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Characterization of a Plant Growth-Promoting Endohyphal *Bacillus subtilis* in *Fusarium acuminatum* from *Spiranthes sinensis*

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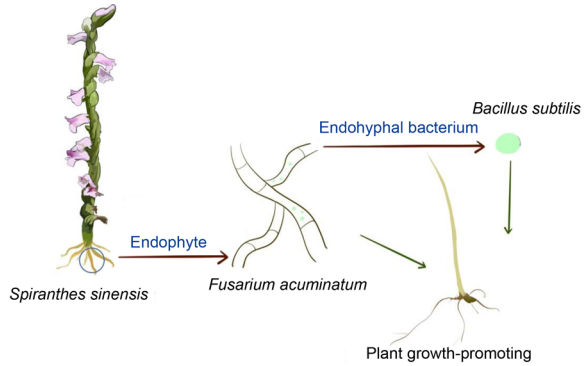
Abstract

Successful seed germination and seedling growth in orchids require an association with mycorrhizal fungi. An endophytic *Fusarium* fungal strain YZU 172038 exhibiting plant growth-promoting (PGP) ability was isolated from the roots of *Spiranthes sinensis* (Orchidaceae). The harboring endohyphal bacteria were detected in the hypha by SYTO-9 fluorescent nucleic acid staining, fluorescence *in situ* hybridization (FISH), and PCR amplification of the 16S rDNA gene's region. Consequently, one endohyphal bacterium (EHB) – a strain YZSR384 was isolated and identified as *Bacillus subtilis* based on morphology, phylogenetic analysis, and genomic information. The results indicated that the strain YZSR384 could significantly promote the growth of rice roots and shoots similar to its host fungus. Its indole acetic acid (IAA) production reached a maximum of 23.361 μg/ml on the sixth day after inoculation. The genome annotation revealed several genes involved in PGP traits, including the clusters of genes encoding the IAA (*trpABCDEFS*), the siderophores (*entABCE*), and the dissolving phosphate (*pstABCS* and *phoABDHPR*). As an EHB, *B. subtilis* was first isolated from endophytic *Fusarium acuminatum* from *S. sinensis*.



K e y w o r d s: endohyphal bacterium, *Fusarium acuminatum*, *Bacillus subtilis*, plant growth-promoting ability

Introduction

Orchid *Spiranthes sinensis* (Pers.) Ames is a Chinese herbal medicine which is rich in flavonoids, phenylpropanoids, and dihydrophenanthrene compounds (Lin et al. 2000; Peng et al. 2007; Peng et al. 2008). It can be used for inflammatory, cancer, diabetic, and other diseases (Gutiérrez 2010; Shie et al. 2015). Because the tiny seeds lack endosperm for successful germination and seedling growth, the orchids, including *S. sinensis*, require an association with endophytic fungi (Zhao et al. 2021). Endophytic fungi enhance nutrient availability to orchid roots and help them survive even upon exposure to pathogens (Sarsaiya et al. 2019). Besides, the fungi provide secondary metabolites promoting seed germination and seedling differentiation (Bhatti and Thakur 2022).

The endophytes in orchids are mostly specific fungi of Basidiomycota, such as Sebacinaceae, Ceratobasidiaceae, and Tulasnellaceae (Zettler and Corey 2018; Li et al. 2021; Wang et al. 2021). In addition, some endophytes from the root, especially *Fusarium* (Ascomycota), are thought to form mycorrhizae with orchids (Tian et al. 2022). Chen et al. (2010) found that *Fusarium* is the dominant endophytic fungi in the medicinal orchid *Dendrobium loddigesii* and could improve its growth rate and biomass. The mycorrhizal fungus *Fusarium oxysporum* of *Bletilla striata* can colonize the root of *B. striata* and *Dendrobium candidum* and promote the vegetative growth of both orchids (Jiang et al. 2019a).

Fusarium has been reported to comprise endohyphal bacteria (EHBs) (Li et al. 2010; Shaffer et al.

