1 **Running title:** Biocontrol bacteria alleviate replanting problem

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3 **Inoculation of biocontrol bacteria alleviated *Panax ginseng* replanting problem**

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12 **Highlight:** Changes in rhizospheric microbial communities driven by ginseng plants

13 of different ages and developmental stages could cause microecological degradation.

14 Biocontrol using microbial antagonists effectively alleviated the replanting problem.

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27 **Linlin Dong and Jiang Xu have equal contributions to this work.**

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30

Abstract

Replanting problem is a common and serious issue hindering the continuous cultivation of *Panax* plants. Changes in soil microbial community driven by plant species of different ages and developmental stages are speculated to cause this problem. Inoculation of microbial antagonists is proposed to alleviate replanting issues efficiently.

High-throughput sequencing revealed that bacterial diversity evidently decreased, and fungal diversity markedly increased in soils of adult ginseng plants in the root growth stage. Relatively few beneficial microbe agents, such as *Luteolibacter*, Cytophagaceae, *Luteibacter*, *Sphingomonas*, Sphingomonadaceae, and Zygomycota, were observed. On the contrary, the relative abundance of harmful microorganism agents, namely, Brevundimonas, Enterobacteriaceae, *Pandoraea*, Cantharellales, *Dendryphion*, *Fusarium*, and Chytridiomycota, increased with pant age. Furthermore, *Bacillus subtilis* 50-1 was isolated and served as microbial antagonists against pathogenic *Fusarium oxysporum* of ginseng root-rot, and its biocontrol efficacy was 67.8% using a dual culture assay. The ginseng death rate and relative abundance of *Fusarium* decreased by 63.3% and 46.1%, respectively, after inoculation with 50-1 in replanting soils. Data revealed that changes in the diversity and composition of rhizospheric microbial communities driven by ginseng of different ages and developmental stages could cause microecological degradation. Biocontrol using microbial antagonists was an effective method for alleviating the replanting problem.

Keywords

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Introduction

Panax ginseng C.A. Meyer demonstrates anti-inflammatory and antitumor effects, and it is commonly used in traditional Chinese medicine (Choo *et al*., 2008; Ernst, 2010). The current annual global market value of this species is approximately 3.5 billion dollars (Hong *et al*., 2006). Wild ginseng resources are becoming scarce because of excessive and predatory exploitation, and wild ginseng is gradually substituted with cultivated ginseng in mainstream market (Li *et al*., 2012; Wu *et al*., 2013). Ginseng is continuously cultivated in fixed plots for 4–5 years, and subsequent replanting usually fails because of continuous cropping obstacles (Ying *et al*., 2012a). Several years of crop rotation is needed in successful replanting. Large-scale deforestation increases to satisfy the market demand for ginseng. Moreover, conflict between the operation of ginseng industry and environmental conservation has worsened. Replanting issue is a severe drawback hindering the development of ginseng industry. Hence, this problem must be addressed urgently.

Factors such as deterioration of soil physicochemical properties, allelopathy/autotoxicity, outburst of soil-borne disease, and soil microbial community changes cause replanting problem (Ogweno & Yu, 2006; Wu *et al*., 2008; Huang *et al*., 2013). Changes in soil microbial community is one of the main factors hindering crop replantation (Nayyar *et al*., 2009; Manici *et al*., 2013). Xiao *et al*. (2016) reported that the lack of balance in rhizospheric microbial communities is one of the key factors causing discontinuous ginseng cultivation. Microbial communities exhibit evident changes during ginseng cultivation, thereby causing an imbalance in microbial community (Ying *et al*., 2012b). Increase in the relative abundance of pathogenic microorganism could lead to the occurrence of soil-borne disease. Taken together, changes in rhizospheric microbial community are suggested to cause the replanting issues.

Many biotic or abiotic factors lead to changes in rhizospheric microbial communities. Plants of different ages can alter microbial community (Wu *et al*., 2015). Differences in diversity and composition of soil microbial community were observed under continuous cropping of *P. notoginseng*; moreover, fungal diversity

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87 and microbial taxa in soil microbial community are related to seedling mortality

88 (Dong *et al*., 2016). Li *et al*. (2012) found that differences between rhizospheric and

89 non-rhizospheric soil microbial community in a particular site tend to become

90 increasingly significant with ginseng growth. *Panax* plants with different ages in a

91 site drive changes in microbial community. Plant developmental stage influences

92 microbial community structure and activity, and marked shifts in diversity and

93 relative activity are observed in soil microbial communities of different

94 developmental stages (Houlden *et al*., 2008). However, the information whether

95 *Panax* plants of different ages and developmental stages mediate microbial

96 community is unclear. We hypothesized that changes in the rhizospheric microbial

97 community driven by ginseng plants of different ages and developmental stages

98 could cause microecological degradation.

99 Root rot is a severe disease that hinders the replantation of *Panax* plants (Guo *et*

100 *al*., 2009). This soil-borne disease is caused by the pathogenic fungus *Fusarium*

101 *oxysporum*, which is the main agent in *Panax* plants (Miao *et al*., 2006; Dong *et al*.,

102 2016). The relative abundance of *F. oxysporum* increased with the notoginseng

103 cultivation and death rate of seedlings was significantly related to its abundance

104 (Dong *et al*., 2016). Thus decrease in the abundance of pathogenic *F. oxysporum*

105 could alleviate the occurrence of root-rot. Biological control using microbial

106 antagonists has attracted interest as an effective method to control plant pathogens

107 due to its non-toxic nature (Zheng *et al*., 2011). Many studies reported that

108 biocontrol bacteria, such as *Pseudomonas fluorescens* and *Bacillus*

109 *amyloliquefaciens* RWL-1, show evident growth inhibition activity against *F.*

110 *oxysporum* in tomato (Kamilova *et al*., 2006; Shahzad *et al*., 2017). Nevertheless,

111 microbial antagonists against ginseng root rot is scarce, and application of biocontrol

112 bacteria could be effective to alleviate the replanting problem.

113 Recently, herbgenomics has been raised for medicinal plants research using

114 genomic tools including investigating rhizospheric environment using metagenomic

115 sequencing (Chen *et al*, 2015). In the present study, high-throughput sequencing

116 analysis of 16S and 18S rRNA genes was used to demonstrate changes in the

4

117 diversity and composition of soil microbial communities in the rhizosphere of

118 ginseng seedlings of different ages and developmental stages. Furthermore,

119 biocontrol bacteria against *F. oxysporum* were isolated using a dual culture technique,

120 and their inhibition activity in a replanting soil was confirmed. These results

121 increased our understanding of the reasons behind replanting issues caused by

122 rhizospheric microbial community. Data provided an effective method for soil

123 bioremediation to alleviate issues in replanting of Chinese herbal medicine.

124

125 **Materials and Methods**

126 **Field experiment and soil extraction**

127 This field experiment was performed in a ginseng plantation in Jingyu, Jilin

128 Province (42°20□N, 126°50□E, 775 m a.s.l.), which is the main ginseng-producing

129 region in China. This region receives a northern temperate continental climate and an

130 annual precipitation of approximately 767 mm. The plough layer in the plantation

131 consists of gray brown soil.

132 Ginseng seedlings are consecutively grown for 4–5 years in a fixed site before

133 their root can be harvested. The disease and mortality of ginseng seedlings generally

134 occur after being consecutively grown for 2 years. Thus, we analyzed the influence

135 of two-, three-, and four-year-old transplanted seedlings on rhizospheric microbial

136 communities. In this experiment, two-, three-, and four-year-old ginseng seedlings

137 were transplanted in each plot in our plantation and denoted as 2-y, 3-y, and 4-y,

138 respectively. In our plantation, field plots were arranged in a completely randomized

139 block design, and three replicate plots (1.7 m × 8.0 m) were used for each plant age.

