Characterization and Variation of the Rhizosphere Fungal Community Structure of Cultivated Tetraploid Cotton

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Abstract

Rhizosphere fungal communities exert important influential forces on plant growth and health. However, information on the dynamics of the rhizosphere fungal community structure of the worldwide economic crop, cotton (*Gossypium* spp.), is limited. Next-generation sequencing of nuclear ribosomal internal transcribed spacer-1 (ITS1) was used to characterize the rhizosphere fungal communities of worldwide cultivated tetraploid cotton using *G. hirsutum* cv. TM-1 (upland cotton) and *G. barbadense* cv. Hai 7124 (island cotton). Plants were grown in field soil (FS) that had been continuously cropped with cotton and nutrient-rich soil (NS) that had not been cropped. Fungal species richness, diversity, and community composition were analyzed and compared among soil resources, cotton genotypes, and developmental stages. We found that the fungal community structure between the rhizosphere and bulk soil of cotton were different and the rhizosphere fungal communities were significantly varied between FS and NS. These results suggest that cotton rhizosphere fungal community structure variation was primarily determined by the interaction of cotton roots with different soil resources. We also found that the community composition of cotton rhizosphere fungi varied significantly during different developmental stages, suggesting that developmental stages were also important factors in the dynamics of rhizosphere fungal communities for the varying dominant fungal genera of the rhizosphere. In addition, we also observed that fungal pathogens were clearly increased at certain developmental stages, suggesting a higher infection rate and a high incidence of corresponding soil-borne disease in each stage. This research illustrates the characteristics of cotton rhizosphere fungal communities and provides important information for understanding the potential influences of rhizosphere fungal communities on cotton growth and health.

Key words: cotton rhizosphere; fungal community; diversity; soil resource; developmental stage

Introduction

Soil microorganisms are a critical component of agroecosystems and play key roles in agricultural ecosystems. The importance of mutual influence between microbial communities and agronomic practices is increasingly being recognized. The rhizosphere is the adjacent soil environment that the plant helps to create and where beneficial and pathogenic microorganisms exert major influential forces on plant growth and health [1]. Rhizosphere microorganisms were thought to be of great importance to plant health due to their involvement in such key processes as the formation of root architecture [2]; formation of soil characteristics [3]; decomposition of organic matter [4, 5]; decomposition and removal of toxins [6, 7]; defense against plant pathogenic microorganisms [2]; and cycling of carbon [8], nitrogen, phosphorus, and sulfur [9-12].

Soil fungi are a critical component of agroecosystems, and the rhizosphere fungal communities play important roles in plant growth and health. In turn, plants largely control rhizosphere fungi through the production of carbon- and energy-rich compounds and bioactive phytochemicals [13]. Some of the beneficial fungi are directly involved in the cycling of nutrients and function as an essential link to soil nutrient availability [14-17]. Some fungi are known for having biocontrol activity against pathogenic microorganisms [17, 18]. These fungi positively influence plant productivity by enhancing plant growth. However, certain rhizosphere fungal species or genera can also negatively influence plant productivity by causing disease, and pathogenic fungi are some of the most serious plant pathogens, for example, stalk rot disease of maize caused by *Fusarium* species [19], *Verticillium* wilt caused by *Verticillium nonalfalfae* on tree-of-heaven [20], and dry root rot caused by *Macrophomina phaseolina,* which affects many crops [21].

It is known that microbial diversity in soil is one of the major components determining soil health [22] and is believed to be one of the main drivers in disease suppression [22-25]. The composition of rhizosphere microbial communities is affected by soil, plant developmental stage, and many other factors [26-30]. Continuous cropping in agricultural production can cause crop yield reduction through soil quality degradation and aggravated plant diseases [31-33]. The fundamental reason for continuous cropping obstacles is related to disorders or deterioration of rhizosphere microorganisms (including rhizosphere fungi) [34, 35].

Cotton (*Gossypium* spp.) is the most important cash crop in the world and provides the most natural textile fibers of the world. Cotton production is threatened by soil-borne plant pathogens such as *Rhizoctonia* spp. [36], *Fusarium moniliforme* [37], *Alternaria alternata* [38], and *Verticillium dahliae* [39]. Understanding the dynamics of the rhizosphere fungal community structure of the worldwide cultivated tetraploid cotton with cotton cultivars in different developmental stages will not only provide basic information on the dynamics of cotton rhizosphere fungal community structure but also help lay a foundation for understanding the mutual influence between rhizosphere fungal communities and the plant health of cotton. Knox *et al.* showed that rhizosphere microbial diversity in cotton is significantly influenced by cultivar type in the field [40]. However, systematic studies on the rhizosphere fungal community structure of cultivated tetraploid cotton are still lacking.

This study characterized the rhizosphere fungal community dynamics across cotton developmental stage growth using two cotton cultivars in continuously mono-cropped cotton field soils (FS) and nutrient-rich soil (NS) that had not been cropped. Our work lays the foundation for more research on cotton rhizosphere fungal communities and may provide insight into further dissection of the structure of rhizosphere fungal communities, which might exert major influential forces on plant growth and health in the agricultural production of cotton.

Materials and methods

Plants and soil

Two cultivars of cultivated allotetraploid *Gossypium* species, *G. hirsutum* cv. TM-1 (upland cotton) and *G. barbadense* cv. Hai 7124 (island cotton with higher disease resistance than upland cotton) were planted in two types of soils FS and NS.

The FS was obtained from 15 to 30 cm below the soil surface in a field that has been continuously planted with cotton for several decades at the Experiment Station of Cotton Research Center of Shandong Academy of Agricultural Sciences (Linqing County, Shandong Province, 36°81′N, and 115.71°13′E), and the NS, which was not influenced by cotton and any other plants, was purchased from Feng Yuan Science and Technology Ltd. (Jinan, China). All visible biota (e.g., weeds, twigs, worms, and insects) were removed, and the soil was then crushed and sifted

97 through a sterile 2 mm sieve. Because the sieved soil drained poorly and was difficult to sample,
98 we mixed sterile sand into the treatment soils at a soil:sand ratio of 2:1 following Lundberg *et al.*
99 [41].

