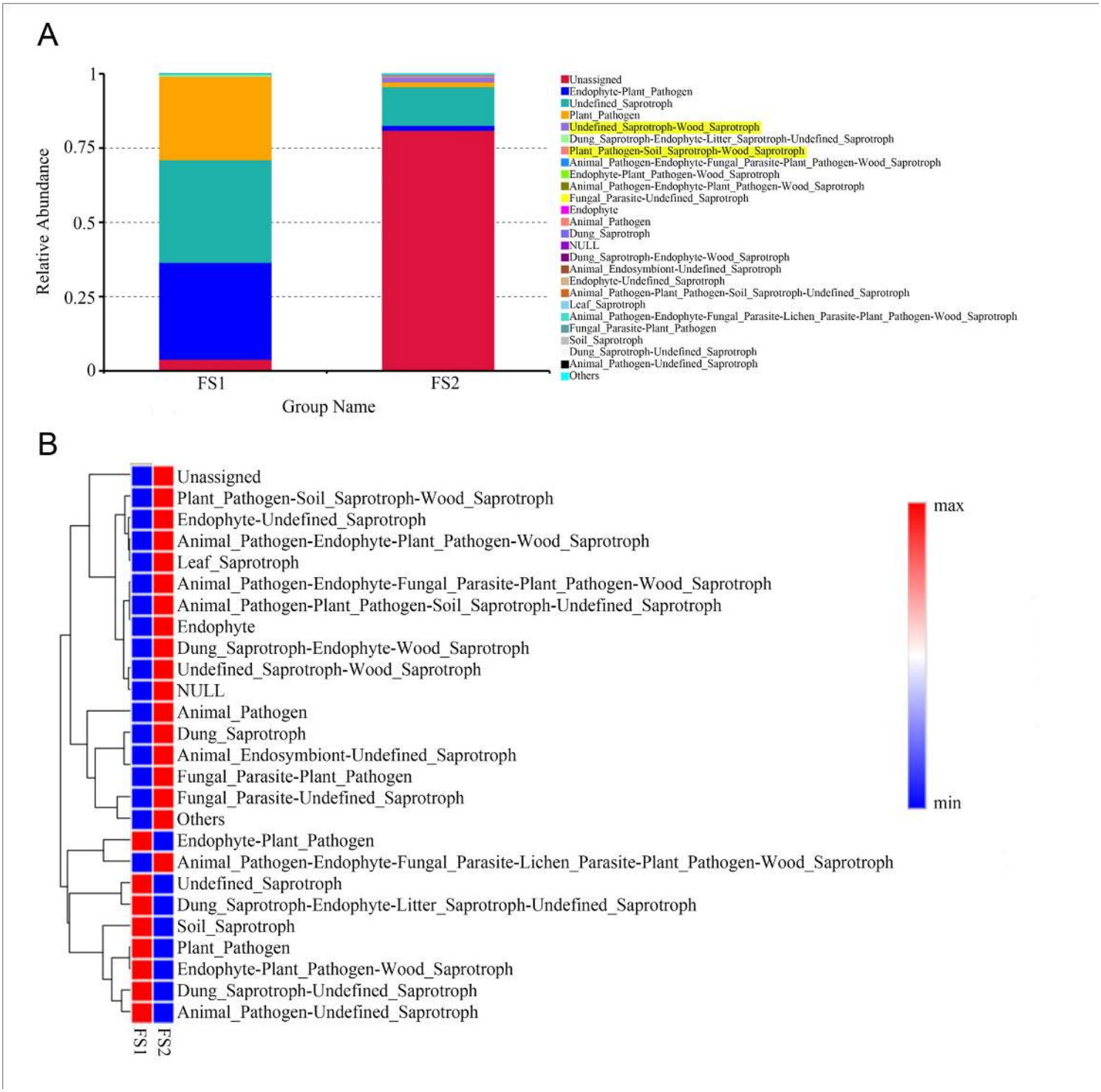


Figure 6

Function prediction of soil fungi. FunGuild analysis based on ITS amplicon can obtain information on the taxonomy and abundance of fungal species present in the samples. According to the annotated results of FunGuild database, the function abundance results of Trophic Mode and Guild were calculated. **(A)**

The histogram demonstrated the abundance of function in Guild. Representative wood saprotroph was highlighted with yellow. **(B)** Clustering diagram of fungi function based on Guild statistics.

