1 ***Trichoderma*, an endophytic fungus isolated from *Codonopsis pilosula*, promotes plant**

2 **growth and active ingredient accumulation**

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Running head

23   Trichoderma isolated from Codonopsis pilosula

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25**Highlights**

26● *Trichoderma* RHTA01 isolated from *Codonopsis pilosula* exhibited the ability of

27   promoting the growth and active ingredients accumulation.

28● The biological effect are explored by co-culture between *C. pilosula* and strain.

29● Antioxidant systems and signaling molecules responded with the strain.

30● Structural equation modeling was used to evaluated the relationship among various

31   factors.

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44**Abstract**

45Fungal endophytes from medicinal plants have the potential to promote plant growth and the

46accumulation of active ingredients via different mechanisms. In this study, an endophytic

47fungus was isolated from the roots of *Codonopsis pilosula*, and was identified as

48*Trichoderma* strain RHTA01 based on morphological analysis, fatty acid composition,

49Fourier infrared spectroscopy and molecular analysis. The strain exhibited good plant growth

50promoting capacity. In *C. pilosula* plants inoculated with *Trichoderma* strain RHTA01, the

51plant defense system, total chlorophyll content and root activity were enhanced, and levels of

52antioxidant enzymes, non-enzymatic ingredients and single molecules including nitric oxide

53(NO), jasmonic acid (JA), salicylic acid (SA) and hydrogen peroxide (H_2O_2) were up-

54regulated in different tissues (Root, Stem and Leave). Activities of enzymes involved in

55polysaccharide and Lobetyolin biosynthesis were up-regulated, thus increasing their

56accumulation in *C. pilosula* plants. The function of endophytic fungi was further clarified by

57structural equation modeling (SEM). Overall, our results provide a strong foundation for

58further investigation of the interaction between endophytic fungi and plants.

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60**Keywords:** *Trichoderma*; Endophytic Fungi; Active Ingredient Accumulation; Biological

61application

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66**1. Introduction**

67   Beneficial microorganisms are increasingly being used as biofertilizers, growth

68enhancers and natural resistance stimulants in agriculture (Meena et al., 2017). Some fungi

69secrete auxin, cytokinin, ethylene, nitric oxide (NO) and other hormones in plants and

70regulate plant metabolic processes, such as growth stimulation, salt tolerance and induction of

71systemic and local resistance to plant pathogens (Deshmukh et al., 2006; Lahrmann et al.,

722013). Endophytes can induce structural changes in plant roots, and thus alter plant nutrient

73uptake and abiotic stress and disease resistance (Andrés et al., 2017; Bertrand at al., 2007;

74Perrine-Walker et al., 2007; Venkatesan et al., 2016). Moreover, endophytes are a valuable

75resource of natural products and have wide application range in medicine and agriculture.

76Fungal endophytes have been in close association with terrestrial plants for approximately

77400 million years (Krings et al., 2007, Zhao et al., 2018). Many studies previously focused on

78the screening of endophytic fungi from medicinal plants under unique environments, and

79these endophytic fungi have been considered novel potential sources of previously

80undescribed bioactive compounds (Ibrahim et al. 2017, Duan et al. 2019). Endophytes

81colonize healthy plants without causing any apparent harm. Endophytic colonization of host

82plants reprograms their gene expression profile and alleviates any physiological and abiotic

83stresses for improving nitrogen utilization and pathogen defense (Chow et al., 2018; Dupont

84et al., 2015; Schiliro et al., 2012; Shoresh et al., 2010; Singh and Singh, 2014). Thus,

85screening fungal endophytes facilitates the discovery of novel microbial resources; however,

86the molecular mechanism underlying the interaction between endophytes and plants is largely

87unknown.

88   *Codonopsis pilosula* is a traditional Chinese herb with high medicinal value, as it has

89been shown to strengthen the physique, invigorate the spleen, and nourish the lung (Liu et al.,

902002). Previously, we isolated 706 endophytic fungi from *C. pilosula* plants collected from

91five counties in the Gansu Province of China. The abundance and bioactivities of 92*Trichoderma* could improve host growth and the accumulation of active ingredients in *C.* 93*pilosula.* The genus *Trichoderma*, which belongs to subdivision *Deuteromycotina*, class 94*Hyphomycetes* and family *Moniliaceae*, is capable of surviving adverse conditions by high 95reproduction, nutrient utilization and rhizosphere modification (Irina et al. 2006, Yuan et al. 962008), and promotes plant growth (Cai et al., 2015; Haque et al., 2010; Hermosa et al., 2012, 97Khan et al., 2017; Masunaka et al., 2011; Mukherjee et al., 2012; Verma et al., 2007; Zhang et 98al., 2013, 2018). *Trichoderma* isolated from soil and plants shows potential for promoting 99plant growth (Harman et al. 2004), However, *Trichoderma* strains produce several bioactive 100secondary metabolites such as auxin (Vinale et al. 2014). In addition, *Trichoderma* spp. can 101interact directly with roots, increasing plant growth potential, resistance to disease and 102tolerance to abiotic stresses (Hermosa et al. 2012). *Trichoderma* is often used as a 103biofertilizer (Bader et al. 2019) for increasing crop yields and promoting the accumulation of 104active ingredients. Although, as revealed by research in recent decades, *Trichoderma* can 105increase the nutrients availability in the plant rhizosphere , reduce the usage of fertilizers and 106resistance the abundance of soil-borne pathogens. However, the response mechanism of plant 107stress induced by *Trichoderma* remains were unclear.

108   In this study, we isolated *Trichoderma* strain RHTA01 from the roots of *C. pilosula*, and 109evaluated its capacity to produce indole-3-acectic acid (IAA). We also examined the effect of 110strain RHTA01 on the accumulation of polysaccharides and Lobetyolin in *C. pilosula* and on 111plant growth. The results of this study provide novel insights into the interaction between 112fungal strains and medicinal plants.

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2. Material and methods

2.1. Plant material and growth conditions

Codonopsis pilosula plants used in this study were sampled in August 2017 from three locations in Dingxi region, Gansu Province, China: LinTao city (103°886'E, 35°222'N, 1883.6±5.2 meters above sea level [masl]), WeiYuan city (104°28'E, 35°091'N, 2067.7±5.2 masl) and MinXian (104°18'E, 34°502'N, 2674.7±5.2 masl). Each plant was randomly selected. Plants were uprooted from the ground, and excess soil was removed by shaking the roots gently. Roots were sealed in plastic wrap, numbered, brought to the laboratory and stored at 4°C. 10 seeds per pot to germinate, pre-germinated healthy *C. pilosula* seeds which were selected the consistent growth for subsequent experiment were sown individually in plastic pots. Hydroponic C. *pilosula* seedlings were grown in an artificial climate chamber (temperature: 25°C; relative humidity: 54%) of the Lanzhou University of Technology (103°46'34"E, '36°3'20"N, 1530.49 ± 5.2 masl) in October 2018.

2.2. Isolation of endophytic fungi

Tissue sectioning and surface sterilization method was used to isolate endophytic fungi from *C. pilosula* (Wang et al, 2011). A 0.5–1.0-cm thick section of the washed root was treated with 75% ethanol for 2 min, followed by 5.0% sodium hypochlorite for 45–60 s, and then washed six times with sterile water. The sterilized root tissue and the last cleaning solution were placed on potato dextrose agar (PDA) medium supplemented with 100 mg L^{-1} ampicillin. The medium were cultured at 28°C in the dark and periodically examined until mycelial growth was observable. Mycelia were picked from the edge of the colony using a

135 sterile toothpick and inoculated onto the same medium multiple times until a single colony,

136 representing a pure fungal culture, was obtained. The strain was stored at 4°C using the

137 puncture preservation method, and the strain was named as RHTA01.

138 **2.3. Identification and biological characterization of *Trichoderma* strain RHTA01**

139 *Trichoderma* strain RHTA01 was identified based on macroscopic and microscopic

140 morphological characteristics and other biological features (Pittand and Hocking 1997,

141 Woudenberg et al., 2013). Genomic DNA of the strain was extracted using the Solarbio's

142 fungal genomic DNA extraction kit (Beijing Solarbio Science &Technology Co., LTD). The

143 isolated DNA was used as a template for PCR amplification of the internal transcribed spacer

144 (ITS) of the 5.8S rRNA gene, nuclear ribosomal small subunit (SSU), nuclear ribosomal large

145 subunit (LSU) and RNA polymerase second largest subunit (RPB2) using ITS1/ITS4,

146 NS1/NS4, LROR/LR5 and fRPB2-5F/fRPB2-7cR primer pairs, respectively (Table S1).

147 Sequence similarity was analyzed by multiple sequence alignment using NCBI BLAST

148 (http://www.ncbi.nlm.nih.gov), and sequences with 97% similarity were selected for

149 phylogenetic analysis. The phylogenetic tree was constructed with MEGA5 (Kumar et al.,

150 2016) and PAUP 4.0 using maximum parsimony (MP) (Cummings. 2004) and maximum

151 likelihood (ML) method (Silvestro et al. 2012), with 1,000 bootstrap (BS) replications. The

152 tree length (TL), consistency index (CI), retention index (RI) and repeat scale consistency

153 index (RCI) were calculated based on the MP analysis, with the heuristic search option, using

154 the Tree Bisection Reconnection (TBR) algorithm with 100 random sequence additions

155 (Swofford 2002). The ML method using PAUP with Kimura2-parameter model, obtained

156 most probable tree via the fast BS analysis of 1,000 pseudoreplications.