100 All plants were grown under the same environmental conditions. Samples were collected at
101 the seedling, budding, and flowering stages. Detailed information about the material and methods
102 were described in our previous report [42].

103 *Greenhouse plant management*

104 Cotton seeds were delinted by sulfuric acid and then surface sterilized with 75% ethanol for 15
105 min, followed by 30% H_2O_2 for 30 min and five rinses with sterile distilled water. The seeds were
106 germinated by incubating at 28 °C in the dark for 2–3 days in petri dishes in which sterile paper
107 was overlaid on 1% water agar. After germination, seedlings were transplanted into the treated soil
108 and raised in a tissue culture room at 28 °C. Plants were moved to a bioclean greenhouse as soon
109 as seedlings developed a second true leaf. The pots were watered every 3 days with sterile water.
110 Control pots contained soil without a cotton plant.

111 *Sampling of cotton rhizosphere and bulk soil*

112 Soil samples were harvested from 4 to 6 July 2015. Well-grown plant individuals in each
113 developmental stage were selected for rhizosphere soil collection. We inverted each pot to remove
114 the soil and plant and then gently shook the plant to remove the soil that did not adhere to the root
115 surface. Rhizosphere soil consisted of ~1 mm of soil that tightly adhered to the root surface and
116 was not easily shaken from the root. To separate the rhizosphere soil, roots were placed in a sterile
117 flask with 50 ml of sterile phosphate buffered saline solution and stirred vigorously with sterile
118 forceps. Samples at the interface or from an unnatural environment were avoided. After cleaning,
119 the roots were removed, and the remaining soil solution was centrifuged for 15 min at 10,000 rpm.
120 The supernatant was discarded to leave the soil fraction. These soil fractions were frozen using
121 liquid nitrogen and stored at −80 °C. We also collected samples from unplanted pots from ~10 cm
122 below the soil surface as bulk soil. There were three biological replicates for each soil treatment
123 (rhizosphere samples of the two cultivars and bulk soil samples in FS and NS were collected at
124 three developmental stages) for a total of 54 replicates.

125 *DNA extraction and detection*

126 The DNA from each soil sample was extracted using the Omega D5625-02 Soil DNA Kit (Omega
127 Biotek Inc., Norcross, GA, USA). DNA concentration and integrity were detected by a microplate
128 reader (Qubit 3.0 Fluorometer; Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel
129 electrophoresis (PowerPac Basic164-5050 and Sub-Cell 96, Bio-Rad Laboratories, Hercules, CA,
130 USA). DNA information for each sample is listed in the Supplementary materials

131 *Preparation of libraries and sequencing*

132 All suitable DNA samples were submitted to BGI Tech Solutions Co., Ltd. (Shenzhen, China) to
133 construct a sequencing library. DNA from 54 soil samples was amplified and sequenced using the
134 Illumina MiSeq platform (Illumina, San Diego, CA, USA). Further details on the subsequent
135 bioinformatics analysis of the sequencing data are listed in the Supplementary materials and
136 methods.

137 *Data analysis*

138 OTU Venn diagram: The presence or absence of operational taxonomic units (OTUs) was
139 determined for each soil sample, and the common and specific OTU IDs were summarized. A
140 Venn diagram was constructed using the package VennDiagram in R (v 3.0.3).

141 Species Annotation: The tag number of each phylum in different soil samples was summarized in
142 a histogram, and all data were used to construct a histogram using R.

143 α-diversity analysis: The species accumulation curves of observed species (Sobs), Chao,
144 Abundance Based Coverage Estimator (ACE), Shannon, and Simpson indices were calculated
145 using the software Mothur (v 1.31.2). The calculation formula of each index can be found at
146 http://www.mothur.org/wiki/Calculators.

147 β-diversity analysis: β-diversity was analyzed using the software QIIME (v 1.80). Normalization
148 was performed to control for sequencing depth differences in different samples. Sequences were
149 extracted randomly according to the minimum sequence number of all samples to generate a new
150 'OTU table biom' file. Then, the β-diversity distance was calculated based on the 'OTU table
151 biom' file. The β-diversity heat map was drawn by the 'aheatmap' function in the 'NMF' package
152 of R.

153 Contribution of each factor: The Bray–Curtis dissimilarity analysis and the information entropy
154 method were used to measure the contribution of the different factors to variability between

155 samples. We then conducted an analysis of variance by the function aov in the R package.
156 Interaction between each of the two factors was considered. For each factor, the contribution rate
157 to fungal community variance was calculated as the mean square of the factor divided by the sum
158 of the mean square of all factors.

159 **Results**

160 Fungal communities were characterized by next-generation sequencing of nuclear ribosomal
161 internal transcribed spacer-1. A total of 5,032,042 high-quality reads were obtained with a median
162 read count of 93,186 per sample (range: 51,752–244,354) (Supplementary Table S1). The
163 high-quality reads were clustered into 1,298 microbial OTUs at 97% similarity after the removal
164 of OTUs that were unassigned or not assigned to the target species.

165 *Fungal communities in bulk soils of FS and NS*

166 Ascomycota, Basidiomycota, and Zygomycota were the most common fungal phyla in both
167 continuously cropped field soil (FS) and nutrient-rich soil (NS) treatments, accounting for
168 59.01–95.81% of all fungal communities (Supplementary Table S2; Supplementary Fig. 1).
169 Excluding unclassified orders (19.39–60.96% of total fungal communities), in both soils,
170 Eurotiales and Hypocreales were dominant in Ascomycota, and Mortierellales was dominant in
171 Zygomycota. The dominant orders of Basidiomycota in FS were Cystofilobasidiales and
172 Sporidiobolales, whereas Thelephorales and Agaricales were dominant in NS.

173 The differences in fungal communities between the FS and NS soils at the genus level were
174 significant. The relative abundance of some fungal genera, such as *Penicillium*, *Gliomastix*, and
175 *Engyodontium*, was significantly lower in FS than in NS ($P < 0.05$), whereas the relative
176 abundance of some fungal genera, such as *Pseudozyma*, *Panaeolus*, and *Lecanicillium* in FS was
177 slightly, but not significantly, higher than in NS (Supplementary Table S2).