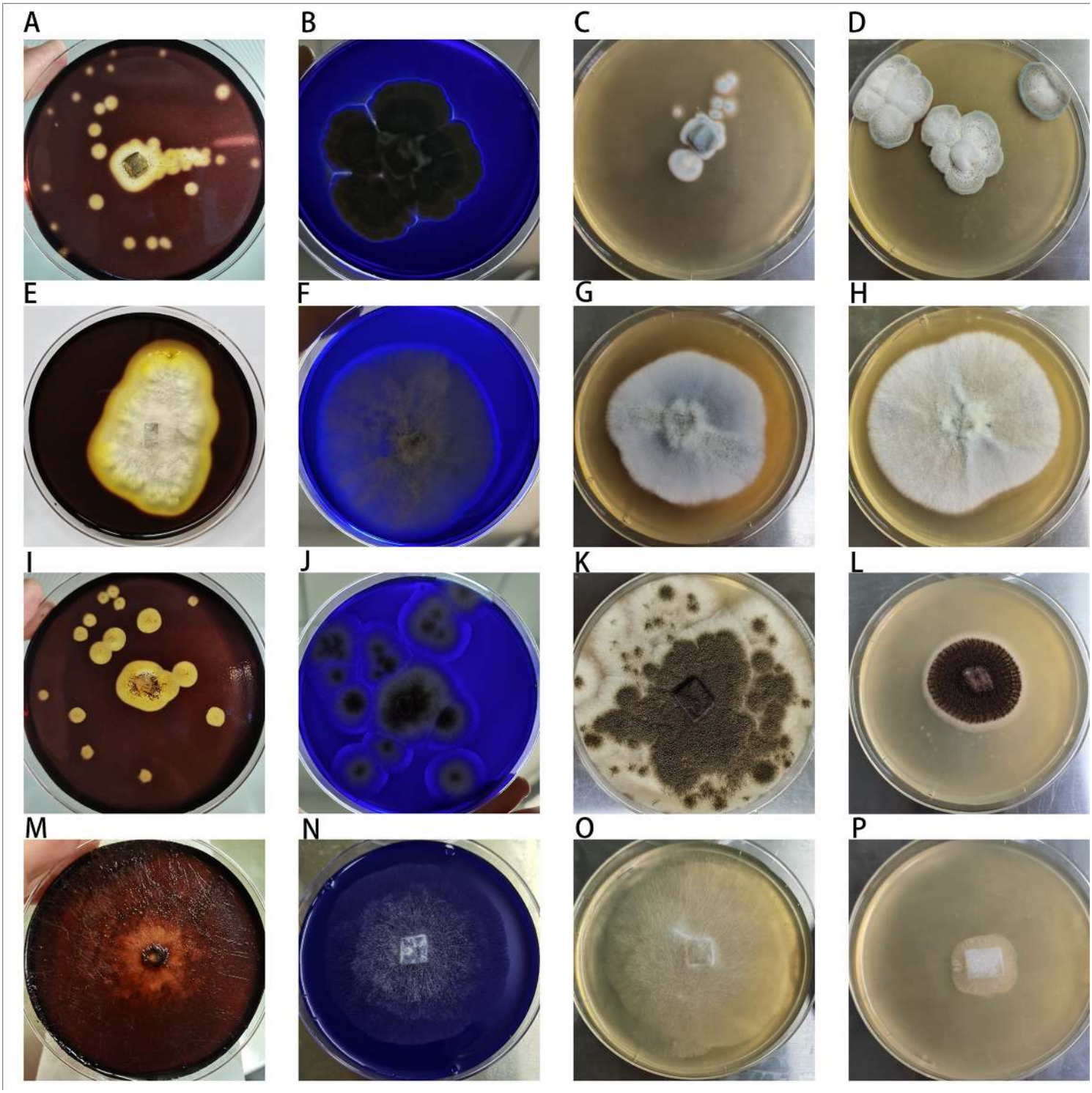


Figure 7

Results of cultivation of purified fungal strains. The four lines from top to bottom are strain 1, strain 2, strain 3 and strain 4; The four columns from left to right are CMC selective medium, PDA medium with aniline blue, PDA medium with Mn^{2+} and PDA medium with guaiacol. The decolorizing circles on plate A, E, I and M suggest that strain 1, strain 2, strain 3 and strain 4 can degrade cellulose; the decolorizing

circles on B, F and J suggest that strain 1, strain 2 and strain 3 can produce lignin degrading enzyme such as laccase. There are no staining circles on all the plates with Mn^{2+} and guaiacol, which demonstrates that all four strains are not able to produce degrading enzyme such as peroxidase or Mn-dependent peroxidase.