157 2.4. Biological analysis of *Trichoderma* strain RHTA01

158 2.4.1. *FT-IR and fatty acid analysis*

159   Fourier transform infrared spectroscopy (FT-IR) has been successfully used for the

160 identification of microorganisms such as bacteria, fungi, yeast, and actinomycetes (Wenning

161 et al., 2002). Dried hyphae powder (1.0 mg) was mixed with 200 mg of KBr, and a tablet was

162 determination from 4000–5000 cm^{-1} by infrared spectrum (Nexus 670 FT-IR, USA Nicolet).

163 A spectral resolution was set at 4 cm^{-1}, with an average scan time of 32 scans.

164   Gas chromatography–mass spectrometry (GC–MS) has been previously used to analyze

165 the fatty acid composition of *Trichoderma* (Ruiz et al, 2007). In this study, 300 mg of dried

166 hyphae powder was mixed with 2 ml of phenyl-petroleum: ether (1:1) solution for 15 min.

167 Then, 2 ml of 0.5 M $NaOH-CH_3OH$ solution was added to sample and water bath at 50°C for

168 15 min, then distilled water was added until the solutions layer. Then, 2 μL of the organic

169 phase was analyzed by GC analysis (Clarus 590 GC with PPC & without Integral Liuid

170 Autosampler).

171 2.4.2. *Physiological characterization*

172   Solid media were prepared using carbon-free Czapek-Dox Agar supplemented with 2%

173 maltose, D-fructose, glucose or sucrose (carbon source). The isolated strain was activated 1–2

174 times on PDA solid medium at 28°C for 5 days, and hyphae were inoculated in the media

175 described above using the point colonization method. Four replicates were prepared for each

176 medium. The inoculum was cultured at 28°C for 36 h, and the diameter of the colony was

177 measured daily. Values of the diameter were plotted against the number of days to generate

178 the growth curves. Additionally, solid media supplemented with 0.3% urea, potassium nitrate,

179sodium nitrate or ammonium sulfate (nitrogen source) also were used to evaluate strain

180growth.

1812.5. Measurement of the growth promoting efficiency of *Trichoderma* strain RHTA01

182   To evaluate the growth promoting capacity of strain RHTA01, hyphae were inoculated

183into Hoagland nitrogen-free medium, National Botanical Research Institute's phosphate

184growth medium (NBRIP), Ashby's nitrogen-free agar (Ashby) and Casman Agar Base (CAS).

185The inoculated media were incubated at 28°C for 7 days, and the growth of the strain was

186monitored daily. To analyze the enzyme production characteristics of the strain, the hyphae

187were inoculated into plasmin screening medium, contact enzyme screening medium,

188chitosanase screening medium, alkaline protease screening medium, complex enzyme

189screening medium and CMC-Na medium. The strain on each medium was cultured for 3–7

190days, and the appearance of a transparent ring around the colony was monitored.

191   To analyze IAA-like substances, 50 mL each of PDA, CzA (Czapek Dox Agar), Ashby,

192NBRIP and Hoagland liquid medium was mixed with trytophan (final concentration: 2 mg

193mL^{-1}). Then, 3 mL of the spore suspension (1×10^7 colony forming units [CFU] mL^{-1}) was

194added to each medium and cultured at 28°C for 7 days on a shaker (120 rpm). Un-inoculated

195media were used as controls. The supernatant from each culture was mixed with Sackowski's

196color reagent and incubated in the dark for 30 min. Each sample was measured with a

197spectrophotometer at 530 nm. The IAA concentration was calculated using the standard curve

198equation ($y = 17.679x + 0.0025$; linear range = 0–0.1 mg mL^{-1}; $R^2 = 0.9991$)

199   Then, 3 mL of the spore suspension (1×10^7 CFU mL^{-1}) was inoculated into five

200different types of demineralized liquid media (PDA, CzA, Ashby, NBRIP and Hoagland), and

201 incubated at the conditions described above. Each culture was filtered through a 0.22-μm

202 filter, and 500 μL of the filtrate was mixed with 500 μL of dye chrome azurol S solution. The

203 mixture was incubated for 1 h, and absorbance was determined at 680 nm. Deionized water

204 was used as a reference (Ar). Siderophore unit (SU) was calculated using the following

205 equation: SU (%) = (Ar-As)/Ar) × 100.

206　　　Next, 3 mL of spore suspension (1×10^7 CFU mL^{-1}) was inoculated into basal liquid

207 medium supplemented with 0.03% 1-aminocyclopropane-1-carboxylate (ACC; sole nitrogen

208 source) and 50 mL of Pikovskaya's Agar (PVK) . The inoculated medium was incubated at

209 30°C and 120 rpm for 5 days. The activity of ACC deaminase was calculated as described

210 previously (Penrose et al., 2003, Viterbo et al., 2010). The total phosphorus content was

211 determined as described previously (Vyas et al., 2007) and calculated according to the

212 standard curve (y = 0.0021x + 0.0785; R^2 = 0.9993). All assays were performed in five

213 replicates.

214 2.6. Plant inoculation assays

215 **2.6.1.** *Experimental design*

216　　　A randomized design was used in this study. *C. pilosula* seedlings in pots were irrigated

217 with spore suspension (1×10^6 CFU mL^{-1}) prepared in Hoagland liquid medium

218 (experimental group) or with an equal volume of sterile Hoagland medium (control group).

219 All plants were grown in soil at 60–65% field water capacity. Hoagland nutrient solution was

220 supplied every 3 d during the co-culture period. Plants were grown for 60 days in an

221 environmentally controlled chamber at 25°C and 54% relative humidity under a 14-h

222 light/10-h dark photoperiod and 50 μEm^{-2} S^{-1} light intensity using fluorescent lamps. Eight

223replicates were performed for each treatment (experimental and control).

224**2.6.2.** *Pathogenicity and mycorrhizal colonization*

225 Leaves of *C. pilosula* plants were injected with 5 μL of spore suspensions at different

226concentrations (1×10^6, 1×10^7 and 1×10^8 CFU mL^{-1}). The injection site on the leaves was

227monitored for spots at 7 days post-inoculation (dpi). Roots of plants in the experimental

228group were sampled every 2 s, cut into 1-cm pieces and placed on glass slides. The roots

229samples were stained with Medan dye for 10 min (Mahmoud et al., 2013), and excess

230staining solution was washed away. The stained root samples were observed under an optical

231microscope (40× objective lens) to evaluate the colonization status of the RHTA01 strain.

232**2.6.3.** *Plant growth measurements*

233 Plants were harvested from the pots after 60 days of growth, and roots were rinsed with

234water to remove the attached soil. Plant height, root diameter and root length were measured

235using a ruler and digital vernier caliper (0.001 mm). Leaf number per plant also was counted.

236Roots, stems and leaves were separated and gently blotted with filter paper. The fresh weight

237of each tissue type was measured immediately after harvest using the Mettler-Toledo

238analytical balance, and samples were stored at -20°C for enzyme activity analysis and other

239measurements.

240**2.6.4.** *Enzyme activity assay*

241 Plant tissue samples (0.1 g fresh weight) were homogenized in 5 mL of cold sodium

242phosphate buffer (50 mM; pH 7.8) using a pre-chilled mortar and pestle under ice-cold

243conditions. After centrifugation at 12,000 × *g* for 20 min, the supernatants were collected and

244stored at 4°C until needed for enzyme activity assays. All tests were carried out in triplicate.

245 Activities of antioxidant enzymes including polyphenol oxidase (PPO), superoxide dismutase

246 (SOD), catalase (CAT) and peroxidase (POD) were measured using kits purchased from

247 Suzhou Keming Biotechnology Co., Ltd. (China).

248 **2.6.5.** *Measurement of non-enzymatic compounds and root vitality*

249 Total chlorophyll (TChl), malondialdehyde (MDA), proline (Pro), soluble protein (Cpr),

250 reducing sugar (ReS), NO, hydrogen peroxide (H_2O_2), salicylic acid (SA) and jasmonic acid

251 (JA) contents were quantified using kits purchased from Suzhou Keming Biotechnology Co.,

252 Ltd. (China). Root vitality was measured using the triphenyl tetrazolium oxide (TTC)

253 method, as described previously (Lindström and Nyström, 1987; Yue, 2019).