178 *Fungal communities of cotton rhizosphere in FS and NS*

179 Ascomycota, Basidiomycota, and Zygomycota were the dominant phyla in the rhizosphere fungal
180 communities, accounting for approximately 33.45–88.51% of the total fungal communities in NS
181 (11.48–66.15% were unclassified) and 85.18–93.88% of the total fungal communities in FS
182 (6.03–14.65% were unclassified) (Fig. 1; Supplementary Table S3; Supplementary Figs. 2, 3).

183 Ascomycota was negatively selected in the rhizosphere in NS but was enriched in the rhizosphere
184 in FS (Fig. 1; Supplementary Table S2–4). The dominant orders of Ascomycota and Zygomycota
185 in the rhizosphere were the same as those in bulk soil (Supplementary Table S3). However, the
186 dominant orders of Basidiomycota in bulk soil from the FS rhizosphere samples were Agaricales
187 and Auriculariales, whereas Sporidiobolales and Agaricales dominated in bulk soil from the NS
188 rhizosphere samples (Supplementary Table S3).

189 **Fig 1. Relative abundance of the fungal community in all treatments.** Two types of soils:
190 nutrient-rich soil (N) and continuous cropping field soil (F). Three cotton plant developmental
191 stages: seedling stage (s), budding stage (b), and flowering stage (f). Two cultivated species:
192 upland cotton (*G. hirsutum* L. cv TM-1) (T) and sea island cotton (*G. barbadense* L. cv Hai7124)
193 (X) and control pots (C) that lacked cotton plants. Each sample was labeled by a three-letter code,
194 such as NsT, which indicates seedlings of sea island cotton grown in nutrient-rich soil.

195 The number of OTUs in the FS rhizosphere (205.33 ± 22.47) was higher than in FS bulk soil
196 (140.67 ± 28.61), whereas in the NS rhizosphere (146.44 ± 40.22), the OTUs were lower than in
197 NS bulk soil (181.11 ± 20.37) (Supplementary Table S5). The α-diversity of fungi was
198 significantly higher in the FS rhizosphere than in FS bulk soil ($P < 0.05$); however, it was
199 significantly lower in the NS rhizosphere than in the corresponding bulk soil ($P < 0.05$). Bulk soil
200 α-diversity of fungi was higher in NS than in FS ($P < 0.05$), but rhizosphere fungal α-diversity
201 was lower in NS than in FS ($P < 0.05$; Fig. 2; Supplementary Table S5).

202 **Fig 2. The α-diversity of rhizosphere fungi.** From left to right and from top to bottom, box plots
203 are Sob, Chao, ACE, Shannon, and Simpson indices.

204 Fungal genera compositions that were enriched or negatively selected in the rhizosphere
205 differed between different soils (Supplementary Table S6; Supplementary Table S7). For example,
206 in NS, the relative abundance of *Mortierella*, *Gliomastix*, and *Engyodontium* was significantly
207 higher in bulk soil compared with rhizosphere soil, where it was much lower or almost
208 undetectable ($P < 0.05$; Supplementary Table S8). In contrast, the relative abundance of
209 *Rhodosporidium* and *Trichoderma* in NS rhizosphere soil was higher than in the respective bulk
210 soil, where it was lower or almost undetectable ($P < 0.05$; Supplementary Table S8). In FS, the
211 relative abundance of *Mortierella*, *Guehomyces,* and *Fusarium* was higher in bulk soil than in

212 rhizosphere soil, where it was lower or undetectable ($P > 0.05$; Supplementary Table S9). The
213 relative abundance of *Penicillium*, *Alternaria*, and *Preussia* was higher in FS rhizosphere soil than
214 in bulk soil, where these genera were almost undetectable ($P < 0.05$; Supplementary Table S9).
215 The abundance of other rhizosphere fungal genera was highly variable and differed between soils.
216 Comparisons of fungal genera whose relative abundance changed inversely in different soils
217 between the rhizosphere and corresponding bulk soil are listed in Table 1.

Table 1 Fungal genera that were affected inversely by cotton root in two soil resources.

Genus	Relative abundance in field soil (mean)			Relative abundance in nutrient soil (mean)		
	Control	Rhizosphere		Control	Rhizosphere	
Paraconiothyrium	0.21	0.00	-	0.00	0.10	+
Rhodosporidium	1.598	0.01	-	0.00	3.45	+
Mrakia	0.50	0.00	-	0.00	0.12	+
Arnium	0.28	0.00	-	0.00	0.08	+
Pseudeurotium	0.77	0.00	-	0.00	0.02	+
Kurtzmanomyces	0.17	0.00	-	0.00	0.02	+
Tomentella	0.05	0.10	+	0.45	0.22	-
Wardomyces	0.00	0.13	+	0.58	0.00	-
Chrysosporium	0.01	0.16	+	0.94	0.00	-
Retroconis	0.00	0.21	+	1.71	0.00	-
Nectria	0.00	0.22	+	1.07	0.00	-
Engyodontium	0.08	0.50	+	3.31	0.07	-
Gliomastix	0.00	0.41	+	2.85	0.00	-
Alternaria	0.17	0.66	+	0.88	0.46	-
Preussia	0.00	0.89	+	2.09	0.03	-
Penicillium	0.71	14.06	+	10.51	4.11	-

"+" denotes fungi with higher relative abundance in rhizosphere soil than in bulk soil, "-" denotes fungi with lower relative abundance in rhizosphere soil than in bulk soil; $P < 0.05$

Variation in rhizosphere fungal communities at different plant developmental stages

In FS, the number of stage-specific OTUs was highest in the seedling stage and decreased gradually through development: upland cotton (T): 90 (seedling stage), 76 (budding stage), and 83 (flowering stage); island cotton (X): 121 (seedling stage), 53 (budding stage), and 48 (flowering stage). In NS, the number of stage-specific OTUs was highest in the budding stage (T: 71, 139, 85; X: 112, 138, 82). In addition, the number of overlapping OTUs in the seedling and budding stages was higher than that in the budding and flowering stages in both FS and NS soil treatments. The number of overlapping OTUs in all three developmental stages was higher in FS than in NS (Supplementary Fig. 4).