140 Ginseng was cultivated strictly in accordance with the standard operating procedures

141 of good agricultural practice (Heuberger *et al*., 2010; Zhang *et al*., 2010).

142 The distinct stages of ginseng development are as follows: vegetative,

143 flowering, fruiting, root growth, and annual dormancy (Table S1). During dormancy,

144 the aboveground plant parts of ginseng wither, and the root becomes dormant. In this

145 stage, the underground root activities weaken, and soil samples obtained during this

146 stage are not included in analyses. This experiment included 36 soil samples

147 obtained from two-, three-, and four-year-old ginseng seedlings under four

148 developmental stages, namely, vegetative (2-Ve, 3-Ve, and 4-Ve), flowering (2-Fl,

149 3-Fl, and 4-Fl), fruiting (2-Fr, 3-Fr, and 4-Fr), and root growth (2-Ro, 3-Ro, and

150 4-Ro). Six ginseng seedlings were randomly obtained from each plot (1.7 m $\times$ 8.0 m);

151 the soils were removed, and rhizosphere fractions were brushed and pooled into one

152 sample. Soil samples were obtained from three replicates per treatment. Thirty-six

153 soil samples were homogenized by passing through a 2 mm sieve for further

154 processing. The characteristics of the soil samples are described in Table S2.

155

Calculation of emergence and survival rates

157 The ginseng emergence rate was analyzed in May. The emergence rate in each plot

158 was calculated as follows: number of emerging ginseng seedlings divided by the

159 total number of transplanted seedlings. The mortality of ginseng seedlings was

160 observed from June to August, and the survival rate was determined at the end of

161 August. The survival rate of ginseng in each plot was calculated as follows: number

162 of living seedlings divided by the total number of emerging seedlings in the area.

163 Three plots per treatment served as three replicates.

164

DNA extraction and PCR amplification

166 Total soil DNA was extracted from 0.1 g of freeze-dried soil using a MoBio

167 Powersoil Kit (MoBio Laboratories Inc., Carlsbad, CA) according to the

168 manufacturer's instructions; the DNA was stored at –20 °C for further processing.

169 For each sample, fragments of 16S and 18S rRNA genes were amplified using the

170 conserved primers 27F/338R (Fierer *et al*., 2008) and 817F/1196R (Rousk *et al*.,

171 2010). The forward and reverse primers contained an eight-base pair barcode (Table

172 S3). Amplification reactions and purification were performed as previously

173 described (Rodrigues *et al*., 2013). Purified PCR products were quantified by

174 Qubit®3.0 (Life Invitrogen, Germany), and these amplicons were pooled in

175 equimolar ratios for sequencing.

176

High-throughput sequencing

177

178 The pooled DNA product was used to construct Illumina pair-end library according

179 to the Illumina's genomic DNA library preparation procedure. The amplicon library

180 was subsequently paired-end sequenced (2×250) on an Illumina HiSeq platform

181 (Shanghai Biozeron Co., Ltd.) according to standard protocols.

182 Raw FASTQ files were demultiplexed and quality-filtered using QIIME with

183 the following pipeline (Caporaso *et al*., 2010). Operational taxonomic units were

184 clustered using 97% similarity cutoff by using UPARSE (version 7.1

185 http://drive5.com/uparse/), and chimeric sequences were identified and removed

186 using UCHIME. The phylogenetic affiliation of each 16S and 18S rRNA gene

187 sequence was analyzed using Ribosomal Database Project (Wang *et al*., 2007) and

188 Silva schemes (Quast *et al*., 2013). Rarefaction analysis based on Mothur v.1.21.1

189 (Schloss *et al*., 2009) was performed to reveal diversity indexes, including Chao 1

190 and Shannon diversity ($H\square$) indexes. We clustered the taxa obtained from the RDP

191 Classifier through complete linkage hierarchical clustering by using *R* package

192 HCLUST (http://sekhon.berkeley.edu/stats/html/hclust.html). PCoA was used to

193 compare groups of samples based on unweighted UniFrac distance metrics in QIIME

194 (Caporaso *et al*., 2010). Linear discriminant analysis effect size (LEfSe)

195 (http://huttenhower.sph.harvard.edu/lefse/) was used to characterize the features

196 differentiating the bacterial and fungal communities in soils according to the

197 methods described by Segata *et al*. (2011). All metagenomic data were submitted to

198 the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov);

199 the accession numbers of 16S rRNA and 18S rRNA genes are SRP103168 and

200 SRP103176, respectively.

201

Quantitative PCR of *Fusarium*

202

203 To compare the relative abundance of *Fusarium* in soils of ginseng seedlings, the

204 copy numbers of *Fusarium* were calculated using the ITS-Fu-F/ITS-Fu-R

205 (Abd-Elsalam *et al*., 2003). Quantitative PCR was performed according to the

206 description of Rousk *et al*. (2010) with minor modification. Standard curves were

207   generated using 10-fold serial dilution of a plasmid containing a full-length copy of

208   *F. oxysporum* 18S rRNA gene to estimate the copy numbers of *Fusarium*. qPCR

209   reactions (25 µL) were performed using a SYBR Green PCR Master mix (Takara,

210   Toyobo, Japan). *Fusarium* copy numbers were generated using a regression equation

211   for each assay relating the cycle threshold value to the known number of copies in

212   the standards.

213

Isolation and selection of antagonistic bacteria

215   Biological control is one of the most remarkable potential approaches for plant

216   disease control. Due to the increase in the abundance of pathogenic agents of

217   ginseng root rot,

218   a dual culture assay was used to screen microbial antagonists against *F. oxysporum*.

219   Rhizosphere soils of three-year ginseng seedlings under root growth stage were used

220   to isolate and select the antagonistic bacteria against *F. oxysporum*. The pathogenic *F.*

221   *oxysporum* was isolated and confirmed in our previous study (Wang *et al*., 2016).

222   Soil (10 g) was homogenized in 100 mL of sterile distilled water, and the bacteria

223   were isolated through serial dilution technique. The isolated single strain was

224   screened on the basis of its antagonistic activity against *F. oxysporum* in a dual

225   culture according to the description of Shahzad *et al*. (2017). The experiment was

226   replicated three times. The zone of inhibition was measured following the

227   description of Kaiser *et al*. (2005) to screen the antagonistic bacterium and examine

228   the antagonistic activity of the candidates.

229

Identification of antagonistic bacterial strain

231   Antagonistic bacterium (named strain 50-1) was identified by morphological and

232   molecular methods. The morphology of strain 50-1 was recorded after it was

233   incubated on Luria–Bertani (yeast extract (5 g), peptone (10 g), NaCl (10 g), and

234   agar (10 g) in 1 L of water) medium for 24 h later. Strain 50-1 was molecularly

235   identified by amplifying 16S rRNA according to the description by Cai *et al*. (2012).

236   The amplified PCR product was analyzed on a 3730 XL sequencer (Applied

237   Biosystems, Foster City, CA, USA), and the generated sequence (accession number

238   KY962803) was submitted to GenBank. Neighbor-joining trees were constructed in

239   MEGA v6.0 to generate Kimura 2-parameter distance matrixes for each sequence

240   following standard parameters. The numbers at the branch knots were bootstrap

241   values based on 1000 resamplings for the maximum likelihood.