254 **2.6.6.** *Extraction and quantification of polysaccharides and Lobetyolin*

255 Plant tissues (0.1 g) were mixed with 20 mL of methanol in a conical flask and subjected

256 to ultrasound treatment (250 W, 40 kHz) for 30 min. The extract from each flask was

257 collected and concentrated to a 5-mL volume in a water bath (80°C). The concentrated extract

258 was transferred into a volumetric flask, and the volume was increased to 10 mL using

259 methanol. The solution was filtered through a 0.45-μm filter, and 20 μL of the filtered sample

260 was subjected to high performance liquid chromatography (HPLC; JASCO, LC-2000, Japan).

261 A dilution series of Lobetyolin-methanol standard (48, 24, 12, 6, 3, 1.5, 0.75 and 0.375

262 mg/L) was prepared and analyzed at 267 nm by HPLC using Agilent C18 column (4.6 mm ×

263 150 mm, 5 μm i.d.) and acetonitrile: water (28:72, v/v) mobile phase at a flow rate of 1 mL

264 min^{-1}. The standard curve was generated by plotting peak area integral value (X-axis) against

265 concentration (Y-axis).

266 To quantify polysaccharides, 0.1 g of plant tissues was added into a conical flask

267containing 30 mL of distilled water and boiled in a water bath for 30 min. The extract was

268transferred to a 50-mL volumetric flask. Total carbohydrate content was measured using the

269phenol-sulphate colorimetry method. The polysaccharide content was calculated as the

270difference between the total carbohydrate content and reducing sugar content (Zou et al,

2712014).

272**2.6.7.** *Determination of active ingredients*

273   Activities of enzymes involved in sucrose metabolism, including sucrose phosphate

274synthase (SPS), sucrose synthase (SS) and soluble acid invertase (AI), and compound

275biosynthesis, including glutamine synthetase (GS), phenylalanine ammonia lyase (PAL) and

276nitrate reductase (NR), were measured using kits purchased from Suzhou Keming

277Biotechnology Co., Ltd. (China).

278**2.7. Structural equation modeling (SEM)**

279   The SEM approach was used to visualize the complex relationships among various

280physiological indicators of *C. pilosula*, and to explore the effects of endophytic fungi on plant

281growth and metabolite biosynthesis. To simplify the model, plant resistance index (PRI),

282signal molecule (SM), carbon metabolism enzyme (CME), nitrogen metabolism enzyme

283(NME), plant growth index (PGI) and polysaccharide content were selected. SEM analyses

284were performed using the *piecewise SEM* package (Smallwaters Corporation, Chicago, IL,

285USA) with the ML method (Lefcheck, 2015).

286**2.8. Statistical analysis**

287   All data were expressed as mean ± standard deviation (SD). Analysis of variance

288(ANOVA) was carried out using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA), and

289significant differences were detected at $P < 0.05$. All plant physiological assays were

290performed in three replicates, and microbiologic properties were measured in five replicates.

291

2923. Results

2933.1. Isolation and identification of endophytic fungi from *C. pilosula*

2943.1.1. *Morphological characteristics of Trichoderma strain RHTA01*

295 An endophytic fungus was isolated from the roots of *C. pilosula* by growing pure

296cultures on the PDA medium. The strain was identified based on hyphae color, spore

297morphology, sporulation structure and the presence or absence of a transverse (Gherbawy et

298al. 2010). Colonies on the PDA medium were circular and white at the center (Fig. 1A).

299Subsequently, green spores were produced at the center of the colony, along with the growth

300of white hyphae, with short branched mycelium and ovoid spores (Fig. 1B). The species was

301initially identified as *Trichoderma*, according to the Fungal Identification Handbook, based

302on macroscopic and microscopic morphological characteristics.

3033.1.2. *Identification of strain RHTA01 by PCR*

304We performed phylogenetic analysis of 16S rRNA sequences of *Trichoderma*,

305*Cephalosporium*, *Penicillium* and *Fusidium* (all belonging to class *Moniliaceae*). Since MP

306and ML trees were almost identical to each other, except for the branching order and branch

307length of the terminal group, we described the phylogenetic results based on the ML and MP

308tree. BLAST searches revealed that ITS sequences amplified from the sample under study

309showed the highest sequence similarity to the ITS sequences of *Trichoderma* (99.85%;

310coverage: 93%), (Fig. 1C). *Penicillium* was used as an outgroup, target strain sequence

311combined with *Trichoderma* 100% ML, MP BS support. BLAST searches using the SSU

312 sequence revealed the highest similarity to *Trichoderma* sequences (99.9%; coverage: 98%).

313 Target strain sequences with *Trichoderma* were grouped together with 80% ML and 73% MP

314 BS support in a multigene tree (Fig. 1D), and combined with *Trichoderma* and *Hypocrea*

315 100% ML, MP BS support. BLAST searches using the LSU sequence revealed the highest

316 similarity to *Trichoderma* sequences (100%; coverage: 95%), (Fig. 1E). Target strain

317 sequences with *Trichoderma* and *Hypocrealutea* 100% ML and MP BS support is combined.

318 BLAST searches using the RPB2 sequence revealed the highest similarity to *Trichoderma*

319 sequences (100%; coverage: 73%), (Fig. 1F). Target strain sequence with *Trichoderma* 99%

320 ML and 100% MP BS support is combined. Since ITS, SSU, LSU and RPB2 sequences

321 showed consistent results, the target strain was identified as *Trichoderma*.

322 *3.1.3 Fatty acid composition*

323　Fatty acids are one of the most stable compounds in microorganisms and are often used as

324 a basis for the classification and identification of microorganisms such as yeast and bacteria

325 (Ozbek et al, 2003). The fatty acid composition of fungi is affected by various factors such as

326 the species, culture media composition and conditions. The fatty acid composition of

327 *Trichoderma* strain RHTA01 was analyzed by GC analysis (Fig. 2A, Table 1). The linoleic

328 acid content of our sample was relatively high (28.41%). Higher amounts of linoleic acid

329 have been reported in other *Trichoderma* species such as *T. reseii* (47%) (Suutari, 1995) and

330 *T. harzianum* (48%) (Serrano-Carreon et al., 1992).

331 Table 1 Fatty acid composition of strain RHTA01 compared with various standards by GC

332 analysis

No.	Component	Standard peak	Sample peak	Sample peak area	Peak area	Fatty acid

		time (min)	Time (min)	(pA*s)	Percentage (%)	content (%)
1	C14:0	30.066	30.075	50.2	0.33	0.31
2	C15:0	32.031	32.044	24	0.16	0.15
3	C16:0	34.198	34.356	2998.7	19.92	18.89
4	C16:1	35.769	35.815	259.5	1.72	1.63
5	C17:0	36.624	36.681	30.1	0.20	0.19
6	C17:1	38.379	38.293	56.3	0.37	0.35
7	C18:0	39.345	39.651	1249.9	8.30	7.91
8	C18:ln9c	41.041	41.42	5605.7	37.23	35.47
9	C18:2n6c	43.919	44.227	4491.4	29.83	28.41
10	C20:0	45.791	45.817	14.7	0.10	0.10
11	C18:3	47.723	47.733	143.2	0.95	0.90
12	C24:0					
		66.54	66.672	131.7	0.87	0.83
13	C20:5					

333 3.1.4. FT-IR analysis

334   FT-IR is a high resolution approach that can sensitively detect changes in molecular

335 groups and their surroundings, thus serving as a perfect tool for the identification of microbial

336 species and status. FT-IR analysis reflects the unique characteristics of the system at a

337 macroscopic level; therefore, it is widely used for the identification of large-scale higher

338 fungi and herbal (Ji et al., 2014; Lecellier et al., 2015). The infrared spectrum can

339 characterize the chemical composition of *Trichoderma*, as different chemical groups in

340 *Trichoderma* will exhibit unique or overlapping absorption peaks in the FT-IR spectrum. The

341 FT-IR spectrum of strain RHTA01 was divided into six absorption peaks (Fig. 2B). The

342 absorption peak at 3700–2996 cm^{-1} represented the stretching vibration region of O-H and N-

343 H (Kumar et al., 2019; Popescu et al., 2010). The absorption peak at 2996–2800 cm^{-1}

344corresponded to the stretching vibration region of the lipid C-H (Kumar et al., 2009; Tandy et

345al., 2010). The absorption peak at 1800–1485 cm^{-1} was mainly derived from guanamine I and

346guanamine II bands, while that at 1485–1185 cm^{-1} corresponded to the symmetric and

347asymmetric stretching vibrations of P=O in proteins, lipids and phosphate compounds such as

348DNA, RNA, phospholipids and phosphate carbohydrates, which are related to the nucleic

349acid content of cells. The absorption peak at 1185–900 cm^{-1} mainly corresponded to the

350stretching vibration band of C-O-C and C-O-P in carbohydrates such as polysaccharides. The

351sugar ring stretching vibration of carbohydrate produced a weak spectral absorption band at

352900–500 cm^{-1}.