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2017), even in orchid root endophytic fungi (Cheng et al. 2022). EHBs inhabiting fungal hyphae were found in Mucoromycota (Desirò et al. 2018), Glomeromycota (Naumann et al. 2010), Ascomycota (Pakvaz et al. 2016), and Basidiomycota (Aslani et al. 2018). EHB can influence the ability of fungal hosts, then the interaction between fungi and their plant hosts by providing bioenergy for fungi (Ghignone et al. 2012; Salvioli et al. 2016). EHB stimulates fungi to produce more phytohormone indole-3-acetic acid (IAA), promoting plant growth (Hoffman et al. 2013; Cheng et al. 2022), regulating fungal secondary metabolism (Partida-Martinez and Hertweck 2005), and enabling fungi to fix nitrogen (Ruiz-Herrera et al. 2015).

During a screening of EHB in the root-associated endophytic fungi of *S. sinensis*, strain YZU 172038 of *Fusarium acuminatum* was found to contain EHB. The aim of this study was to verify the phenomenon of EHB harboring, the EHB isolation and identification, and to determine the PGP ability of the strain YZU 172038 and its EHB.

Experimental

Materials and Methods

EHB detection. The endophytic *F. acuminatum* YZU 172038 of *S. sinensis* was obtained from Pei et al. (2019). It was inoculated on potato dextrose agar (PDA) and cultured at 28°C to collect the mycelia. To determine the presence of bacteria in fungal hyphae, fresh mycelia were picked from two-day-old PDA colonies, stained with 100 µl of 10 µmol/l SYTO-9 green fluorescent nucleic acid solution, well mixed, and incubated for 15–30 min in the dark. Finally, the stained hyphae were observed under the Nikon ECLIPSE Ni-U fluorescence microscope (Nikon, Japan) (Obasa et al. 2017). Fluorescence *in situ* hybridization (FISH) was used to detect further the presence of bacteria in hyphae of *F. acuminatum* YZU 172038. The sample was fixed in 500 µl 4% formalin, dissolved in phosphate-buffered saline (PBS), incubated at 4°C for 5–6 h, washed twice with 500 µl PBS solution in 1.5 ml centrifuge tube, and then dehydrated in solutions of anhydrous ethanol (50%, 70%, and 95%) for 3 min. Dehydrated mycelium was mixed with 10–100 µl probe solution (400 µl formamide, 350 µl diethyl pyrocarbonate (DEPC) water, 250 µl EDTA and 1 µl 10 µM EUB338 probe) and hybridized at 46°C for 1.5 h. A universal 16S rRNA gene oligonucleotide probe of EUB338 (5'-GCTGCCTCC CGTAGGAGT-3') was labeled at the 5-end with FAM (green). Each sample was rinsed in 100 µl wash buffer (460 µl 5 M NaCl, 1 ml 1 M Tris, 50 µl 10% SDS, DEPC water, filled to 50 ml) and warmed to 46°C twice (Hoffman and Arnold 2010). Finally, the mycelium was placed on a slide with the anti-fluorescence quenching agent and observed under the fluorescence microscope.

Meanwhile, the fungal DNA was extracted by the CTAB method (Stenglein and Balatti 2006). The PCR amplification of 16S rDNA region for the genomic DNA was performed using primers of ER10 (5'-GGCGGACGGGTGAGTAA-3') and ER11 (5'-ACTGCTGCCTCCCGTAG-3') to examine its harboring endohyphal bacteria. It was carried out in a T100TM thermal cycler (BIO-RAD, USA) with the following parameters: initial denaturing for 30 s at 94°C, then 30 cycles at 94°C for 10 s, annealing at 55°C for 45 s, then at 72°C for 30 s, with the final extension for 10 min at 72°C (Cheng et al. 2022). The PCR products were visualized and displayed on 1% agarose electrophoresis gel under ultraviolet illumination.