242   To further identify the strain 50-1, total genomic DNA from strain 50-1 was

243   extracted (Tiangen, Beijing, China) and purified by RNase-free DNase I (Takara,

244   Kyoto, Japan) to analyze its genome. The complete genome sequence was assembled

245   using a hybrid sequencing strategy combining PacBio RS II and Illumina HiSeq

246   sequencing platforms (Koren *et al*., 2012). Genome sequencing was performed with

247   Illumina HiSeq2500 using the PE250 strategy following the manufacturer's protocol.

248   The reads obtained with Illumina PCR adapter and the filtered low-quality reads

249   were assembled by SOAPdenovo (Li *et al*., 2008; Li *et al*., 2010) to generate

250   scaffolds. Gene prediction genome assembly was performed using Glimmer and

251   gene functions were annotated by BLASTP against NR, COG and KEGG databases.

252   GeneMarkS (Besemer *et al*., 2001) with an integrated model combining the

253   GeneMarkS-generated parameters and Heuristic model parameters. A genome

254   overview with annotation information was created using Circos (Krzywinski *et al*.,

255   2009). The whole genome was deposited in NCBI (BioProject ID PRJNA383782).

256

257   **Evaluation of the biocontrol efficacy of antagonists in replanting soils**

258   A pot experiment was performed to assess the biocontrol efficacy of bacterium 50-1

259   in replanting soils in our phytotron. Each pot contained 1 kg of soils cultivated with

260   ginseng seedlings for three years and happened root rot. Two-year-old ginseng

261   seedlings (three plants) were transplanted in each pot. The pots were placed in the

262   phytotron under the following conditions: 26±2 °C, 60% humidity, and 14 h of light

263   alternating with 10 h of darkness. The rhizospheres of uniform ginseng seedlings

264   were inoculated with 1 mL (10^6 cfu mL^{-1}) of cultures containing biocontrol strains

265   when the seedlings were cultivated for one week. Pots were inoculated with cultures

266   containing inactivated strains as controls. Five pots served as one replicate, and three

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267   replicates were prepared. After a two months following inoculation, the death rate of

268   ginseng seedlings was calculated as follows: number of dead seedlings divided by

269   the total number of transplanted seedlings in each treatment. The rhizosphere soils of

270   three seedlings were randomly selected from each treatment, and they served as one

271   sample for the analysis of the relative abundance of *Fusarium.* The experiment was

272   replicated three times.

273

274   **Statistical analyses**

275   SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical

276   analyses. Variables were considered for all treatment replicates and subjected to

277   ANOVA. Mean values were compared by calculating the least significant di erence

278   at 5% level.

279

280   **Results**

281   **Emergence and survival rates of ginseng seedlings**

282   The ginseng plants grown on field showed a healthy appearance with no sign of

283   disease during the growing season (Figs. 1A–1C). The emergence and survival rates

284   exhibited no significant difference among two-, three-, or four-year-old ginseng

285   seedlings (Figs. 1D, 1E). The emergence rate of two-, three-, or four-year-old

286   ginseng seedlings ranged from 82.3% to 86.9% (Fig. 1D), and their survival rate was

287   94.1%–97.1% (Fig. 1E). The growth of ginseng seedlings of different ages was

288   favorable, which guaranteed the success of the field experiment.

289

290   **Taxonomic diversity of bacterial and fungal communities**

291   A total of 2,296,684 classifiable 16S rRNA sequence reads were obtained from 36

292   soil samples for analysis (Table S3). The mean number of classifiable sequences per

293   sample was 63,796 (dominant length: 283–293 bp). The bacterial diversity indexes

294   (*H'* and Chao 1) markedly changed in the rhizosphere of ginseng seedlings of

295   different ages in late developmental stages (Fig. 2). *H'* and Chao 1 values were

296   significantly higher in the rhizosphere of 4-y seedlings than those in the rhizosphere

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297   of 2-y seedlings in fruiting stage (Figs. 2A, 2B). During root growth, *H'* and Chao 1

298   values evidently decreased in the rhizospheres of 3-y and 4-y seedlings than those in

299   the rhizosphere of 2-y seedlings.

300   A total of 1,321,521 classifiable fungi sequences with a mean of 36,709

301   sequences were obtained per soil sample (dominant length: 411–414 bp; Table S3).

302   *H'* and Chao 1 values markedly decreased in the rhizosphere with increasing ginseng

303   age under vegetative stage (Figs. 2C, 2D). During root growth, *H'* and Chao 1 values

304   were evidently higher in the soils of 3-y and 4-y seedlings than those in the soil of

305   2-y seedlings.

306

307   **Variation in bacterial and fungal community compositions**

308   PCoA ordination and Bray–Curtis distance matrix revealed evident difference in

309   bacterial communities in soils of ginseng plant of different ages and developmental

310   stages (Figs. 3, S1). In vegetative and flowering stages, the second principal

311   components (14.41% and 14.22% contributions, respectively) demonstrated that the

312   bacterial communities in soils of 2-y seedlings differed from those in soils of 4-y

313   seedlings (Figs. 3A, 3B). In fruiting stage, the first principal component axis (22.00%

314   contributions) indicated that the bacterial communities in soils of 2-y seedlings

315   significantly differed from those in soils of 3-y seedlings (Fig. 3C). Additionally, the

316   first principal component axis (22.29% contributions) indicated that the bacterial

317   communities in soils of 3-year-old seedlings significantly differed from those in soils

318   of 2-y and 4-y seedlings; furthermore, the second principal component (13.98%

319   contributions) demonstrated that the bacterial communities in soils of 2-y seedlings

320   differ from those in soils of 4-y seedlings (Fig. 3D). LEfSe revealed differences in

321   the rhizospheric bacterial communities of ginseng seedlings of different ages and

322   developmental stages (Fig. S2).

323   PCoA ordination and Bray–Curtis distance matrix revealed that fungal

324   communities obviously differed in the soils of ginseng seedlings of different ages

325   and developmental stages (Figs. 3, S1). In vegetative stage, the first principal

326   component axis (33.94% contribution) of the fungal communities in soils of 4-y

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327   seedlings markedly differed from those of the fungal communities in soils of 2-y

328   seedlings. Moreover, the second principal component axis (17.55% contribution) of

329   the fungal communities in soils of 2-year-old seedlings considerably differed from

330   those of the fungal communities in the soils of 3-y seedlings (Fig.3E). At the

331   flowering and fruiting stages, the first principal components (25.71% and 38.96%

332   contributions, respectively) in the fungal communities in soils of 2-y ginseng

333   seedlings significantly differed from those of the fungal communities in the soils of

334   3-y and 4-y seedlings (Figs. 3F, 3G). During root growth, the first principal

335   component (26.97% contribution) of fungal communities in soils of 3-y seedlings

336   considerably differed from those of fungal communities in soils of 2-y and 4-y

337   seedlings, and the second principal component (14.93% contribution) in fungal

338   communities in soils of 2-y seedlings differed from those of fungal communities in

339   soils of 4-y seedlings (Fig. 3H). According to LEfSe analysis, fungal composition

340   differed in the soils of ginseng seedlings of different ages and developmental stages

341   (Fig. S3).

342

343   **Changes in the relative abundance of bacterial taxa**

344   The relative abundance of bacterial groups changed in soils of ginseng plants of

345   different ages and developmental stages (Fig. 4). The relative abundance of

346   Chthoniobacteraceae, Chthonomonadales, Chthonomonadetes, *Chthoniobacter*,

347   *Granulicella*, and *Blastocatella* declined with plant age in vegetative stage (Fig. 4A).