353**3.2. Characteristics and biological significance of *Trichoderma* strain RHTA01**

354   The growth of *Trichoderma* strain RHTA01 was analyzed on different culture media,

355and the microscopic features of the colonies were observed. The strain exhibited variation in

356colony size and oval spore number (Fig. S1, Table S2). Additionally, the strain showed a

357certain preference toward the nutrient substrates and displayed different growth rates,

358physiological activities, and metabolic pathways on different media. According to the

359mycelial dry weight on different culture substrates, *Trichoderma* strain RHTA01 showed the

360highest utilization of glucose and urea, and the lowest utilization of maltose and potassium

361nitrate.

362   It has been shown that *Trichoderma* secretes a variety of cell wall degrading enzymes

363(CWDEs), such as chitinases, cellulases, xylanases, glucanases and proteinases, during

364parasitism (Vinale et al., 2010). In this study, the *Trichoderma* strain RHTA01 was inoculated

365on various media, and colony morphology was observed after 7 days (Table S3). The strain

366showed different growth rates on PDA, Hoagland, NBRIP and Ashby media (Fig. S2a). The

367nitrogen fixation, phosphorus dissolution and IAA-like substrate production capacity of the

368strain as well as its enzyme activities are shown in Fig. S2b. The strain did not grow on

369Congo red medium and only grew transparent hyphae with no spores on Ashby medium,

370indicating that the nitrogen fixation ability of the strain was weak. Additionally, the strain

371could grow without a transparent circle on the NBRIP, plasmin screening, contact enzyme

372screening, chitosanase screening, complex enzyme screening and alkaline protease screening

373media, which suggests that the strain exhibits weak ability to dissolve phosphorus and

374produce fibrinolytic enzyme, contact enzyme, chitosanase, complex enzyme and alkaline

375protease. However, transparent circles were produced on skim milk medium, CAS medium

376and CMC-Na medium, indicating that the strain has a stronger ability to secrete protease,

377siderophore and cellulase than other enzymes (Fig. S2c).

378   Next, we performed qualitative analysis of IAA and siderophore produced by

379*Trichoderma* strain RHTA01 in various liquid media. When *Trichoderma* strain RHTA01 was

380cultured in Hoagland and Ashby liquid media at 120 rpm and 28°C for 7 days, the

381concentration of IAA reached at 1 μg mL^{-1} and 24.2 μg mL^{-1}, respectively; however, no IAA

382could be detected in other media. The content of siderophore was 2.27%, 33.99% and 72.27%

383in CzA, Ashby and NBRIP liquid media, respectively. When the strain was cultured for 5

384days in liquid media supplemented with ACC as the sole nitrogen source, the ACC deaminase

385activities were 2.91, 2.95, 3.24, 2.92 and 2.80 μmol α-ketobutyrate mg protein^{-1}·h^{-1},

386respectively. Based on these results, we deduced that *Trichoderma* strain RHTA01 is a

387beneficial microorganism and therefore could be used as a biofertilizer, growth enhancer and

388stimulant of natural resistance.

3893.3. Effect of *Trichoderma* strain RHTA01 on plant growth

390*3.3.1. Pathogenicity and colonization of strain RHTA01*

391   *Trichoderma* strain RHTA01 showed no pathogenicity on *C. pilosula* leaves. No brown

392or necrotic spots were observed at the injection site on *C. pilosula* leaves compared with the

393control (Fig. 3A.). After co-cultivation of *C. pilosula* seedlings with *Trichoderma* for 2

394weeks, the roots of seedlings were sectioned and stained with cotton-dyeing solution (Fig.

3953D, E). A large number of hyphae and spores was observed in the root tissues of inoculated

396seedlings compared with the control group, thus confirming that the strain had colonized the

397roots of *C. pilosula*. The responsiveness of the strain to root exudates was considered a major

398factor in colonization (MILLS et al., 1994), and root exudates likely induced the strain to

399colonize the root surface (Somers et al., 2004).

400*3.3.2. Chlorophyll content and the activity of root system*

401   When the strain RHTA01 was used as a fertilizer on *C. pilosula* seedlings, the fresh

402weight, root length and leaf number of *C. pilosula* seedlings increased (Fig. 3B, C).

403Additionally, the total chlorophyll content and root vitality of *C. pilosula* seedlings in the

404experimental group increased significantly (by ~2-fold) compared with the control group

405(Fig. 3F). These results indicate that the strain could improve the photosynthetic efficiency of

406plants as well as the root respiration, metabolism and biosynthesis of amino acids,

407phytohormones and other compounds (Stierle et al., 1993). This may be because *Trichoderma*

408secretes extracellular enzymes such as chitinase, xylanase and glucanase to dissolve the cell

409wall of other fungi and to produce a variety of secondary metabolites such as gypsum and

410antibacterial peptides that kill other pathogenic fungi. This explains why *Trichoderma* is the

411dominant genus in *C. pilosula* (Saravanakumar et al., 2016).

4123.4. Effect of RHTA01 on the content of signaling molecules in *C. pilosula*

413   Fungi act as inducers to activate the Ca^{2+}, H_2O_2, NO, JA and SA signaling pathways in

414the host plant to complete colonization and commensalism and to increase stress resistance

415and secondary metabolite production (Rojo et al., 2007; Wang et al., 2011). When *C. pilosula*

416plants were treated with strain RHTA01, the content of NO in roots decreased significantly (*p*

417< 0.05), but no difference was detected in stems and leaves. However, the content of JA

418increased significantly in roots and showed minor differences in stems and leaves. The

419content of H_2O_2 increased slightly in root and stem tissues but decreased significantly in

420leaves (*p* < 0.05). The content of SA increased significantly in root and stem tissues (*p* <

4210.05) but only slightly in leaves.

422   In summary, contents of SA and JA were the highest in C. *pilosula* roots, followed by

423stems and leaves, implying that SA and JA mainly affect the metabolism of roots in *C.*

424*pilosula*. Additionally, *Trichoderma* strain RHTA01 reduced the H_2O_2 content in leaves,

425whereas SA, JA and NO contents were increased in all three tissues of *C. pilosula* tested in

426this study. Contents of NO and H_2O_2 were the highest in leaves, followed by roots and stems

427(Fig. 3G), suggesting that H_2O_2 is the upstream molecular of NO. It is possible that

428*Trichoderma* strain RHTA01 affected the production of H_2O_2 in mesophyll cells, which in

429turn would affect the NO content of leaves, eventually leading to the closure of mesophyll

430cells (Praveen et al., 2019) and consequently affecting photosynthesis.

4313.5. Effect of RHTA01 on the antioxidant system of *C. pilosula*

432   Inoculation of *C. pilosula* plants with *Trichoderma* strain RHTA01 activated the

433signaling molecules and altered their contents, as shown above. Additionally, RHTA01 also

434affected the activity of antioxidant enzymes and the amount of non-enzymatic compounds.

435The content of MDA decreased in the roots and leaves of *C. pilosula* but increased

436significantly in stems ($p < 0.05$) (Fig. 3). Reactive oxygen species (ROS) oxidative burst may

437occur in the stem, resulting in an increased amount of lipid peroxidation products such as

438MDA (Yalcinkaya et al., 2019). These results clearly suggest that *Trichoderma* inoculation

439could prevent oxidative damage in roots. After colonization, the Pro content decreased

440significantly in roots ($p < 0.05$) but only slightly in stems; however, the Pro content of leaves

441increased, implying that the strain completely colonized the host plant, reaching a steady

442state. The content of reducing sugar (Res), one of main products of photosynthesis, was the

443highest in the leaves of *C. pilosula*, followed by roots and stems; chlorophyll content showed

444the same trend, suggesting that the strain could improve the photosynthetic efficiency of

445plants

446   Cpr was associated with the activities of enzymes, including PPO, POD, CAT and SOD,

447and other stress resistance indicators. *Trichoderma* inoculation increased the Cpr content of

448*C. pilosula* (Fig. 4), possibly because Cpr acts as an antioxidant and protects plant cells from

449oxidative damage.by regulating the content of various enzymes. In this study, the content of

450SOD decreased significantly in roots ($p < 0.05$), but no significant change was detected in

451stems and leaves. PPO also decreased significantly in roots and leaves ($p < 0.05$) but showed

452a minor increase in the stem. POD showed a marked decrease in the root ($p < 0.05$), a slight

453decline in the stem and an increase in the leaf ($p < 0.05$). CAT increased significantly in the

454root ($p < 0.05$) but showed no difference in the stem and leaf. Thus, the application of

455*Trichoderma* decreased the SOD, POD and PPO activities in the roots of *C. pilosula*, CAT

456and POD activities in stems and CAT and PPO activities in leaves. However, CAT activity in

457roots, SOD and PPO activities in stems and SOD and POD activities in leaves were

458improved. This suggests that different oxidases played tissue-specific roles because of the

459strain RHTA01; for example, SOD activity was inhibited for reducing the production of

460H_2O_2, and CAT activity was increased to scavenge H_2O_2. These results indicate that the strain

461RHTA01 could reduce oxidative damage by regulating the activities of antioxidant enzymes

462in plant roots, and H_2O_2 is an important factor affecting plant growth and secondary

463metabolite synthesis.