EHB isolation and observation. For the EHB isolation, the endophytic *F. acuminatum* YZU 172038 was inoculated in potato dextrose broth (PDB) to shake at 200 rpm for 72 h at 28°C referring to Obasa et al. (2017). The mycelia were collected and washed twice with sterile distilled water. The mycelial pellets were surface-sterilized with 75% ethanol for 2 min and rinsed with sterile water five times. The fifth washing liquid was spread on Luria-Bertani (LB) agar medium cultured at 28°C for 48 h to examine the possible bacterial contamination. The resulting mycelia were re-suspended in 0.5 ml sterile water and ground well using a sterile mortar. The 100 µl abrasive solution was transferred to 5 ml LB medium containing 100 µg/ml hygromycin B, and then grown at 150 rpm at 28°C for 4 h. The obtained LB broth was diluted and spread on LB agar placed at 28°C to obtain single bacterial colonies. The experiment was repeated twice. The pure cultures were stored in glycerol solution at –80°C.

The purified EHB was inoculated on LB agar, cultured at 28°C for 24 h, and the colony morphology, color, and other traits were observed. Scanning electron microscopy (SEM) was used to observe the morphology and determine the size of EHB. The EHB was inoculated in LB medium, cultured at 28°C, 200 rpm, for 20 h, and the cultures were centrifuged at 8,000 rpm for 5 min to collect the cells. After fixing in 2.5% glutaraldehyde solution for 2–4 h, EHB's cells were washed three times with 0.1 M phosphate buffer (pH 6.8), dehydrated with 30%, 50%, 70%, 90%, and 100% ethanol in turn, for 15–20 min each, replaced twice with isoamyl acetate, and processed in the vacuum freeze dryer for 3 h. After drying, the samples were mounted on stubs, sputter-coated with gold-palladium, and finally examined with SEM (VEGA 3 SBU, Tescan, China) (Jiang et al. 2019b).

EHB identification and verification. Only one EHB strain (YZSR384) was isolated from the mycelia of endophytic *F. acuminatum* YZU 172038 and used

for further assays. It was transferred to the LB medium and incubated at 28°C for 24 h at 200 rpm. The cells were collected by centrifuging, and DNA was extracted with a bacterial genomic DNA extraction kit (OMEGA, China). Its 16S rDNA region was amplified with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3') using the condition of Galkiewicz and Kellogg (2008). The successful PCR products were sent to the TSINGKE company (Beijing, China) for purification and sequencing. The resulting sequence underwent the BLAST analysis in the NCBI database. The present EHB strain and their relevant strains derived from GenBank were aligned in by the ClustalW method implemented in MEGA software (version 7.0). The phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1,000 bootstrapped replications to estimate evolutionary distances (Kumar et al. 2016). *Pseudomonas aeruginosa* JCM 5962 was used as an outgroup.

Since the EHB appeared as *Bacillus subtilis* after the sequence analysis, a specific primer pair of EN1F (5'-CCAGTAGCCAAGAATGGCCAGC-3') and EN1R (5'-GGAATAATCGCCGCTTTGTGC-3') (Ashe et al. 2014) specific for *B. subtilis* was used to verify the existence of EHB in the endophytic fungus. Both previous fungal and EHB's genomic DNA were amplified with initial denaturing for 5 min at 94°C, then 10 cycles for 30 s at 94°C, annealing at 70°C for 20 s with the temperature decrease of 1°C every cycle, then 45 s at 74°C, followed by 25 cycles for 30 s at 94°C, annealing at 60°C for 20 s, than 45 s at 72°C, with the final extension for 10 min at 72°C. The PCR products were electrophoresed in 1% agarose gel and visualized under UV transillumination.

Plant growth-promoting (PGP) assays. To determine the PGP ability of the endophytic fungal strain YZU 172038, a mycelial plug (6 mm in diam.) was inoculated into 100 ml PDB medium incubated at 28°C for 7 d with 200 rpm rotation. After centrifugation, the supernatant was filtered with a bacterial filter of 0.22 μm pore size. Plump, healthy rice seeds were selected and sterilized with 2% sodium hypochlorite for 1 min, followed by 70% ethanol solution for 30 s, and rinsed with sterile water three times. The surface sterilized seeds were soaked in the fermentation supernatant for one day (Gholamalizadeh et al. 2017). Rice seeds (20 per plate) were transferred to sterile Petri dishes (n = 3) containing two layers of sterilized filter papers moistened with distilled water, kept in the dark incubator at 28°C for five days. PDB treatment was used as a control. The experiment was repeated two times.