348   The relative abundance of Arthrobacter, Brevundimonas, Micrococcaceae,

349   Rhodobiaceae, Intrasporangiaceae, and Micrococcales was significantly higher in

350   soils of 3-y and 4-y seedlings than that in soils of 2-y seedlings. The relative

351   abundance of *Luteolibacter*, Phyllobacteriaceae, *Acidovorax*, *Moheibacter*, and

352   Cytophagaceae in soils of 3-y and 4-y seedlings evidently decreased, and the

353   abundance of Elusimicrobia and Armatimonadetes was markedly increased in the

354   flowering stage (Fig. 4B). The relative abundance of *Bacillus*, Enterobacteriales,

355   Enterobacteriaceae, *Brevundimonas*, and Anaerolineae was significantly higher in

356   soils of 3-y and 4-y seedlings than that in soils of 2-y seedlings; additionally, the

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357   abundance of *Luteibacter*, Clostridia, and Clostridiales declined in soils of 3-y and

358   4-y seedlings in fruiting stage (Fig. 4C). The relative abundance of *Paralcaligenes*,

359   Sphingomonadaceae, Saccharibacteria, *Sphingomonas*, and Alcaligenaceae

360   significantly decreased with plant age, whereas the relative abundance of *Pandoraea*,

361   Chlamydiales, and Chlamydiae was obviously higher in soils of 3-y and 4-y

362   seedlings than that in soils of 2-y seedlings in root growth stage (Fig. 4D).

363

364   **Changes in the relative abundance of fungal taxa**

365   The relative abundance of fungal taxa changed in soils of ginseng plants of different

366   ages and developmental stages (Fig. 5). The relative abundance of

367   Cystofilobasidiales, Ophiostomataceae, *Ophiostoma*, Ophiostomatales, and

368   Cystofilobasidiaceae significantly declined with seedling age, and the abundance of

369   Pezizales, Cantharellales, *Dendryphion*, Pezizomycetes, and Tubeufiaceae was

370   evidently higher in soils of 4-y seedlings than those in soils of 2-y and 3-y seedlings

371   in the vegetative stage (Fig. 5A). The relative abundance of Microascales, *Helicoma*,

372   and Tubeufiaceae was obviously higher in soils of 4-y seedling than those in soils of

373   2-y and 3-y seedlings in the flowering stage (Fig. 5B). The relative abundance of

374   Tremellales, Acrospermales, *Occultifur*, *Acrospermum*, Cystobasidiales,

375   Cystobasidiaceae, and Cystobasidiomycetes was markedly higher in the soils of 4-y

376   seedlings than that in the soils of 2-y and 3-y seedlings in fruiting stage (Fig. 5C).

377   The relative abundance of Zygomycota was obviously lower in soils of 3-y and 4-y

378   seedlings than that in soils of 2-y seedlings, and the abundance of Tremellomycetes,

379   Chytridiomycota, and Sordariales significantly increased in soils of 4-y seedlings

380   than those in soils of 2-y and 3- y seedlings in root growth stage (Fig. 5D).

381   The relative abundance of *Fusarium* showed increasing trends in the soils of the

382   adult ginseng plants under later developmental stages (Figs. 5E, S4). According to

383   the high-throughput sequencing analysis, the relative abundance of *Fusarium*

384   increased by 22.5%–25.0%, 35.7%–50.0%, and 18.2%–36.4% in soils of 3-y and 4-y

385   seedlings in the flowering, fruiting, and root growth stages, respectively (Fig. 5E).

386   The copy numbers of *Fusarium* also showed similar trends based on the analysis of

13

387 quantitative PCR (Fig. S4). These results revealed that the relative abundance of

388 pathogenic agent increased in soils during ginseng growth.

389

390 ***Bacillus subtilis* 50-1 were responsible for the biocontrol of *F. oxysporum***

391 Due to the relative abundance of pathogenic agent (*Fusarium*) increase in the

392 ginseng soils, dual culture techniques were used to isolate microbial antagonists

393 against *F. oxysporum* to control root rot. Antagonistic bacterium, namely, *B. subtilis*

394 50-1, was isolated and confirmed using a dual culture technique (Fig. 6). Strain 50-1

395 exhibited a broad spectrum of growth inhibition activity against *F. oxysporum*,

396 thereby resulting in 67.8% inhibition percentage (Fig.6A). Strain 50-1 is a

397 Gram-positive, oxidase- and catalase-positive, rod-shaped bacterial species (Fig.6B,

398 Table S4). Analysis of the 16S rRNA sequences revealed that strain 50-1 belonged to

399 *B. subtilis* with the bootstrap value of 100% (Fig. 6C). Strain 50-1 was further

400 confirmed according to the genome sequencing. The complete genome was

401 composed of a circular chromosome of 4,040,837 bp with 43.86% GC content (Fig.

402 6D, Table S5). The total numbers of genes were 4,193, which covered 88.6% of the

403 genome encoding 3,176 proteins. The function of these annotated genes was mainly

404 energy production and conversion, amino acid transport and metabolism,

405 transcription, carbohydrate transport and metabolism, inorganic ion transport and

406 metabolism, and signal transduction mechanisms based on the COG function

407 classification (Fig. S5).

408

409 **Inoculation of biocontrol bacteria reduced ginseng death rate in a replanting**

410 **soil**

411 The pot experiment analysis revealed that ginseng death rate and the relative

412 abundance of *Fusarium* significantly decreased by 63.3% and 46.1% in the

413 replanting soils inoculated with strain 50-1, respectively (Fig. 7). Furthermore, the

414 height and leaf area increased by 62.7% and 22.5%, respectively, after inoculation

415 with strain 50-1 (Fig. S6). These results revealed that inoculation of biocontrol

416 bacteria alleviated the ginseng replanting problem.

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417

Discussion

419

420 Replanting problem is a common and serious issue in cultivation of medicinal plants.

421 Following replantation, *Rehmannia glutinosa* suffers from disease, and its biomass

422 and tuber quality decline (Qi *et al*., 2009; Wu *et al*., 2011; Yang *et al*., 2011). The

423 survival rate of ginseng seedlings is lower than 25% after replantation for three years

424 (Zhao *et al*., 2001). Multiple factors have caused this replanting problem, and

425 researchers suggested that changes in microbial communities influence soil health

426 and crop yield (Bisseling *et al*., 2009; Bulgarelli *et al*., 2013). In addition,

427 rhizospheric microbial community is governed by plant species and growth (Incroğlu

428 *et al*., 2011). Thus, we hypothesized that changes in the rhizospheric microbial

429 community were driven by ginseng seedlings of different ages and development

430 stages, which caused the replanting problem of *Panax* plants. Reducing the

431 occurrence of the disease is a useful strategy to alleviate the replanting issues.

432 Biological control is one of the most remarkable potential approaches for plant

433 disease control because of its safety and environmental friendliness.

434 Diversity in bacterial and fungal communities changed in the rhizosphere of

435 ginseng seedlings of different ages and developmental stages. *H'* and Chao1 values

436 revealed that bacterial diversity was obviously low, whereas fungal diversity was

437 significantly higher in the rhizosphere of adult ginseng seedlings in the root growth

438 stage. A similar study reported that increase in ginseng cultivation ages reduced

439 bacterial diversity and increased fungal diversity (Xiao *et al*., 2016). Moreover,

440 diversity of microbial community in the rhizosphere of *Pseudostellaria heterophylla*

441 decreases with increased number of cropping years (Zhao *et al*., 2016). Additionally,

442 the developmental stage of crops is an important driver of microbial community

443 structure (Houlden *et al*., 2008). Plant ages and developmental stages alter the

444 microbial diversity during growth. The diversity of soil microorganisms is critical in

445 the maintenance of soil health and quality, and it serves as a sensitive bioindicator

446 (He *et al*., 2008). The death rate of notoginseng and fungal diversity are significantly

15

447   negatively correlated, which suggest that fungal diversity is a potential bioindicator

448   of soil health (Dong *et al*., 2016). A relationship exists between microbial diversity

449   and root disease suppression (Nitta, 1991; Workneh & van Bruggen, 1994). Mazzola

450   (2004) reported that reduced soil microbial diversity is responsible for the

451   development of soil-borne diseases. Reduced bacterial diversity in response to adult

452   plants is possibly the key indicator of ecological variations and functional

453   impairment in a root growth stage.