464**3.6. Effect of strain RHTA01 on enzyme activities regulating carbon metabolism and**

465**polysaccharide content**

466   Polysaccharide is one of the active ingredients in *C. pilosula* (Ming et al., 2017). The

467polysaccharide content decreased significantly in the root but increased notably in the stem

468and leaf ($p < 0.05$) (Fig. 5). The activity of sucrose biosynthesis enzymes (SPS, SS and AI)

469were evaluated to explore the accumulation of polysaccharides (Fig. 5A). The activity of SPS

470was significantly reduced in root, stem and leaf tissues ($p < 0.05$), whereas that of SS and AI

471was significantly increased in root and leaf ($p < 0.05$), with only minor changes in the stem.

472AI hydrolyzes sucrose to glucose and fructose, whereas SPS catalyzes sucrose into UDP-

473glucose and fructose in the form of uridine diphosphate (UDP). SS degrades sucrose into

474hexose or its derivatives, which are used in various physiological metabolic pathways,

475possibly as the main transportable product of photosynthesis, and accumulated in leaves

476(Wang et al., 2014). Relatively high activities of AI and SPS have been shown to improve

477plant growth in rice, tobacco and cotton (Cui et al, 2007). According to our results, the

478application of *Trichoderma* significantly increased the activities of SS and AI, resulting in

479increased accumulation of polysaccharides in stems and leaves of *C. pilosula*. However, the

480decreased activity of SPS in roots decreased the polysaccharide content of *C. pilosula* roots.

481These results suggest that SPS is the most important enzyme in the polysaccharide

482biosynthesis pathway. It is possible that *Trichoderma* strain RHTA01 increased the

483expression of genes involved in photosynthesis and respiration in stem and leaf cells, which

484is consistent with our previous results on photosynthesis.

485**3.7. Effect of strain RHTA01 on the activity of key enzymes involved in nitrogen**

486**metabolism**

487   Three nitrogen metabolism-related enzymes (GS, NR and PAL) are reportedly involved

488in the anabolism of active compounds. GS regulates nitrogen metabolism in plants

489(Vijayalakshmi et al., 2015). NR acts as both a regulating enzyme and rate-limiting enzyme,

490catalytically reduces nitrate to nitrite ($NO^{3-} \rightarrow NO^{2-}$) and affects the metabolic rate of NO^{3-}-N.

491NR participates in the biosynthesis and degradation of NO, thus controlling the homeostasis

492of NO, a key signaling molecule in plant cells, and plays a central role in the overall biology

493of plants (Chamizo-Ampudia et al. 2016, 2017). PAL is a key enzyme involved in the first

494step of the phenylpropanoid pathway and is responsible for the biosynthesis of plant

495phenylpropanoids and phenolics (Delledonne 1998; Huang et al., 2010). Change in PAL

496activity is a common response of plant cells to biotic and abiotic stresses (Kim et al., 2014).

497in soybean (*Glycine max*) cell culture, sodium nitroprusside (SNP), an NO donor, induces the

498expression of genes encoding PAL and CHS (the first enzyme in the phenylpropane pathway)

499(Delledonne 1998). In this study, the application of *Trichoderma* decreased the activities of

500GS, NR and PAL in the roots and leaves of *C. pilosula* but significantly increased their

501activities in stems. These results indicate that the strain with weak nitrogen fixation ability

502could inhibit the activity of these three enzymes in roots and reduced the content of NO to

503protect the host plant, which is consistent with the quantitative analysis of NO. The increased

504relatively activity in the stem implies that some active compounds are synthesized, such as

505the highest content of Lobetyolin. Lobetyolin is another important bioactive compound

506widely distributed in the stems and leaves of C. *pilosula*. After colonization by *Trichoderma,*

507the contents of Lobetyolin in all three tissues of *C. pilosula* plants decreased significantly.

508This suggests that the strain regulates nutrient transport to promote plant growth.

509**3.8. Correlation among various biological indicators**

510   The inoculation of C. *pilosula* with *Trichoderma* strain RHTA01 changed the biological

511indicators of various tissues of the host plant (Fig. 6A). Signal transduction is a common

512physiological and biochemical phenomenon that plays an important role in plant growth and

513development, stress resistance and resistance to diseases and insect pests (Barazani et al.,

5142007). After inoculation with the strain, receptors on the plant cell surface were activated and

515combined with strain secretion selectively (Fig. 6B). Then, the expression of related genes

516involved in complex signal transduction pathways was regulated to increase plant resistance

517and secondary metabolite accumulation (Fig. 6B). Zhai et al. (2017) proposed the

518polysaccharide biosynthesis pathway of *C. pilosula* (Fig. 6C), and SS was a key enzyme in

519this pathway. Redundancy analysis (RDA) analysis showed that plant indices (TChl and LN)

520were positively correlated with plant stress resistance indices (MDA, Pro, Cpr, ReS, PPO,

521SOD, CAT and POD) but negatively correlated with stress resistance indicators and signaling

522molecules (NO, H_2O_2, JA and SA) (Fig. 6D). This result showed that MDA, Pro, Cpr, ReS,

523PPO, SOD, CAT, POD could protect plant tissues and cells from oxidative damage. SA was

524negatively correlated with stress resistance indicators, indicating that SA plays an important

525role in plant cell growth and metabolism. Many studies show that endophytic fungi can be

526used as an inducer to participate in the process of secondary metabolic from herbs (Fouda et

527al., 2015). In this study, signaling molecules were negatively correlated with the contents of

528polysaccharides (CPP) and Lobetyolin (CL) (Fig. 6E). The results of correlation analysis

529among CPPs, CL and metabolic enzymes is shown in Fig. 6F. The CPP content was positively

530correlated with SS and AI activities but negatively correlated with SPS activity. In addition,

531CPP was positively correlated with GS and PAL activities but negatively correlated with NR

532activity. Sucrose metabolism plays a key role in plant development, stress response and

533biosynthesis of essential compounds by the production of various sugars as metabolites (Tang

534et al., 2009). Sucrose metabolism confers abiotic stress tolerance in plants (Ruan et al., 2010).

535This suggests that *Trichoderma* strain RHTA01 enhances the yield and biotic stress tolerance

536of the host plant by regulating signal transduction of NO, H_2O_2, JA and SA, although further

537investigation is needed.

538**3.9. SEM analysis**

539   To further understand the correlation among the physiological indexes of *C. pilosula*,

540bioactive components (polysaccharides and Lobetyolin) and key carbon and nitrogen

541metabolic enzymes, SEM analysis was conducted (Fig. 7). The ellipse indicates the latent

542variable, and the rectangular box indicates the observed variable. The dashed one-way arrow

543is the model parameter for the estimation process, and the number on the two-way arrow

544pointing to the different variables represents the correlation coefficient of the two variables.

545The green and red arrows indicate positive and negative correlations, respectively. Arrow

546width indicates the strength of the causal effect. PRI and SM showed a positive correlation in

547all three tissues of *C. pilosula*, although the correlation coefficient (1.25) was more

548significant in roots than in stems and leaves. The effects of the remaining variables varied

549significantly among different tissues. In roots, no significant difference was detected between

550PRI and CME, NME and PGI, whereas PRI showed a significant negative correlation with

551CL. Notably, the interaction among variables was the most significant in the stem of *C.*

552*pilosula*. PRI and CME showed a significant negative correlation ($r = 1.18$). Additionally,

553CME and NME showed a significant negative correlation with PGI and PGI, respectively,

554whereas PRI showed a positive correlation with CL ($r = 0.95$). In the leaves of *C. pilosula*,

555the correlation among variables was significantly weak, but the correlation between CME and

556TChl was significant. Thus, by determining the biological indicators of different tissues of *C.*

557*pilosula* and comprehensively predicting the interaction among variables, we could better

558understand the impact of changes in different variables on plants.