To determine the PGP ability for the EHB strain YZSR384, it was inoculated into the demineralized LB medium at 28°C, 200 rpm overnight, and adjusted to an appropriate concentration ($OD_{600} = 1.0$). The previously mentioned sterile rice seeds were soaked in the bacterial culture for one day, and the PGP ability was assayed. The demineralized LB medium was used as a control.

IAA production of EHB. To determine the IAA production in EHB strain YZSR384, the Salkowski colorimetric method was used to screen (Bhutani et al. 2018). The EHB was inoculated into the LB medium containing L-tryptophan (100 mg/l) and grown at 28°C with 200 rpm rotation. 3 ml of culture broth (n = 3) was taken every day. After centrifugation, 2 ml of supernatant was treated with 2 ml of Salkowski reagent (1 ml of 0.5 mol/ml $FeCl_3$ and 50 ml 35% $HClO_4$). After incubation at room temperature in the dark for 30 min, the optical densities were measured at 530 nm with a spectrophotometer (BIO-RAD, USA). The LB medium containing 10 mg/ml L-tryptophan was used as a control. The test was repeated twice, and IAA concentration was evaluated with the standard curve resulting from different IAA concentrations.

EHB genomic analysis. The genomic DNA of EHB strain YZSR384 was extracted using the same method previously for the EHB identification. The whole-genome sequencing was completed by the BENAGEN company (Wuhan, China) using the third-generation of Nanopore sequencer and the second-generation Illumina HiSeq technology (Cock et al. 2010; Büttner et al. 2019). Freely available software Unicycler (0.4.8) (https://github.com/rrwick/Unicycler) was used to assemble the obtained data. The coding genes of the assembled genome were predicted by the Prokka software (1.1.2), and the predicted gene sequences were compared with COG, KEGG, Swiss-Prot, Refseq, and other functional databases by BLAST to obtain the gene function annotation results (Seemann 2014).

Statistical Analysis. The data were statistically analyzed by using SPSS for Windows 17.0 (SPSS Inc., USA). One-way ANOVA was applied to confirm variability and validity of the results. Duncan's multiple range test was performed to determine the significance between the treatments at $p = 0.05$.

Results

EHB existence in *F. acuminatum* YZU 172038 hyphae. Under the fluorescence microscope, many bacterial nuclei were found in the hyphae stained with the fluorescent SYTO-9 (Fig. 1A and 1B). In addition, the hybridized bacteria were also clearly observed in the hyphae of *F. acuminatum* YZU 172038 (Fig. 1C and 1D). A 255 bp PCR fragment was obtained after amplifying endophytic *F. acuminatum* YZU 172038 genomic DNA using primers ER10 and ER11 designed for a partial sequence of 16S rDNA region (Fig. 1E). These results indicated that EHB existed in the hyphae of *F. acuminatum* YZU 172038.