454   The bacterial and fungal compositions evidently differed in the rhizospheres of

455   ginseng plants of different ages and developmental stages. Variation in bacterial and

456   fungal community composition is observed during continuous cropping of

457   notoginseng (Dong *et al*., 2016). Dynamics of microbial species is occurred in the

458   rhizosphere during ginseng growth (Li *et al*., 2012). Sugiyama *et al*. (2014) reported

459   that soybeans affected the bacterial communities under vegetative stage, with further

460   alterations possibly occurring during later growth stages. The most divergent

461   community structures occur in the young plant stage of tomato, and the flowering

462   and senescence stages show increasingly similar community structure (Inceoğlu *et*

463   *al*., 2011). The composition of microbial community was affected by plant age and

464   development stage. In our study, the agents of beneficial microbes were relatively

465   few, whereas those of harmful microorganisms increased with plant age. The agents

466   of beneficial microbes include the microorganisms that degrade compounds

467   (*Luteolibacter*, Cytophagaceae, *Luteibacter*, and *Sphingomonas*), and resist disease

468   and improve growth (Sphingomonadaceae and Zygomycota). The agents of harmful

469   microorganisms mainly infect plants (*Brevundimonas*, Enterobacteriaceae,

470   *Pandoraea*, Cantharellales, *Dendryphion*, and Chytridiomycota). Another study has

471   showed similar results, i.e., the population of beneficial microbe decreases, whereas

472   that of harmful microorganisms increases with increasing number of cropping years

473   (Zhao *et al*., 2016). Soil microbial community is an important bioindicator of soil

474   function (Zuppinger-Dingley *et al*., 2014). Changes in functional groups revealed

475   that micro-ecological environment was degraded in the rhizosphere with increasing

476   age of ginseng.

477   Rhizospheric microbial community can be influenced by soil characteristics and

478   plant species (Lauber *et al*., 2008; Berg and Smalla, 2009). In our study, the pH and

479   available K and organic matter contents exhibited no significant difference in

480   ginseng plants of different ages and developmental stages (Table S2). Plant species

481   could be one of the most important factors that influence rhizobacterial communities

482   (Micallef *et al*., 2009). Root exudates offer substrates for microbial metabolism and

483   act as intermediates for biogeochemical reactions in rhizosphere (Uren, 2000; Berg

484   and Smalla, 2009). In the early developmental stages of *Arabidopsis*, cumulative

485   secretion levels of sugars and sugar alcohols are high, which enhanced the richness

486   of rhizospheric microbial community (Chaparro *et al*., 2014). By contrast, root

487   exudates contain allelochemical that disturbs the balance in a microbial community

488   (Wu *et al*., 2015). These results imply that root exudates are one of the main drivers

489   of changes in rhizospheric microbial communities during ginseng growth. Moreover,

490   our results showed that the diversity of microbial communities markedly changed in

491   the rhizosphere of ginseng plants with different ages in root growth stage. This

492   phenomenon is possibly a result of different root types and root exudates. The

493   composition of *Arabidopsis* root exudates changes throughout developmental

494   gradient in plants, and tomato root exudates in reproductive stage are more

495   phytotoxic than those in vegetative stage (Yu and Matsui, 1994; Chaparro *et al*.,

496   2013). Ginseng root rapidly grows after cropping for three years before harvest,

497   especially in root growth stage. Ginseng root growth stage is possibly characterized

498   by a specific but different root exudation pattern that drives different bacterial

499   communities.

500   Biological control using microbes is an environmentally friendly approach of

501   controlling disease (Bargabus *et al*., 2003; Tjamos *et al*., 2005). In our study, we

502   isolated *B. subtilis* 50-1 as an effective antagonist against *F. oxysporum*; the results

503   of antagonist inoculation revealed that biocontrol bacterium reduced the ginseng

504   morbidity and alleviated the replanting problem. *Bucillus* strains, as potent biological

505   control agents of plant diseases, have been reported in many studies (Zouari *et al*.,

506   2016; Shahzad *et al*., 2017). The biocontrol efficacy of *B. amyloliquefaciens* 54

17

507 against bacterial fruit blotch is evident in a greenhouse (Jiang *et al*., 2015). *B.*

508 *megaterium* (B5), *B. cereus* sensu lato (B25), and *Bacillus* sp. (B35) display the

509 highest antagonistic activity against *Fusarium verticillioides*, and antagonistic

510 activity reveals that these strains produce glucanases, proteases, chitinases,

511 siderophores, and auxins (Figueroa-López *et al*., 2016). Furthermore, data suggest

512 that microbial strains producing bioactive constituents can help the inoculated plant

513 to reduce the negative effects of pathogenesis and abiotic stresses (Shahzad *et al*.,

514 2017). The microbial antagonists could be used for efficient control of plant

515 pathogens and comprise an environmentally friendly approach. Additionally, genome

516 sequence analysis provides insights into the pathways of functional bacteria and

517 facilitates their exploration. The genome sequence analysis of *Ganoderma lucidum*

518 revealed key genes encoding cytochrome P450s in secondary metabolism (Chen *et*

519 *al*., 2012). In our study, the genome sequence of strain 50-1 helps accelerate the

520 development and application of biological inoculant.

521

522 **Supplementary data**

523 The details were online.

524 **Table S1** Description of developmental stages of ginseng and the sampling times.

525 **Table S2** Soil chemical characteristics in soils of ginseng cropping.

526 **Table S3** Barcodes, numbers of bacterial and fungal sequences and average length in

527 each sample.

528 **Table S4** Physiological and biological characteristics of *Bacillus subtilis* 50-1.

529 **Table S5** Genome features of *Bacillus subtilis* strain 50-1.

530 **Figure S1** Changes in bacterial and fungal community in rhizosphere of ginseng

531 seedlings of different ages and developmental stages.

532 **Figure S2** LEfSe identified the taxa in the soil bacterial communities of plants of

533 different ages in vegetative (A), flowering (B), fruiting (C), and root growth stages

534 (D).

535 **Figure S3** LEfSe identified the taxa in the fungal communities of plants of different

536 ages in vegetative (A), flowering (B), fruiting (C), and root growth stages (D).

537 **Figure S4** Relative abundance of *Fusarium* in the soils based on the analysis of

538 real-time PCR.

539 **Figure S5** COG function classification in the *Bacillus subtilis* strain 50-1.

540 **Figure S6** The height (A) and leaf area (B) of ginseng seedlings after inoculation of

541 strain 50-1 in a replanting soil.

542

543 **Acknowledgements**

546

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547

548   **Figure captions**

549

550   **Figure 1** Growth status of ginseng seedlings. (A–C) Two-, three-, and four-year-old

551   ginseng seedlings, respectively. (D) Emergence rate of ginseng seedlings. (E)

552   Survival rate of ginseng seedlings. 2-y, 3-y, and 4-y represents the transplanted two-,

553   three-, and four-year-old seedlings, respectively. Data are presented as mean ± SD (*n*

554   = 3). Identical letters denote nonsignificant difference at 0.05 significance level.

555

556   **Figure 2** Bacterial and fungal diversities in the rhizosphere of ginseng seedlings of

557   different ages and developmental stages. (A, B) show the Chao 1 and Shannon

558   diversity (*H'*) of bacterial community. (C, D) show the Chao 1 and *H'* of fungal

559   community. Ve, Fl, Fr, and Ro represent vegetative, flowering, fruiting, and root

560   growth stages, respectively. Data are presented as mean ± SD (*n* = 3). Non-identical

561   letters denote significant difference in the same developmental stage of ginseng plant

562   with different ages at 0.05 significance level.