559**4. Conclusions**

560   In this study, we isolated an endophytic fungus from *C. pilosula* roots and identified it as

561*Trichoderma* strain RHTA01 by molecular biology, fatty acid composition and infrared

562spectroscopy analyses. In addition, *Trichoderma* exhibited the ability to produce numerous

563extracellular enzymes, and its ability to produce proteases, siderophores and cellulases was

564slightly stronger than other enzymes. *C. pilosula* plants treated with *Trichoderma* showed an

565increase in IAA, TChl content and root vitality and produced specific secondary metabolites,

566which potentially influence plant growth through various strategies. *Trichoderma* inoculation

567significantly increased the net activity of SS via a complicated signal transduction pathway

568and promoted the accumulation of polysaccharides and alkynes in *C. pilosula.* Finally, the

569role of endophytic fungi in host plant response, plant growth and active ingredient

570accumulation was further clarified by SEM analysis. Overall, our results suggest that

571*Trichoderma* strain RHTA01 has tremendous potential in biotechnological and ecological

572applications.

573

578

579**AUTHOR CONTRIBUTIONS**

580YG Wang and QW Yang contributed equally to this work. YG Wang, FF Leng, JX Chen

581designed and revised the manuscript. QW Yang and YL Li performed the experiments and

582analyzed data. QW Yang wrote the manuscript. K Chen, LM Yu, S Hu and M Ding collected

583data. SW Li and FF Leng provided the SEM analysis. All authors have read and approved the

584manuscript.

585

586**CONFLICT OF INTEREST**

587   All authors declare that they have no conflict of interest.

588

ETHICAL APPROVAL

590   This article does not contain any studies with human participants or animals performed
591 by any of the authors.

592

REFERENCES

594 Andrés M.F., Diaz C.E., Giménez C., et al.. Endophytic fungi as novel sources of biopesticides: the

595   macaronesian laurel forest, a case study. Phytochemistry Reviews, **2017**, 16, 1-14.

596   https://doi.org/10.1007/s11101-017-9514-4.

597 Bader A.N., Salerno G.L., Covacevich F., Consolo V.F.. Native *Trichoderma harzianum* strains from

598   Argentina produce indole-3 acetic acid and phosphorus solubilization, promote growth and control

599   wilt disease on tomato (*Solanum lycopersicum* L.). Journal of King Saud University - Science, **2019**,

600   S1018-3647(18), 30815-2. https://doi.org/10.1016/j.jksus.2019.04.002

601 Barazani O., Von Dahl C.C., Baldwin I.T.. *Sebacina vermifera* Promotes the Growth and Fitness of

602   Nicotiana attenuata by Inhibiting Ethylene Signaling. Plant Physiology, **2007**, 144, 1223-1232.

603   https://doi.org/10.1104/pp.107.097543.

604 Bertrand A., Prévost D., Bigras F.J., Castonguay Y.. Elevated atmospheric CO_2 and strain of rhizobium

605   alter freezing tolerance and cold-induced molecular changes in alfalfa (*medicago sativa*). Annals of

606   Botany, **2007**, 99(2), 275-284. https://doi.org/10.1093/aob/mcl254.

607 Cai F., Chen W., Wei Z., et al.. Colonization of *trichoderma harzianum* strain sqr-t037 on tomato roots and

608   its relationship to plant growth, nutrient availability and soil microflora. Plant & Soil, **2015**, 388, 337-

609   350. https://doi.org/10.1007/s11104-014-2326-z.

610 Chamizo-Ampudia A., Sanz-Luque E., Llamas A., et al.. Nitrate Reductase Regulates Plant Nitric Oxide

611 Homeostasis. Trends in Plant Science, **2017**, 22(2):163-174.

612 https://doi.org/10.1016/j.tplants.2016.12.001.

613 Chamizo-Ampudia A., Sanz-Luque E., Llamas ángel, et al.. A dual system formed by the ARC and NR

614 molybdoenzymes mediates nitrite-dependent NO production in,*Chlamydomonas*. Plant, Cell &

615 Environment, **2016**, 39, 2097-2107. https://doi.org/10.1111/pce.12739.

616 Chow Y.Y., Rahman S., Ting A.S.Y.. Evaluating the host defense responses in oil palm to complex

617 biocontrol endophyte-pathogen-host plant interaction via Fluidigm® real-time polymerase chain

618 reaction (RT-PCR). Biological Control, **2018**, 129, 148-157.

619 https://doi.org/10.1016/j.biocontrol.2018.10.011.

620 Cui N., Li T.L., Li Y.. The main action of 14-3-3 proteins in plants. Biotechnology, **2007**, 17(2): 86-89.

621 https://doi.org/10.1360/biodiv.070080.

622 Deshmukh S., Huckelhoven R., Schafer P., et al.. The root endophytic fungus piriformospora indica

623 requires host cell death for proliferation during mutualistic symbiosis with barley. Proceedings of the

624 National Academy of Science, **2006**, 103(49), 18450-18457.

625 https://doi.org/10.1073/pnas.0605697103.

626 Druzhinina I.S., Kopchinskiy A.G., Kubicek C.P.. The first 100 trichoderma species characterized by

627 molecular data. Mycoscience, **2006**, 47(2): 55-64. https://doi.org/10.1007/s10267-006-0279-7.

628 Duan X.X., Qin D., Song H.C., et al.. Irpexlacte A-D, four new bioactive metabolites of endophytic fungus

629 *Irpex lacteus* DR10-1 from the waterlogging tolerant plant *Distylium chinense.* Phytochemistry

630 Letters, **2019**, 32, 151-156. https://doi.org/10.1016/j.phytol.2019.06.001.

631 Dupont P.-Y., Eaton C.J., Wargent J.J., et al.. Fungal endophyte infection of ryegrass reprograms host

632 metabolism and alters development. New Phytologist, **2015**, 208(4): 1227-1240.

633 https://doi.org/10.1111/nph.13614.

634 Fouda A.H., Hassan S.E.D., Eid A.M., Ewais E.E.D.. Biotechnological applications of fungal endophytes

635   associated with medicinal plant *Asclepias sinaica* (Bioss.). Annals of Agricultural Sciences, **2015**,

636   60(1): 95-104. https://doi.org/10.1016/j.aoas.2015.04.001.

637 Gherbawy Y., Kesselboth C., Elhariry H., Hoffmann K.. Molecular Barcoding of Microscopic Fungi with

638   Emphasis on the Mucoralean Genera Mucor and Rhizopus. Molecular Identification of Fungi, **2010**,

639   213-250. https://doi.org/10.1007/978-3-642-05042-8_11.

640 Haque M.M., Haque M.A., Ilias G.N.M., Molla A.H.. *Trichoderma*-enriched bio-fertilizer, a prospective

641   substitute of inorganic fertilizer for mustard (*Brassica campestris*) production. Agriculturists, **2010**, 8,

642   66-73. https://doi.org/10.3329/agric.v8i2.7579.

643 Harman G., Howell C., Viterbo A., et al.. *Trichoderma* species- opportunistic, avirulent plant symbionts.

644   Nature Reviews Microbiology, **2004**, 2(1):43-56. https://doi.org/10.1038/nrmicro797.

645 Hermosa R., Viterbo A., Chet I., Monte E.. Plant-beneficial effects of Trichoderma and of its genes.

646   Microbiology, **2012**, 158(1):17-25. https://doi.org/10.1099/mic.0.052274-0.

647 Huang J.L., Gu M., Lai Z.B., et al.. Functional analysis of the Arabidopsis PAL gene family in plant

648   growth, development, and response to environmental stress. Plant Physiology, **2010**, 153(4): 1526-

649   1538. https://doi.org/10.1104/pp.110.157370.

650 Ibrahim A., Sørensen D., Jenkins H.A., et al.. Epoxynemanione a, nemanifuranones A-F, and

651   nemanilactones A-C, from, Nemania serpens, an endophytic fungus isolated from *Riesling*

652   *grapevines*. Phytochemistry, **2017**, 140, 16-26. https://doi.org/10.1016/j.phytochem.2017.04.009.

653 Ji P., Wei Y.M., Xue W.X., et al.. Characterization and antioxidative activities of polysaccharide in Chinese

654   angelica and its processed products. International Journal of Biological Macromolecules, **2014**, 67,

655   195-200. https://doi.org/10.1016/j.ijbiomac.2014.03.025.

656 Khan M.Y., Haque M.M., Molla A.H., et al.. Antioxidant compounds and minerals in tomatoes by

657 Trichoderma-enriched biofertilizer and their relationship with the soil environment. Integr. Agric,

658 **2017**, 16, 691-703. https://doi.org/10.1016/S2095-3119(16)61350-3.