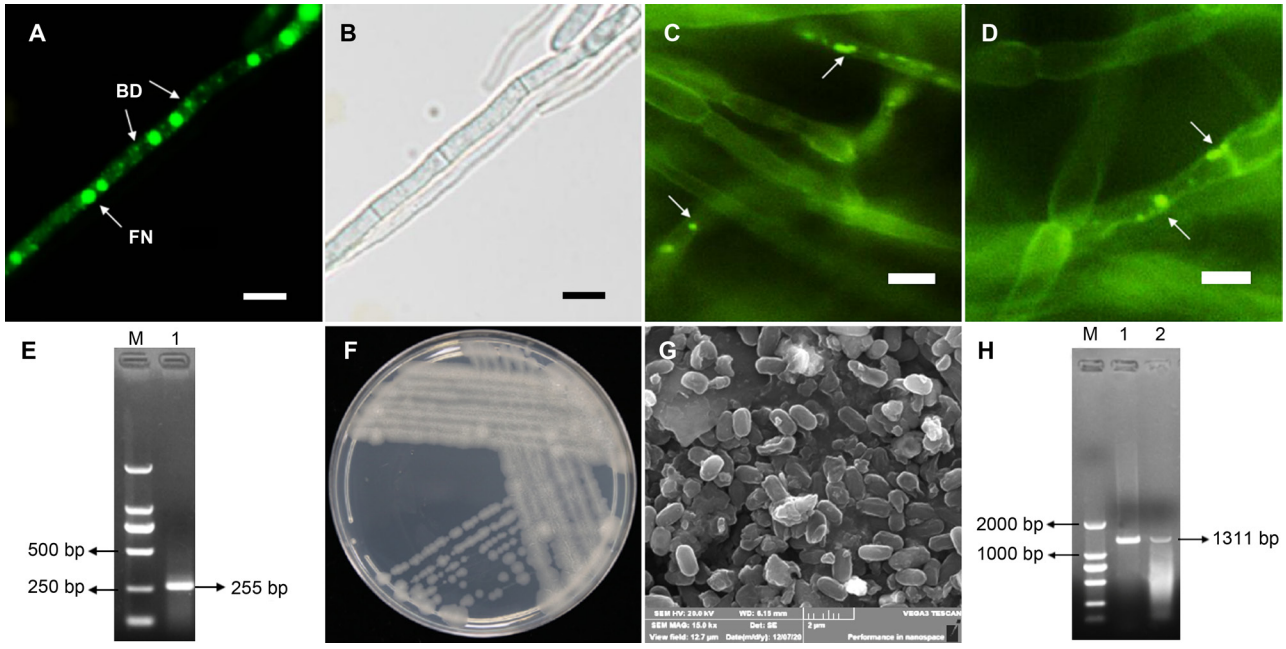

Fig. 1. The endohyphal bacteria in *Fusarium acuminatum* YZU 172038.
(A, B) Fluorescent SYTO-9 nucleic acid stained hyphae under fluorescent microscopy (FN – fungal nucleus, BD – bacterial DNA), scale bars = 5 μm; (C, D) bacteria (green signals pointed by white arrows) in hypha detected by the FISH method, scale bars = 5 μm; (E) PCR amplification of the 16S RNA gene (M – 2000 bp maker, 1 – YZU 172038); (F) colony morphology of EHB YZASR384 on LA medium; (G) scanning electron microscopy of EHB YZASR384; (H) PCR amplification of endoglucanase gene (1 – EHB YZSR384, 2 – YZU 172038).

EHB identification and verification. The colonies of EHB YZSR384 grown on the LA plate were round, with irregular edges, and milky white (Fig. 1F). Under scanning electron microscope (SEM), the EHB cells were rod-shaped, with 1–1.5 × 0.6–0.8 μm in size (Fig. 1G). The resulting 16S rDNA sequence (accession no. OP108437) had 98% identity to *B. subtilis* after BLAST searches, with a high homology to the type strain IAM 12118 of *B. subtilis* (MK267098). The phylogenetic tree (Fig. 2) showed that YZSR384 and IAM 12118 form a clade with a high bootstrap value of 98%. Therefore, based on morphological and phylogenetic analysis, the EHB YZSR384 was identified as *B. subtilis*. To further confirm the EHB existence, the genomic DNA of fungal strain YZU 172038 and its EHB YZSR384 were amplified with specific endoglucanase primer pairs of EN1R and EN1F for *B. subtilis*. The exact length of positive bands around 1,311 bp was clearly visualized under UV transillumination after electrophoresis (Fig. 1H).

Plant growth-promoting (PGP) assays and the EHB IAA production. During PGP assays upon rice seed ger-