563

564   **Figure 3** Changes in bacterial and fungal communities in the rhizosphere of ginseng

565   seedlings of different ages and developmental stages. (A–D) PCoA ordination plots

566   display the relatedness of samples separated using Unweighted UniFrac distance of

567   classified 16S rRNA gene sequences at vegetative, flowering, fruiting, and root

568   growth stages, respectively. (E–H) PCoA ordination plots show the relatedness of

569   samples separated using Unweighted UniFrac distance of classified 18S rRNA gene

570   sequences at the Ve, Fl, Fr, and Ro stages, respectively. Red, green, and blue

571   represent the samples in the rhizosphere of 2-y, 3-y, and 4-y transplanted ginseng

572   seedlings, respectively.

573

574   **Figure 4** Relative abundance of the bacterial taxa detected by the linear discriminant

575   analysis effect size (LEfSe) as biomarker. (A–D) represent the vegetative, flowering,

576   fruiting, and root growth stages, respectively. 2-, 3- and 4- represent the samples in

577   the rhizosphere of 2-y, 3-y, and 4-y transplanted ginseng seedlings. Data represent

27

578   the mean values of $n = 3$.

579

580   **Figure 5** Relative abundance of the fungal taxa detected by LEfSe as biomarker and

581   *Fusarium.* (A–D) represent the vegetative, flowering, fruiting, and root growth

582   stages, respectively. (E) Relative abundance of *Fusarium.* Data are presented as

583   mean $\pm$ SD ($n = 3$).

584

585   **Figure 6** *Bacillus subtilis* 50-1 antagonized *Fusarium oxysporum.* (A) Colony

586   diameter measured in a dual culture. (B) Morphological features of bacterium 50-1.

587   (C) Relationships of 16S rRNA sequences between *B. subtilis* strain 50-1 (black

588   body) and published 16S rDNA sequences. (D) Genome map of strain 50-1. The six

589   circles (outer to inner) represent the scale line, forward strand CDSs (color by COG

590   categories), reverse strand CDSs (color by COG categories), RNA genes, GC content,

591   and GC skew. From outside to center: genome size, genes on the forward strand

592   (color by COG categories), genes on the reverse strand (color by COG categories),

593   RNA genes (tRNAs, orange; rRNAs, red), GC content (red and blue), and GC skew.

594   Only bootstrap values higher than 70% are shown. Bars represent the mean $\pm$ SE (n

595    $= 3$). Asterisks denote significant differences between the colony diameters of *F.*

596   *oxysporum* and *F. oxysporum* +strain 50-1 at *P*< 0.05.

597

598   **Figure 7** Inoculation of strain 50-1 in ginseng replanting soils. (A) Death rate of

599   ginseng after inoculation with strain 50-1. (B) Copy numbers of *Fusarium* in soils.

600   No-inoculation and inoculation represent the samples inoculated with cultures

601   containing inactivated and activated strain 50-1, respectively. Data are presented as

602   mean $\pm$ SD ($n = 3$). Asterisks denote significant di□erences between the