659 Kim D.S and Hwang, B.K. An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in

660 salicylic acid-dependent signaling of the defence response to microbial pathogens. Journal of

661 experimental botany, **2014**, 65(9): 2295-2306. https://doi.org/10.1093/jxb/eru109.

662 Krings M., Taylor T.N., Hass H., et al.. Fungal endophytes in a 400-million-yr-old land plant, infection

663 pathways, spatial distribution, and host responses. New Phytologist, **2007**, 174, 648-657.

664 https://doi.org/10.1111/j.1469-8137.2007.02008.x.

665 Kumar R., Mago G., Balan V., Wyman, C.E. Physical and chemical characterizations of corn stover and

666 poplar solids resulting from leading pretreatment technologies. Bioresource technology, **2009**,

667 100(17): 3948-3962. https://doi.org/10.1016/j.biortech.2009.01.075.

668 Kumar V. and Dwivedi, S.K.. Hexavalent chromium stress response, reduction capability and

669 bioremediation potential of *Trichoderma* sp. isolated from electroplating wastewater. Ecotoxicology

670 and Environmental Safety, **2019**, 185: 109734. https://doi.org/10.1016/j.ecoenv.2019.109734.

671 Kumar S., Stecher G., Tamura K.. MEGA7, Molecular evolutionary genetics analysis version 7.0 for

672 bigger datasets. Molecular Biology and Evolution, **2016**, 33, 1870-1874.

673 https://doi.org/10.1093/molbev/msw054.

674 Lahrmann U., Ding Y., Banhara A., et al.. Host-related metabolic cues affect colonization strategies of a

675 root endophyte. Proc Natl Acad Sci USA, **2013**, 110, 13965-13970.

676 https://doi.org/10.1073/pnas.1301653110.

677 Lecellier A., Gaydou V., Mounier J., et al.. Implementation of an FTIR spectral library of 486 filamentous

678 fungi strains for rapid identification of molds. Food Microbiology, **2015**, 45, 126-134. https://doi.org/

679 10.1016/j.fm.2014.01.002.

680 Lee Y.C., Johnson J.M., Chien C.T., et al,. Growth promotion of Chinese cabbage and *arabidopsis* by

681 *piriformospora indica* is not stimulated by mycelium-synthesized auxin. Molecular plant-microbe

682 interactions: MPMI, **2011**, 24(4), 421. https://doi.org/10.1094/MPMI-05-10-0110.

683 Lefcheck J S. piecewise SEM: Piecewise structural equation modelling in R for ecology, evolution, and

684 systematics. Methods in Ecology & Evolution, **2016**, 7(5). https://doi.org/10.1111/2041-210X.12512.

685 Lindström A., Nyström C.. Seasonal variation in root hardiness of container-grown Scotspine,

686 Norwayspruce, and lodgepole pine seedlings. Canadian Journal of Forest Research, **1987**, 17(8):787-

687 793. https://doi.org/10.1139/x87-126.

688 Liu G., Anderson C., Scaltreto H.,et al.. QS-21 structure/function studies: effect of acylation on adjuvant

689 activity. Vaccine, **2002**, 20(21-22), 2808-2815. https://doi.org/10.1016/s0264-410x(02)00209-8.

690 Mahmoud R.S., Kazuhiko N., Murad G.. A New Fungal Endophyte, Scolecobasidium humicola, Promotes

691 Tomato Growth under Organic Nitrogen Conditions. Plos One, **2013**, 8(11):e78746.

692 https://doi.org/10.1371/journal.pone.0078746.

693 Masunaka A., Hyakumachi M., Takenaka S.. Plant growth-promoting fungus, *Trichoderma koningi*

694 suppresses isoflavonoid phytoalexin vestitol production for colonization on/in the roots of *Lotus*

695 *japonicus*. Microbes & Environments, **2011**, 26, 128. https://doi.org/10.1264/jsme2.ME10176.

696 Meena S.K., Rakshit A., Singh H.B., Meena V.S.. Effect of nitrogen levels and seed bio-priming on root

697 infection, growth and yield attributes of wheat in varied soil type. Biocatalysis & Agricultural

698 Biotechnology, **2017**, 12:172-178. https://doi.org/10.1016/j.bcab.2017.10.006.

699 MILLS., Aaron L.. Soil microbial ecology-applications in agricultural and environmental management.

700 Soil Science, **1994**, 157, 195-197. https://doi.org/10.1097/00010694-199403000-00010.

701 Ming K., Chen Y., Yao F., et al.. Phosphorylated *Codonopsis pilosula* polysaccharide could inhibit the

702 virulence of duck hepatitis A virus compared with *Codonopsis pilosula* polysaccharide. International

703   journal   of   biological   macromolecules,   **2017**,   94:   28-35.

704   https://doi.org/10.1016/j.ijbiomac.2016.10.002.

705 Mukherjee M., Mukherjee P.K., Horwitz B.A., et al.. *Trichoderma*-plant-pathogen interactions, advances in

706   genetics of biological control. Microbiol, **2012**, 52, 522-529. https://doi.org/10.1007/s12088-012-

707   0308-5.

708 Ozbek A., Aktas O.. Identification of three strains of mycobacterium species isolated from clinical samples

709   using fatty acid methyl ester profiling. Journal of International Medical Research, **2003**, 31, 133.

710   https://doi.org/10.1177/147323000303100210.

711 Passari A.K., Chandra P., Zothanpuia, et al.. Detection of biosynthetic gene and phytohormone production

712   by endophytic actinobacteria associated with *solanum lycopersicum* and their plant-growth-promoting

713   effect.   Research   in   Microbiology,   **2016**,   167(8),   692-705.

714   https://doi.org/10.1016/j.resmic.2016.07.001.

715 Penrose D.M., Glick B.R.. Methods for isolating and characterizing ACC deaminase-containing plant

716   growth-promoting   rhizobacteria.   Physiologia   Plantarum,   **2003**,   118,   10-15.

717   https://doi.org/10.1034/j.1399-3054.2003.00086.x.

718 Perrine-Walker F.M., Gartner E., Hocart C.H., et al.. Rhizobium-initiated rice growth inhibition caused by

719   nitric oxide accumulation. Mol Plant Microbe Interact, **2007**, 20(3), 283-292. https://doi.org/10.1094/

720   MPMI-20-3-0283.

721 Ping G.J., Dong W., Ya C.L., et al.. *Transcriptome* Sequencing of *Codonopsis pilosula* and Identification of

722   Candidate Genes Involved in Polysaccharide Biosynthesis. PLOS ONE, **2015**, 10(2): e0117342.

723   https://doi.org/10.1371/journal.pone.0117342.

724 Pitt J.I., Hocking A.D.. Fungi and food spoilage. New York: Springer, **2009**. https://doi.org/10.1007/978-0-

725   387-92207-2.

726 Popescu C.M., Popescu M.C., Vasile C.. Structural changes in biodegraded lime wood. Carbohydrate

727   Polymers, **2010**, 79(2): 362-372. https://doi.org/10.1016/j.carbpol.2009.08.015.

728 Praveen A., Pandey A., Gupta M.. Nitric oxide alters nitrogen metabolism and PIN gene expressions by

729   playing protective role in arsenic challenged *Brassica juncea* L. Ecotoxicology and Environmental

730   Safety, **2019**, 30,176:95-107. https://doi.org/10.1016/j.ecoenv.2019.03.054.

731 Rojo F.G., Reynoso M.M., Ferez M., et al.. Biological control by Trichoderma species of *Fusarium solani*

732   causing peanut brown root rot under field conditions.Crop protection, **2007**, 26, 549-555.

733   https://doi.org/10.1016/j.cropro.2006.05.006.

734 Ruan Y.L., Jin Y., Yang Y.J., et al.. Sugar Input, Metabolism, and Signaling Mediated by Invertase: Roles in

735   Development, Yield Potential, and Response to Drought and Heat. Molecular Plant, **2010**, 3(6):942-

736   955. https://doi.org/10.1093/mp/ssq044.

737 Ruiz N., Dubois N., Wielgosz-Collin G., et al.. Lipid content and fatty acid composition of a marine-

738   derived *trichoderma longibrachiatum* strain cultured by agar surface and submerged fermentations.

739   Process Biochemistry, **2007**, 42, 676-680. https://doi.org/10.1016/j.procbio.2006.09.017.

740 Saravanakumar K., Yu C., Kai D., et al.. Synergistic effect of trichoderma-derived antifungal metabolites

741   and cell wall degrading enzymes on enhanced biocontrol of *fusarium oxysporum* f. sp. cucumerinum,

742   theory and applications in pest management. Biological Control, **2016**, 94, 37-46.

743   https://doi.org/10.1016/j.biocontrol.2015.12.001.