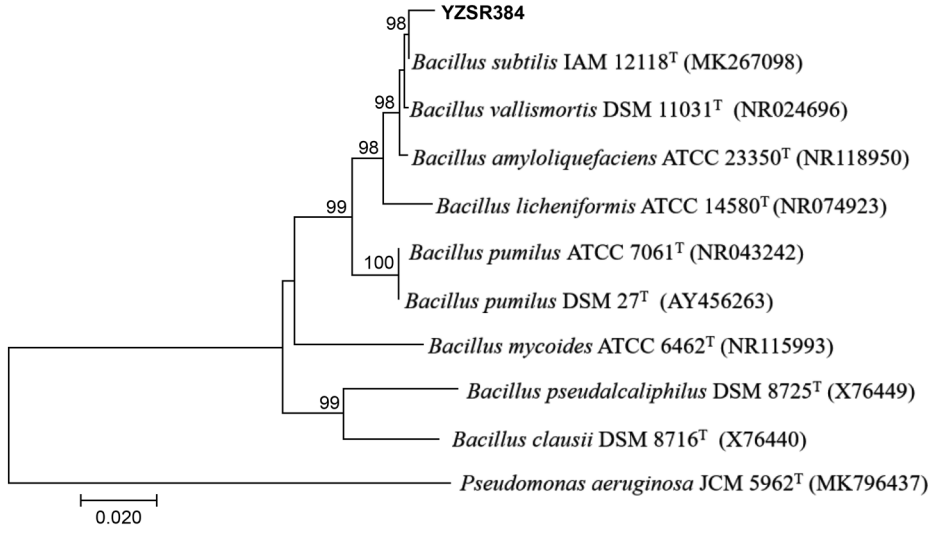

Fig. 2. Phylogenetic tree of endohyphal strain YZSR384 using the neighbor-joining method based on the 16S rRNA gene sequences. Bootstrap values (%) presented at the branches were calculated from 1,000 replications.

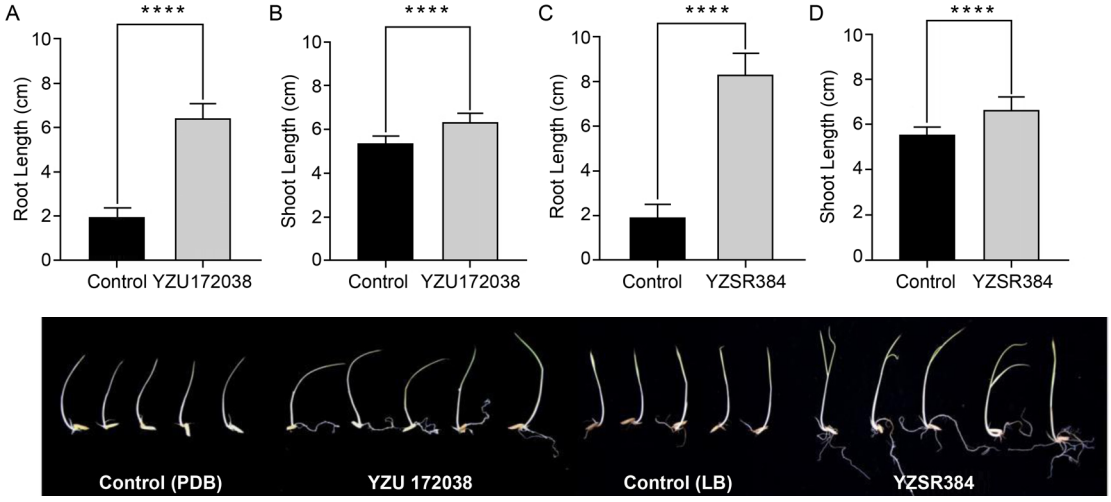

Fig. 3. Plant growth promoting (PGP) assays for rice seeds using the culture broth of endophytic *Fusarium acuminatum* YZU 172038 (A, B) and its EHB *Bacillus subtilis* YZSR384 (C, D). The controls were PDB broth (A, B) and demineralized LB liquid (C, D).

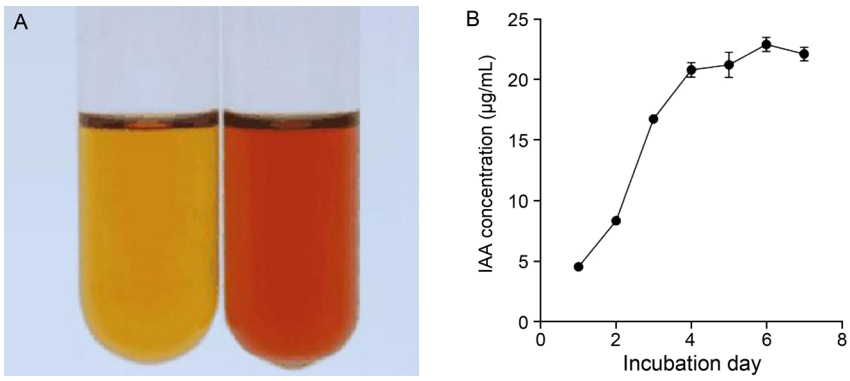

Fig. 4. Detection of the IAA production in EHB YZSR384 culture broth.
(A) Salkowski test of the negative control (left) and the EHB YZSR384 broth (right).
(B) The IAA concentration in different incubation times.