Analysis of α-diversity indicated that in FS, the Sobs, Chao, and ACE indices were higher in the cotton rhizosphere fungal communities during all three developmental stages compared with bulk soil. The Sobs index decreased gradually from the seedling to the flowering stage in bulk soil, but no significant difference was found in the rhizosphere sample between different

234 developmental stages (except for the difference between the seedling stage and budding stage in
235 the rhizosphere of island cotton) ($P < 0.05$; Fig. 2; Supplementary Table S5). In NS, the
236 rhizosphere harbored a fungal community of higher α-diversity than bulk soil. We compared the
237 α-diversity of different samples from NS to that of FS. The Sobs, Chao, and ACE indices
238 indicated that the α-diversity of bulk soils from FS was generally lower than those from NS, but
239 not significantly. In contrast, rhizosphere soils from FS were significantly higher than those from
240 NS ($P < 0.05$; Fig. 2; Supplementary Table S5).

241 Each developmental stage had dominant fungal genera found with high relative abundance.
242 We determined the genera that had high relative abundance (relative abundance >0.5) in the
243 different developmental stages. In the rhizosphere soils, *Penicillium*, *Fusarium*, and *Mortierella* in
244 FS and *Penicillium*, *Fusarium*, and *Talaromyces* in NS presented a higher relative abundance in
245 all three developmental stages. In addition, each developmental stage harbored the specific
246 dominant rhizosphere fungal genera (Supplementary Table S10). The number of dominant genera
247 was highest in the budding stage.

248 We also analyzed how the fungal community was affected by the presence of cotton. A large
249 change was defined as a difference in relative abundance between rhizosphere and bulk soil that
250 was >1 or <-1. The difference between rhizosphere and bulk soil fungal genera relative abundance
251 differed at different developmental stages. We defined a genus for which relative abundance was
252 greater in rhizosphere soil compared with bulk soil as an enriched fungal genus (EFG) and a genus
253 for which abundance was lower in rhizosphere soil compared with bulk soil as a depleted fungal
254 genus (DFG). EFGs were most abundant in the budding stage, whereas DFGs were most abundant
255 in the seedling stage. The number of DFGs in NS was higher than in FS, in accordance with our
256 finding that the α-diversity of fungal communities was higher in NS than in FS, and many fungi
257 were depleted under the influence of cotton root (Supplementary Table S10).

258 We analyzed the β-diversity of the samples based on Bray–Curtis dissimilarity analysis.
259 Cluster analysis indicated that samples from the same soil resources were clustered into one group
260 (Fig. 3A). The β-diversity of different soils (mean Bray–Curtis: 0.97) was significantly higher
261 than the β-diversity of different developmental stages (mean Bray–Curtis N: 0.66, F: 0.60) ($P <$
262 0.01; Supplementary Table S11; Fig. 3B). Statistical analyses were conducted to assess the

263 contribution of each factor to the structure of the fungal community in the cotton rhizosphere and
264 found that species-level soil factors contributed approximately 42.27% to the fungal community
265 structure in the cotton rhizosphere, which was higher than other factors ($P < 0.05$; Supplementary
266 Table S11).

267 **Fig 3. β-diversity analysis of different treatments.** A: Cluster analysis of different treatments. B:
268 Bray–Curtis distance analysis of different treatments.

269 *Potential pathogenic and phosphate-solubilizing fungi in the cotton rhizosphere*

270 Pathogenic fungi were mainly distributed in the genera *Alternaria, Fusarium, Gibberella* [43],
271 *Rhizoctonia, Thanatephorus* [44], and *Verticillium*. We analyzed the dynamics of those genera in
272 different soils and found that the relative abundance of each genus in the rhizosphere was higher
273 in bulk soil in pots containing FS but lower in pots containing NS (Supplementary Table S12). In
274 addition, the relative abundance of these genera differed during different plant developmental
275 stages. In FS, the greatest difference in the relative abundance between bulk soil and rhizosphere
276 was present in *Alternaria* and *Rhizoctonia* at the seedling stage, and *Fusarium*, *Thanatephorus*,
277 *Verticillium*, and *Gibberella* at the budding stage (Fig. 4; Supplementary Table S12). The
278 rhizosphere relative abundance of *Fusarium* was lower than bulk soil at the seedling stage, and
279 *Rhizoctonia* was lower than bulk soil at the budding stage. We conclude that in continuously
280 cotton-cropped soil, those genera were suppressed by the cotton root at different stages. In NS, the
281 relative abundance of most of these genera was lower in rhizosphere soil than in bulk soil, with the
282 exception of the seedling stage for *Alternaria* and *Fusarium*, the budding stage for *Fusarium* and
283 *Rhizoctonia* and the flowering stage for *Gibberella* (Fig. 4; Supplementary Table S12). Cotton
284 growth in soil that had not been cropped might have a high infection rate at each stage by those
285 genera. The difference in these genera between the two genotypes was not significant
286 (Supplementary Table S12). The relative abundance of disease-associated fungal genera, with the
287 exception of *Fusarium* (FS: 2.02–43.19; NS: 3.17–7.40), was higher in NS than in FS ($P < 0.05$),
288 such as *Verticillium* (FS: 0.14–1.19; NS: 3.89–5.29) and *Alternaria* (FS: 0.04–0.85; NS: 2.01–3.27;
289 Supplementary Table S2).

290 **Fig 4. Variations of potential pathogenic and phosphate-solubilizing fungal genera.** The

X-axis shows different values of relative abundance between rhizosphere soils and bulk soils.

Aspergillus and *Penicillium,* the potential phosphate-solubilizing fungal genera, were detected in our research. In NS, the relative abundance of both fungal genera was lower in the rhizosphere than in bulk soil ($P < 0.05$). In FS, the relative abundance of the two fungal genera was higher in the rhizosphere than in bulk soil, but this difference was not statistically significant ($P > 0.05$). In addition, the relative abundance of the two genera in rhizosphere soil was higher in FS than in NS (*Aspergillus*: $P < 0.01$; *Penicillium*: $P > 0.05$; Supplementary Table S12).