744 Schilirò E., Ferrara M., Nigro F., Mercado-Blanco J.. Genetic responses induced in olive roots upon

745   colonization by the biocontrol endophytic bacterium *pseudomonas fluorescens* PICF7. Plos One,

746   **2012**, 7(11), e48646. https://doi.org/10.1371/journal.pone.0048646.

747 Serrano-Carreon L., Hathout Y., Bensoussan M., Belin J.M.. Lipid accumulation in *Trichoderma* species.

748   Fems Microbiol Letters, **1992**, 93, 181-7. https://doi.org/10.1111/j.1574-6968.1992.tb05087.x

749 Shoresh M., Harman G. E., Mastouri F.. Induced systemic resistance and plant responses to fungal

750   biocontrol agents. Annual Review of Phytopathology, **2010**, 48(1), 21-43.

751   https://doi.org/10.1146/annurev-phyto-073009-114450.

752 Silvestro D., Michalak I.. raxmlGUI, a graphical front-end for RAxML. Organisms Diversity & Evolution,

753   **2012**, 12, 335-337. https://doi.org/10.1007/s13127-011-0056-0.

754 Singh S.P, Singh H.B.. Effect of mixture of Trichoderma isolates on biochemical parameters in leaf of

755   *Macrophomina phaseolina* infected brinjal. Journal of Environmental Biology, **2014**, 35(5):871-876.

756   https://doi.org/10.1007/s11367-014-0772-8.

757 Somers E., Vanderleyden J., Srinivasan M.. Rhizosphere bacterial signalling, a love parade beneath our

758   feet. Critical Reviews in Microbiology, **2004**, 30(4), 205-240.

759   https://doi.org/10.1080/10408410490468786.

760 Stierle A., Strobel G., Stierle D.. Taxol and taxane production by taxomyces andreanae, an endophytic

761   fungus of pacific yew. Science, **1993**, 260(5105), 214-216. https://doi.org/10.1126/science.8097061.

762 Suutari M.. Effect of growth temperature on lipid fatty acids of four fungi *aspergillus niger, neurospora*

763   *crassa, penicillium chrysogenum, andtrichoderma reesei*. Archives of Microbiology, **1995**, 164, 212-

764   216. https://doi.org/10.1007/bf02529973.

765 Cummings M.P.. PAUP* [Phylogenetic Analysis Using Parsimony (and Other Methods)]. Dictionary of

766   Bioinformatics and Computational Biology. John Wiley & Sons, Inc, **2004**.

767   https://doi.org/10.1002/0471650129.dob0522.

768 Tandy S., Healey J.R., Nason M.A., et al.. FT-IR as an alternative method for measuring chemical

769   properties during composting. Bioresour Technol, **2010**, 101:5431–5436.

770   https://doi.org/10.1016/j.biortech.2010.02.033.

771 Tang T., Xie H., Wang Y.X., et al.. The effect of sucrose and abscisic acid interaction on sucrose synthase

772   and its relationship of grain filling of rice (*Oryza sativa* L.). Exp Bot, **2009**, 60(9): 2641–2652. https://

773   doi.org/10.1093/jxb/erp114.

774 Venkatesa S., Ramar G., Kathirvelu B., et al.. Biological properties of endophytic fungi. Brazilian Archives

775   of Biology and Technology, **2016**, 59, 387. https://doi.org/10.1590/1678-4324-2016150436.

776 Verma M., Brar S.K., Tyagi R.D., et al.. Antagonistic fungi, *Trichoderma* spp.:panoply of biological

777   control. Biochemical Engineering Journal, **2007**, 37:1-20. https://doi.org/10.1016/j.bej.2007.05.012.

778 Vijayalakshmi P., Vishnukiran T., Kumari B.R., et al.. Biochemical and physiological characterization for

779   nitrogen use efficiency in aromatic rice genotypes. Field Crops Research, **2015**, 179:132-143.

780   https://doi.org/10.1016/j.fcr.2015.04.012.

781 Vinale F., Sivasithamparem K., Ghisalberti E.L., et al.. *Trichoderma* secondary metabolites active on plants

782   and   fungal   pathogens.   The   Open   Mycology   Journal,   **2014**,   8(1)1544.

783   https://doi.org/10.2174/1874437001408010127.

784 Vinale F., Marra R., Scala F., et al.. Major secondary metabolites produced by two commercial trichoderma

785   strains active against different phytopathogens. Letters in Applied Microbiology, **2010**, 43, 143-148.

786   https://doi.org/10.1111/j.1472-765X.2006.01939.x.

787 Viterbo A., Landau U., Kim S., et al.. Characterization of acc deaminase from the biocontrol and plant

788   growth-promoting agent *Trichoderma asperellum* t203. FEMS Microbiology Letters, **2010**, 305, 42-

789   48. https://doi.org/10.1111/j.1574-6968.2010.01910.x.

790 Vyas P., Rahi P., Chauhan A., Gulati A. Phosphate solubilization potential and stress tolerance of

791   *Eupenicillium   parvum*   from   tea   soil.   Mycological   research,   **2007**,   111,   931-938.

792   https://doi.org/10.1016/j.mycres.2007.06.003.

793 Wang L., Cui N., Zhao X.C., et al.. Accumulations of carbohydrate and the preliminary study of regulation

794   of 14-3-3 protein on sucrose phosphate synthase (SPS) activity in two tomato species. Journal of

795   integrative agriculture, **2014**, 13(2): 358-364. https://doi.org/10.1016/S2095-3119(13)60373-1.

796 Wang Y., Dai C.C., Zhao Y.W., Peng Y.. Fungal endophyte-induced volatile oil accumulation in

797   *Atractylodes lancea* plantlets is mediated by nitric oxide, salicylic acid and hydrogen peroxide.

798   Process Biochemistry, **2011**, 46(3):730-735. https://doi.org/10.1016/j.procbio.2010.11.020.

799 Wenning M., Seiler H., Scherer S. Fourier-transform infrared microspectroscopy, a novel and rapid tool for

800   identification of yeasts. Applied & Environmental Microbiology, **2002**, 68, 4717-21.

801   https://doi.org/10.1128/AEM.68.10.4717-4721.2002.

802 Woudenberg J.H.C., Groenewald J.Z., Binder M., Crous P.W.. Alternaria redefined. Studies in mycology,

803   **2013**, 75: 171-212. https://doi.org/10.3114/sim0015.

804 Yalcinkaya T., Uzilday B., Ozgur R., et al.. Lipid peroxidation-derived reactive carbonyl species (RCS):

805   Their interaction with ROS and cellular redox during environmental stresses. Environmental and

806   Experimental Botany, **2019**, 165, 139-149. https://doi.org/10.1016/j.envexpbot.2019.06.004.

807 Yuan Z.L., Chen Y.C., Zhang C.L., Lin F.C. *Trichoderma chlorosporum*, a new record of endophytic fungi

808   from *dendrobium nobile* in China. Mycosystema, **2008**, 27, 608-610.

809   https://doi.org/10.3969/j.issn.1672-6472.2008.04.021.

810 Yue L., Lian F., Han Y., et al.. The effect of biochar nanoparticles on rice plant growth and the uptake of

811   heavy metals: Implications for agronomic benefits and potential risk. Science of the Total

812   Environment, **2019**, 656:9-18. https://doi.org/10.1016/j.scitotenv.2018.11.364.

813 Zhai X., Jia M., Chen L., et al.. The regulatory mechanism of fungal elicitor-induced secondary metabolite

814   biosynthesis in medical plants. CRC critical reviews in microbiology, **2017**, 43(2):238-261.

815   https://doi.org/10.1080/1040841X.2016.1201041.

816 Zhang F.G., Huo Y.Q., Xu X.X., et al.. Trichoderma improves the growth of *Leymus chinensis*. Biology

817   and Fertility of Soils, **2018**, 54, 685-696. https://doi.org/10.1007/s00374-018-1292-7.

818 Zhang F.G., Yuan J., Yang X.M., et al.. Putative *Trichoderma harzianum* mutant promotes cucumber

819   growth by enhanced production of indole acetic acid and plant colonization. Plant Soil, **2013**, 368,

820   433-444. https://doi.org/10.1007/s11104-012-1519-6.

821 Zhao M., Yuan L.Y., Guo D.L., et al.. Bioactive halogenated dihydroisocoumarins produced by the

822   endophytic fungus *lachnum palmae* isolated from *przewalskia tangutica*. Phytochemistry, **2018**, 148,

823   97-103. https://doi.org/10.1016/j.phytochem.2018.01.018.

824 Zou Y.F., Chen X.F., Malterud K.E., et al.. Structural features and complement fixing activity of

825   polysaccharides from *Codonopsis pilosula* Nannf. var. modesta LT Shen roots. Carbohydrate

826   polymers, **2014**, 113: 420-429. https://doi.org/10.1016/j.carbpol.2014.07.036.

827