603   No-inoculation and inoculation at *P*< 0.05.
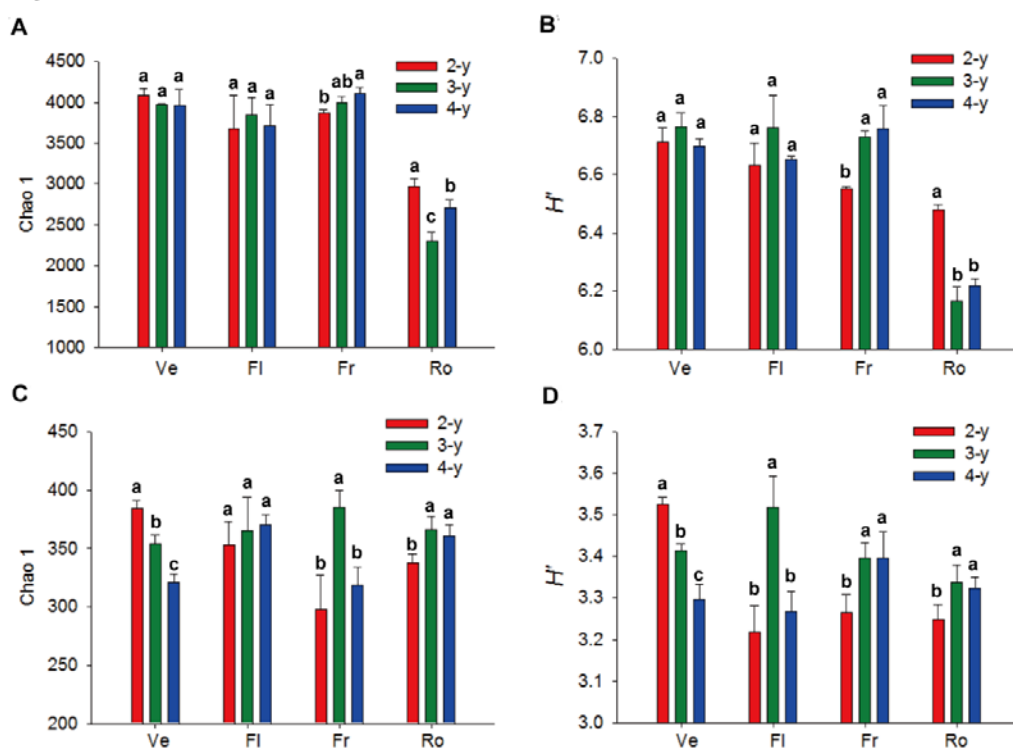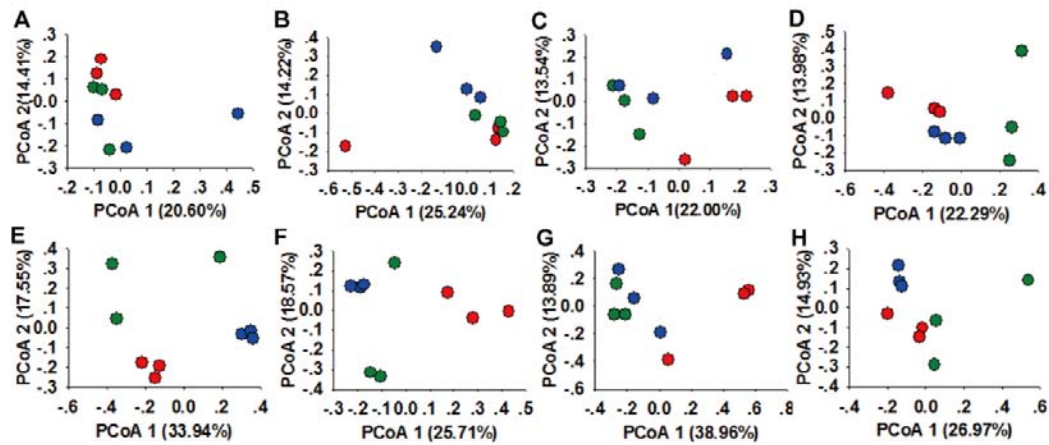
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606 **Figure 1**
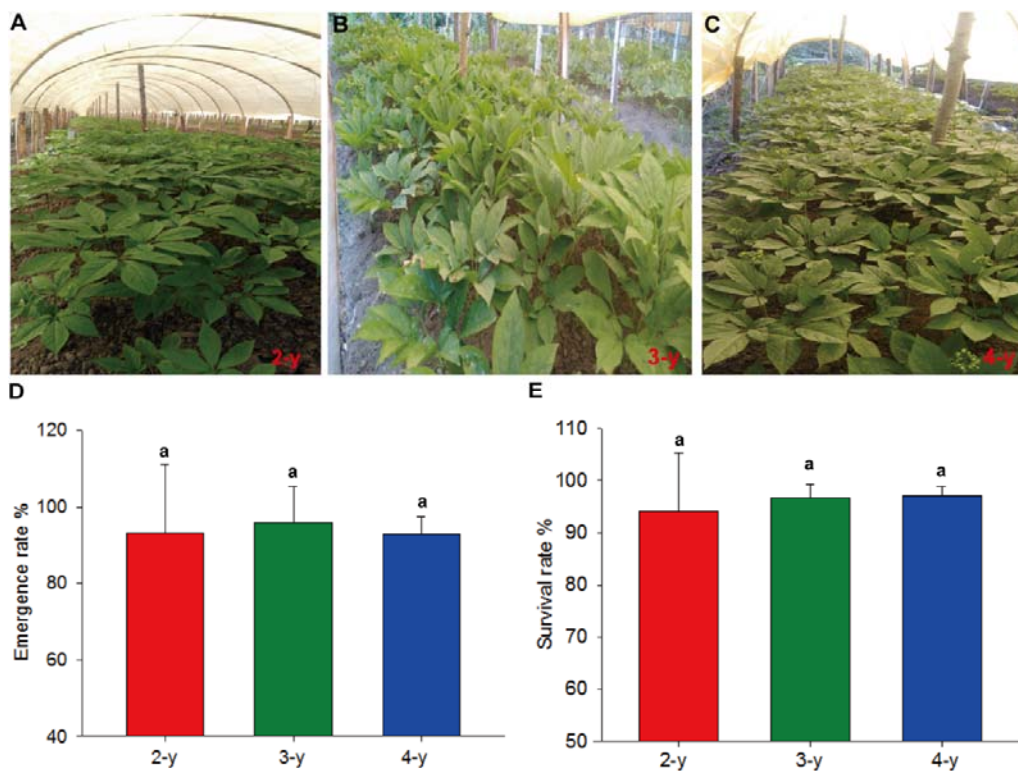
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609 **Figure 2**
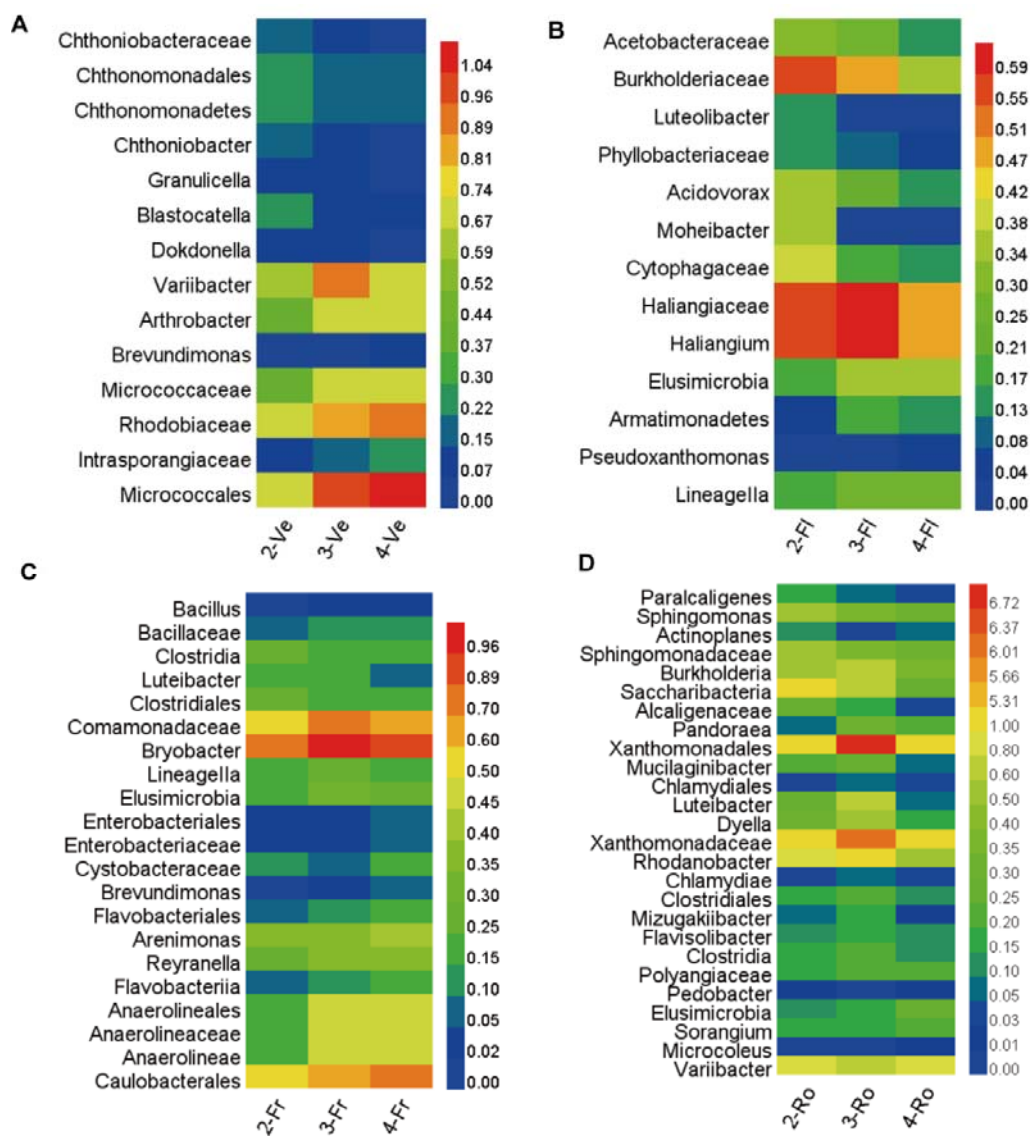