Discussion

The difference in fungal community structure between the rhizosphere and bulk soil of cotton

Plant roots have a remarkable effect on the physical and chemical characteristics of soil, such as its structure and water retention [45-47]. The physical and chemical characteristics of the root-associated soil are important because they determine both the physiological aspects of root function, such as water and nutrient uptake, and the microbial activity that is most relevant to root growth [48-50]. Plant roots also release root exudates, volatile substances, border cells, and polymers into the soil environment and regulate the community structure of the rhizosphere microbiome through complex interactions with soil microorganisms [51-57], promoting the colonization of beneficial microorganisms and inhibiting the colonization of harmful microorganisms [58]. Many studies have confirmed the existence of differences in the microbial communities of rhizosphere soil and the surrounding bulk soil of *Arabidopsis,* rice and *Populus* [30, 41, 59].

In the present study, the dominant fungal phyla in the rhizosphere of the two cultivars of cultivated allotetraploid *Gossypium* species were Ascomycota, Basidiomycota, and Zygomycota, which is the same as that in bulk soils. The relative abundance of each phylum in rhizosphere soil differed from that of bulk soil to different degrees. Fungal communities influenced by cotton roots were mainly distributed in Basidiomycota. The dominant orders of Ascomycota and Zygomycota were the same in rhizosphere and bulk soils, but Basidiomycota was different. The dominant orders of Basidiomycota were Agaricales and Auriculariales in FS and Agaricales and Trechisporales in NS, which differed from that of bulk soil. Thus, we speculate that the

soil-derived fungal community composition determines the rhizosphere fungal community of cotton, whereas cotton root affects the soil fungal community composition to a large extent. The β-diversity analysis and contribution analysis of each factor based on Bray–Curtis dissimilarity confirm the conclusion that the soil resource in this study is the main factor that determines the rhizosphere fungal community.

Rhizosphere fungal communities varied in FS and NS

The characteristic of the soil itself is an important factor affecting the community structure of plant rhizosphere microorganisms. Moreover, the microorganism composition of soil is the main cause of variation in the community structure of the rhizosphere microbiome [60, 61]. In this study, significant differences were presented in rhizosphere fungal communities between different sources of soil. The difference was presented in two aspects: 1) The influences of cotton root on different fungal species were different. For example, in NS, the relative abundance of *Engyodontium*, *Mortierella*, and *Penicillium* was lower in pots containing cotton plants, whereas the relative abundance of *Clitopilus*, *Fusarium*, and *Rhodosporidium* was higher in pots containing cotton plants; 2) The influence of cotton root on some fungal communities differed substantially between NS and FS soil. For example, the relative abundance of *Mrakia*, *Rhodosporidium*, and *Talaromyces* in rhizosphere soil compared to bulk soil was higher in NS but lower in FS. This difference might be attributed to the different characteristics of the two soil resources. Thus, we conclude that the cotton rhizosphere fungal community structure variation was mainly determined by the interaction of cotton root with different sources of soil.

Rhizosphere microbial diversity can improve a plant's resistance to soil-borne disease [17]. Previous studies have shown that continuous cropping can decrease the structural and functional diversity of the soil microbiome [62, 63]. In the present study, pots that did not contain plants had lower fungal α-diversity in FS than in NS, corroborating that long-term continuous cropping of cotton decreases fungal α-diversity, which in turn may be one of the important factors inducing continuous cotton-cropping obstacles. However, after planting with cotton, the fungal α-diversity of rhizosphere soils from FS was increased compared with bulk soil and higher than that of NS. We speculate that fungal communities in continuously cotton-cropped field soils might contain an abundance of fungi that are closely linked to cotton growth, nutrient absorption, and stress

348 tolerance, and the functional limitation of such fungal communities is the main reason for
349 continuous cotton-cropping obstacles.

350 *Developmental stages contributed to the variation of the fungal community in the cotton*
351 *rhizosphere*

352 Baudoin *et al.* proposed that the quantity and quality of root exudate input into the rhizosphere
353 differ at different plant developmental stages, leading to differences in the composition of
354 rhizosphere microbial communities between plant developmental stages [64]. Other studies have
355 also demonstrated that rhizosphere microbes are significantly affected by the developmental
356 stages of plants [65-69]. Our results indicate that the community composition of cotton
357 rhizosphere fungi varied significantly during different developmental stages. The species richness
358 of rhizosphere fungal communities was highest in the seedling stage in FS and in the budding
359 stage in NS. In addition to the common dominant fungal genera of all three developmental stages,
360 the rhizosphere fungal communities had a stage-specific dominant genus. The number of
361 dominant genera and EFGs were the highest in the budding stage, which may be related to the
362 plant requiring specific materials or releasing certain hormones into the soil during this stage.

363 *Alterations of potential pathogenic and phosphate-solubilizing fungal genera in the rhizosphere of*
364 *cotton*

365 Incidence rates of soil-borne disease are affected by many factors, such as the soil environment
366 [70, 71], soil fungal community structure and function [17, 72, 73], relative abundance of
367 pathogenic fungi, resistance of cotton cultivars, and developmental stage of cotton. Our results
368 show that the relative abundance of disease-associated fungal genera in the bulk soil of FS and NS
369 differed significantly. The relative abundance of potential pathogenic fungal genera (besides
370 *Fusarium*) was lower in bulk soil of FS compared with that of NS. However, the relative
371 abundance of these potentially pathogenic fungal genera in the rhizosphere was higher in FS and
372 lower in NS compared with the corresponding bulk soil treatments.

373 The effect of cotton root on potentially pathogenic soil fungal genera also differed in
374 different plant developmental stages. In FS, the relative abundance of *Alternaria* and *Rhizoctonia*
375 at the seedling stage and *Fusarium*, *Gibberella*, *Thanatephorus*, and *Verticillium* at the budding
376 stage in the cotton rhizosphere had the highest enrichment compared with bulk soil. In NS, the

377 potentially pathogenic fungal genera were suppressed in rhizosphere soil, with the exception of the
378 seedling stage for *Alternaria* and *Fusarium*, the budding stage for *Fusarium* and *Rhizoctonia* and
379 the flowering stage for *Gibberella*. We speculate that potentially pathogenic fungal genera
380 enriched in a developmental stage have a high infection rate of cotton root and thus cause a high
381 incidence of soil-borne disease. The incidence rate was higher in FS than in NS and highest in the
382 budding stage.