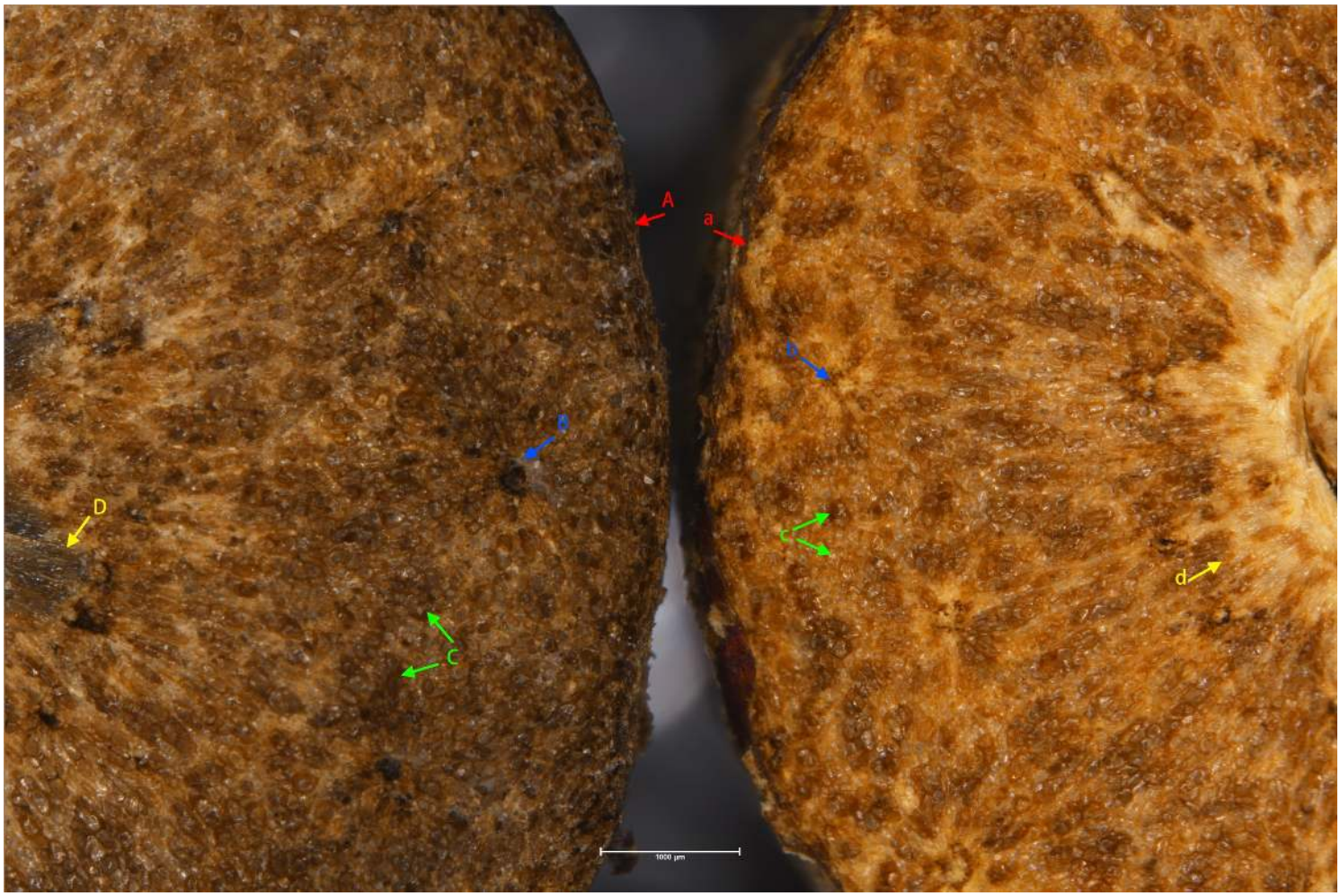


Figure 8

The fruit cultivated with strain 1 (left, annotated with capital letters) and negative control (right, annotated with lowercase letters). The after-processing pericarp close to exocarp (A) pretend to be more fragile than that in the negative control (a). The vascular tissues in the pericarp (B) become pitted and browned compared with the negative control (b). After processing, the tissues in the pericarp become darker and distinction between tissues becomes weaker (C and c). Properties of endocarp (D and d) also changed after processed with strain 1.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.tif
- FigureS2.tif

- FigureS3.tif
- FigureS4.tif
- TableS1.docx
- TableS2.docx
- TableS3.docx
- TableS4.docx
- TableS5.docx
- TableS6.docx
- TableS7.docx
- TableS8.docx