610
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612 **Figure 3**
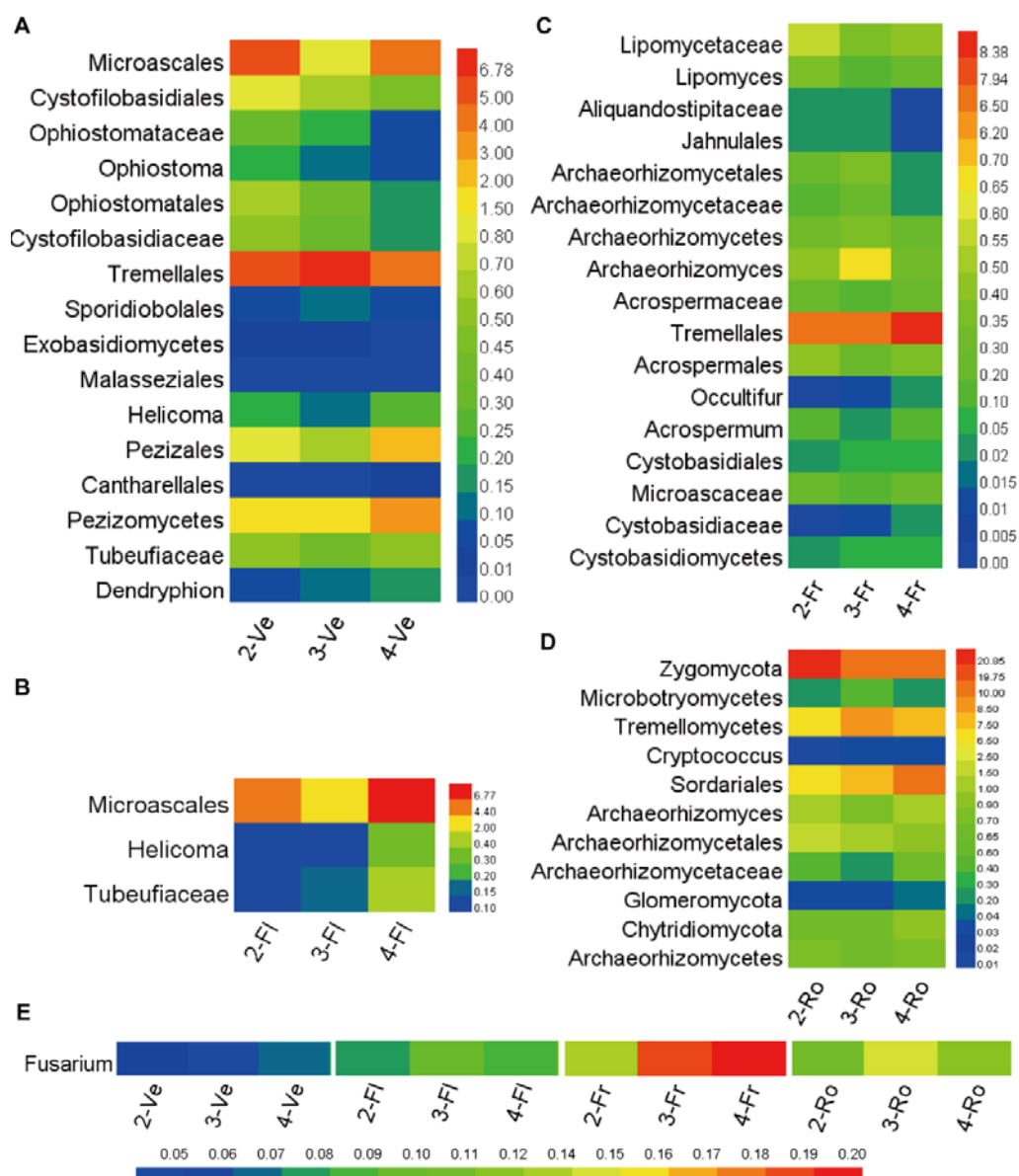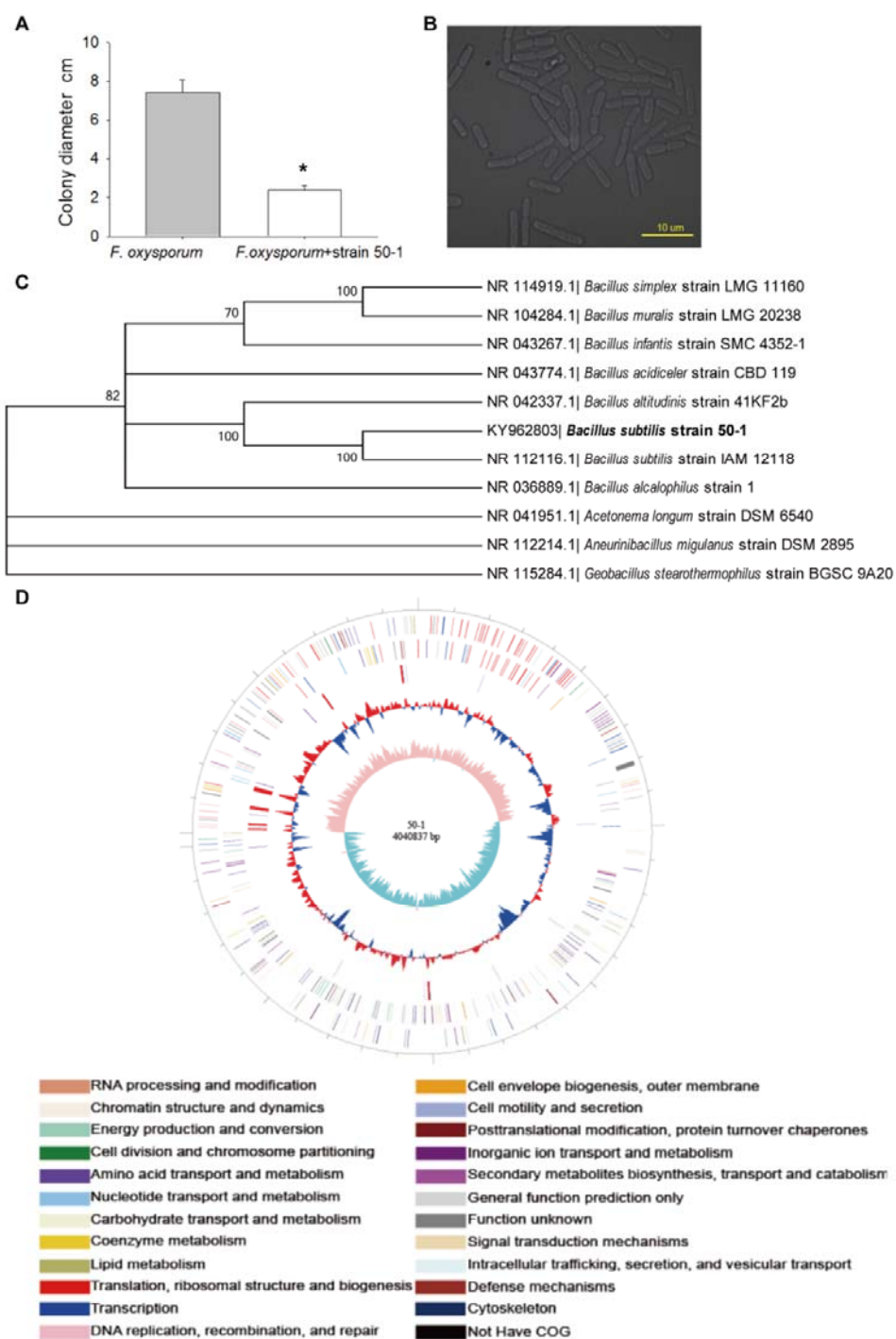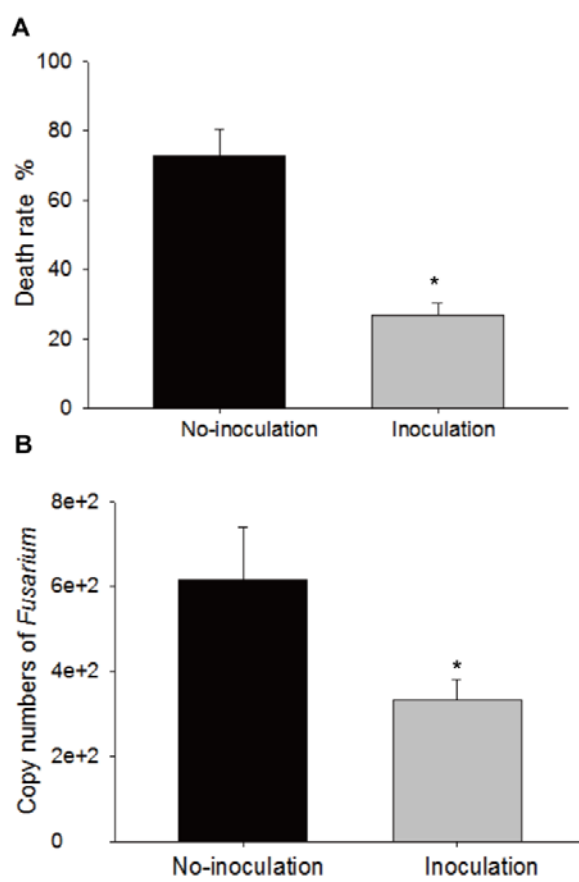

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615   **Figure 4**

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618 **Figure 5**

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621 **Figure 6**

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624 **Figure 7**

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