383 Diseases associated with fungal genera also differed by cotton genotype. Upland cotton
384 (TM-1) was more susceptible to disease than island cotton (Hai7124), but this difference was not
385 significant.

386 Fungi play an important role in the absorption and transformation of nutrients, especially
387 phosphate-solubilizing fungi [14-16, 74]. Fungal species of *Aspergillus* and *Penicillium,* such as
388 *Aspergillus tubingensis*, *Aspergillus niger* [75], *Aspergillus awamori, Penicillium citrinum* [15],
389 *Penicillium albidum* [76], and *Penicillium oxalicum* [77], play an important role in phosphate
390 solubility. We analyzed the dynamics of the two potential phosphate-solubilizing fungal genera. In
391 cotton rhizosphere soils, the relative abundance of the two genera was higher in FS than in NS.
392 This may be attributed to differences in physical and chemical properties and utilization of
393 nutrient substances.

394 Our study provides insights into the structural variation of rhizosphere fungal communities
395 under the influence of soil resources, developmental stage, and genotype, which might play key
396 roles in cotton growth and health. The soil resources, cotton developmental stage, and cotton
397 genotype all impacted cotton rhizosphere fungal community composition. The composition of the
398 cotton rhizosphere fungal community was primarily determined by soil resources and regulated to
399 a certain degree by plant developmental stage. A limited effect was found for the cotton genotype.

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595

596 **Supporting information:**

597 **Supplementary Fig S1. Relative abundance of fungal phyla in bulk soil of both soils.**

598 **Supplementary Fig S2. Relative abundance of fungal phyla in the rhizosphere of cotton**
599 **planted in field soil that has been continuously cotton-cropped.**

600 **Supplementary Fig S3. Relative abundance of fungal phyla in the rhizosphere of cotton**
601 **planted in nutrient-rich soil.**

602 **Supplementary Fig S4. Total number of OTUs of specific and common fungi in different**
603 **treatments.**

604 **Supplementary materials and methods S1**

605 **Supplementary Table S1. Statistics and analyses of sequencing data.**

606 **Supplementary Table S2. Relative abundance of fungi in bulk soil.**

607 **Supplementary Table S3. Relative abundance of fungi in rhizosphere soil.**

608 **Supplementary Table S4. Relative abundance increases multiples in rhizosphere fungal**
609 **phyla compared with bulk soils.**

610 **Supplementary Table S5. OTU numbers and α-diversity of each sample.**

611 **Supplementary Table S6. Fungal genera that were increased or decreased in relative**
612 **abundance in the rhizosphere compared with bulk soil in field soil.**

613 **Supplementary Table S7. Fungal genera that were increased or decreased in relative**
614 **abundance in the rhizosphere compared with bulk soil in nutrient-rich soil.**

615 **Supplementary Table S8. Relative abundance of fungal genera that were affected by the**
616 **presence of cotton root in nutrient-rich soil.**

617 **Supplementary Table S9. Relative abundance of genera that were affected by the presence of**
618 **cotton root in field soil.**

619 **Supplementary Table S10. Analysis of fungal genera found during different plant**
620 **developmental stages.**

621 **Supplementary Table S11. Beta-diversity between samples.**

622 **Supplementary Table S12. Analysis of potential pathogenic and phosphate-solubilizing**
623 **fungal genera.**

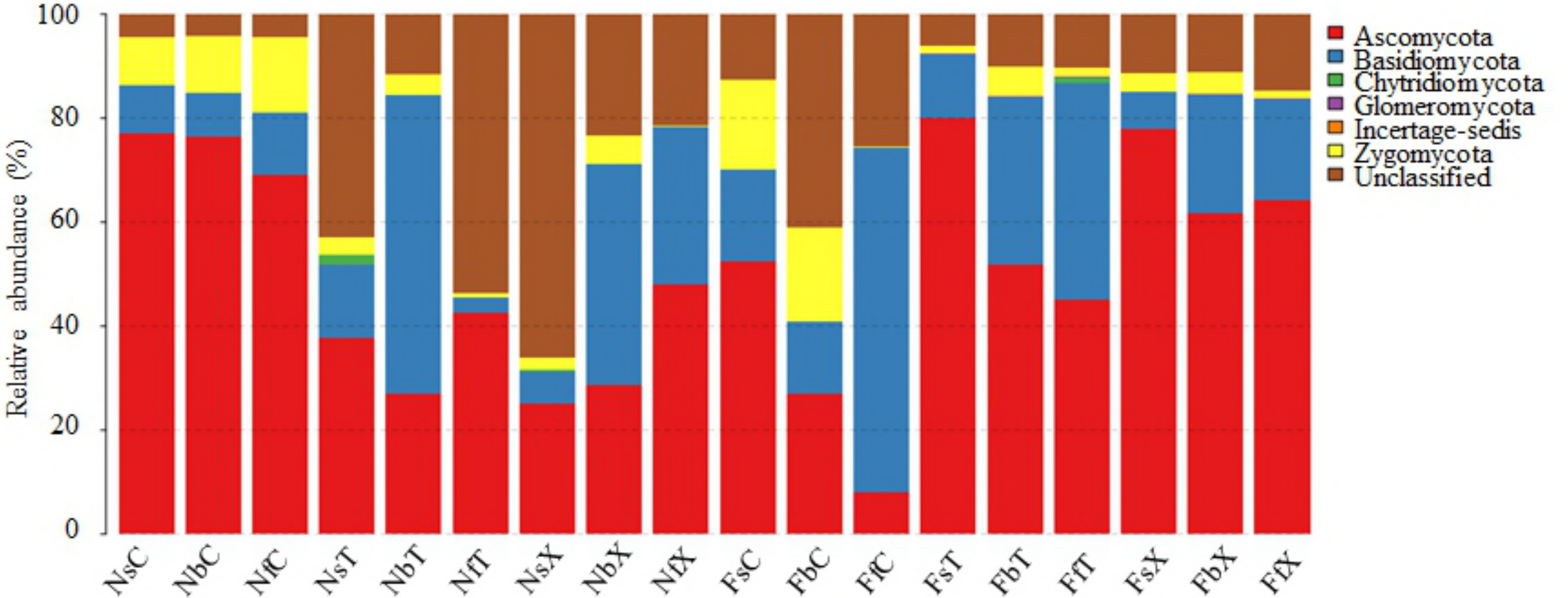
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Figure

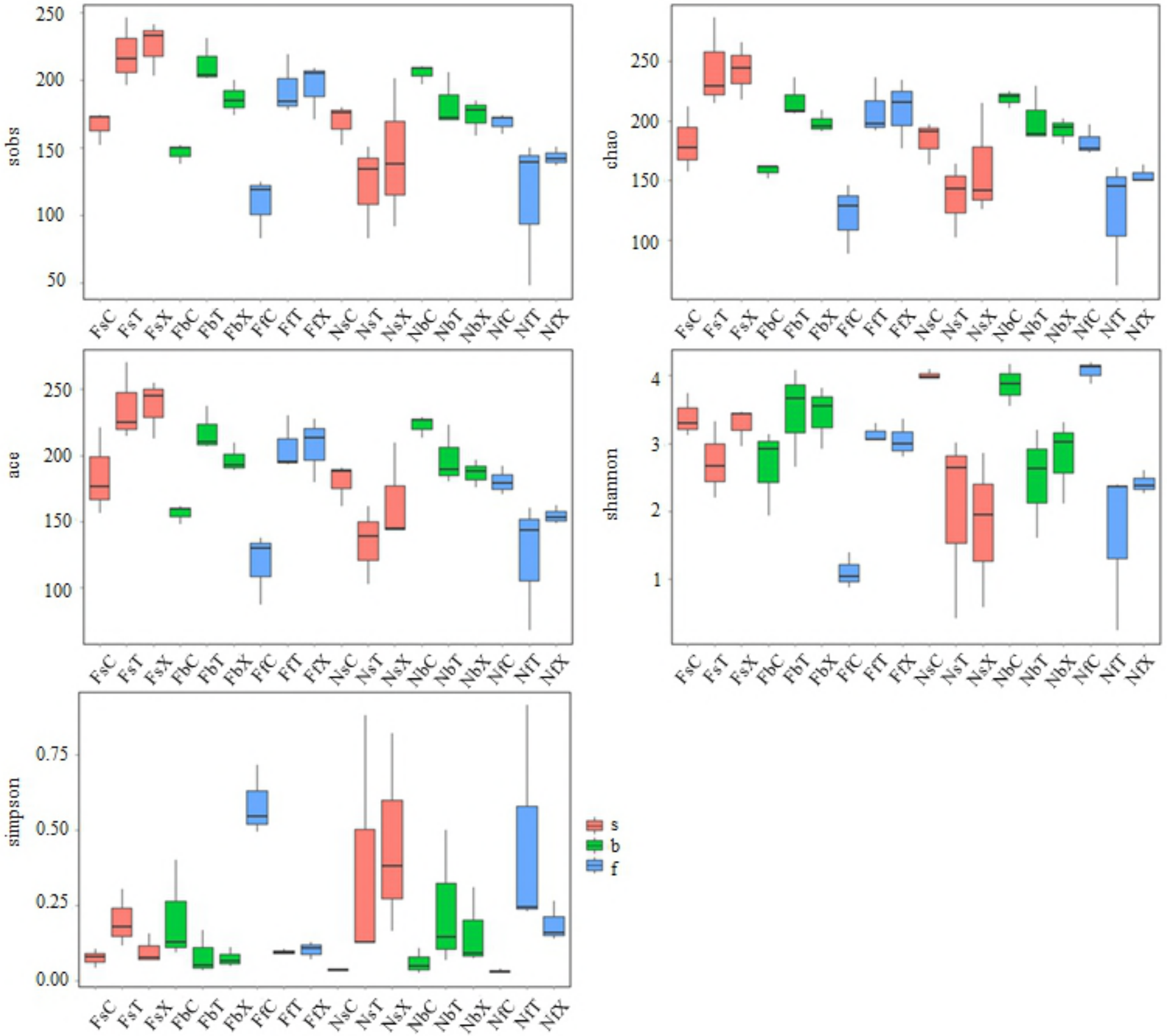
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Figure

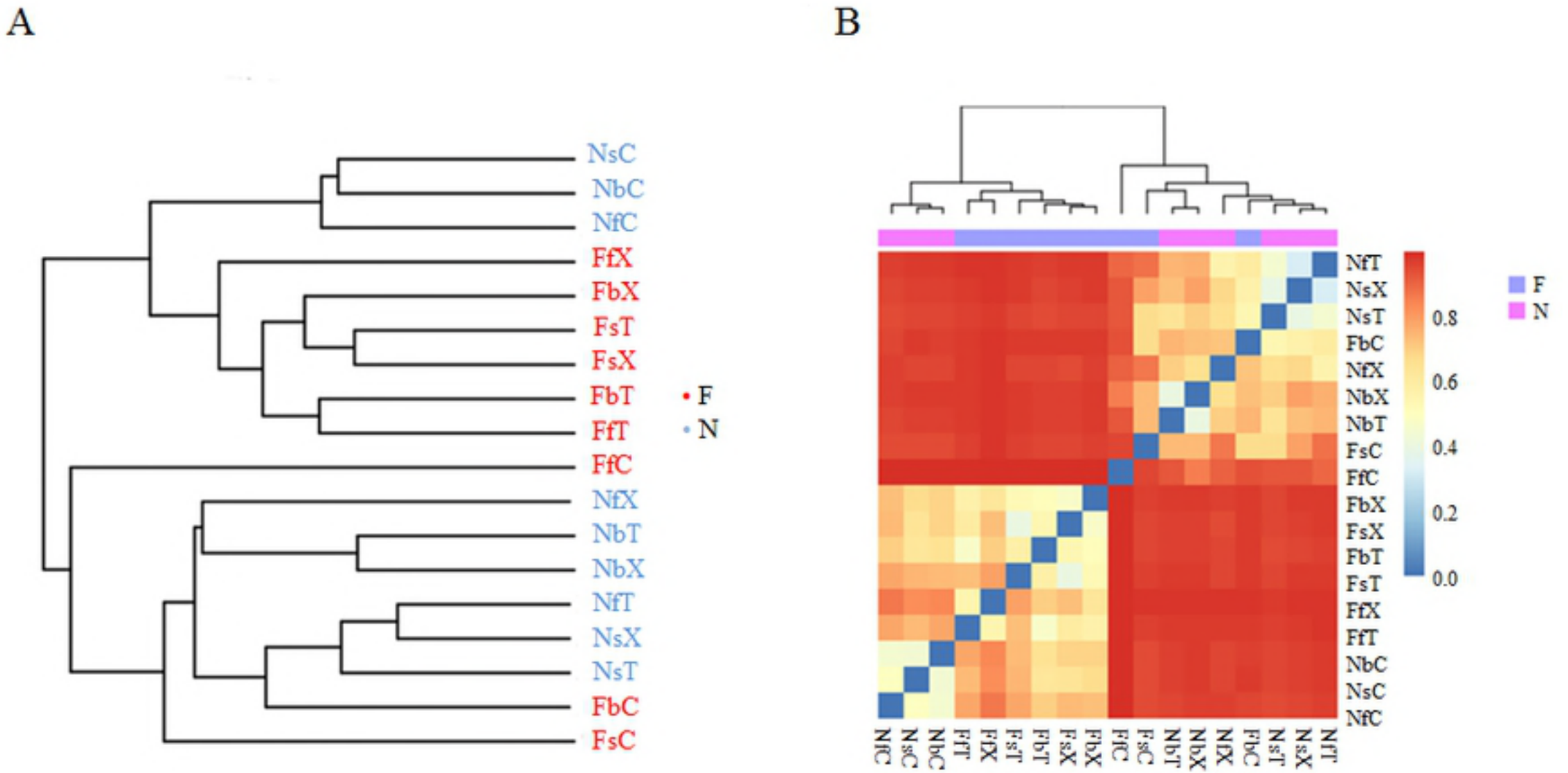
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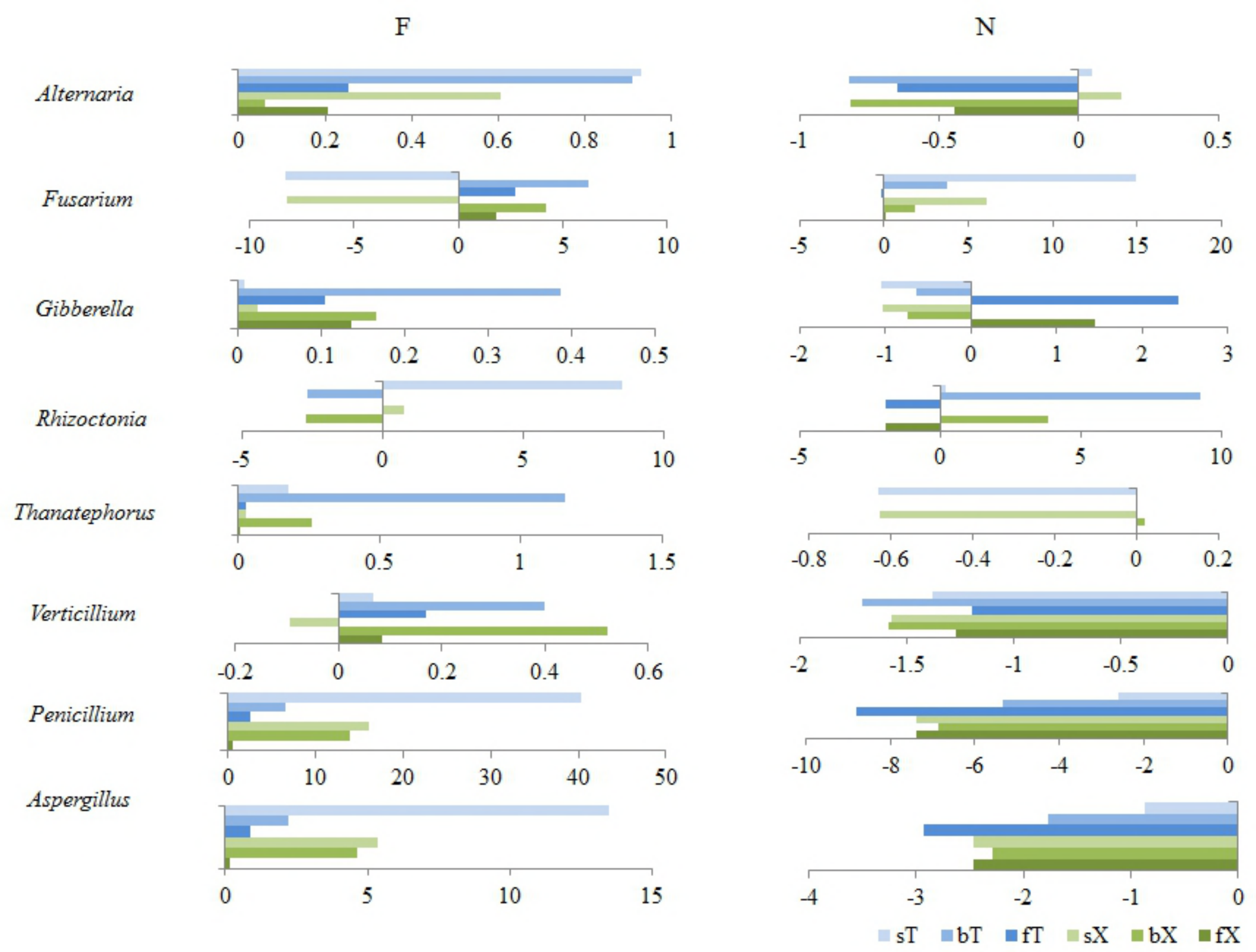
Figure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Figure