mination, the endophytic *F. acuminatum* YZU 172038 and its EHB YZSR384 cultures could promote the root and shoot growth. For the fungal strain YZU 172038, the root (6.44 ± 1.27 cm) and shoot (6.34 ± 0.82 cm) length was significantly increased compared to negative controls (1.99 ± 0.81 cm and 5.36 ± 0.57 cm, respectively, $p < 0.01$). The roots and shoots (8.32 ± 1.32 cm and 6.69 ± 0.78 cm) treated with EHB YZSR384 were significantly longer than the controls (1.93 ± 0.72 cm and 5.56 ± 0.48 cm, respectively; $p < 0.01$). The development and growth of rice roots were promoted in both strains compared to the rice shoots (Fig. 3).

The PGP-related IAA production was determined by the Salkowski method, which resulted in the color changes of the EHB broth (Fig. 4A). The results indicated that EHB YZSR384 could produce IAA. In the quantitative analysis of IAA production, it appeared that IAA was produced in the highest concentration on the sixth day, reaching 23.361 μg/ml after inoculation. Then the concentration began to decline (Fig. 4B).

Genome sequencing, assembly, and annotation. The genomic analysis of the EHB YZSR384 (accession no. CP102769) indicated that the strain's genome had a size of 4,215,636 bp with 43.51% GC content, contained 4,444 genes, 4,216 coding genes, and 118 RNA genes (including 30 rRNA genes, 87 tRNA genes, and 1 tmRNA gene). The other gene prediction is shown in Table I. These results confirmed that the EHB is *B. subtilis*.

Table I
Genome properties of EHB *Bacillus subtilis* YZSR384.

Feature	*Bacillus subtilis* YZSR384
Genome size (bp)	4,215,636
GC content (%)	43.51
Total number of genes	4444
Number of CDSs	4216
tRNA genes	87
rRNA genes	30
tmRNA genes	1
Genomic islands	4
Genes allocated to Uniprot	4,157
Genes allocated to Refseq	4,180
Genes allocated to KEGG	2,330
Genes allocated to Pfam	3,761
Genes allocated to GO	3,359
Genes allocated to COG	2,816

Table II
Genes associated with plant growth-promoting (PGP) traits for EHB *Bacillus subtilis* YZSR384.

PGP activity	Gene	Function	Chromosome location
IAA production	*trpS*	Tryptophanyl-tRNA synthetase	1218699–1217707 –
	trpA	Tryptophan synthase subunit alpha	2371911–2371108 –
	trpB	Tryptophan synthase subunit beta	2373106–2371904 –
	trpF	Phosphoribosylanthranilate isomerase	2373734–2373087 –
	trpC	Indole-3-glycerol phosphate synthase	2374491–2373739 –
	trpD	Anthranilate phosphoribosyltransferase	2375500–2374484 –
	trpE	Anthranilate synthase	2377019–2375472 –
Phosphate solubilization	*phoD*	Alkaline phosphatase	283609–285360 +
	phoB	Alkaline phosphatase	621083–619695 –
	phoA	Alkaline phosphatase	1018051–1016675 –
	phoH	Phosphate starvation-inducible protein	2615049–2614090 –
	PhoP	Alkaline phosphatase synthesis transcriptional regulatory protein	2978117–2977395 –
	phoR	Two-component sensor histidine kinase	3236753–3238354 +
	pstB	Phosphate ABC transporter ATP-binding protein	2577585–2576803 – 2578405–2577596 –
	pstA	Phosphate ABC transporter permease	2579310–2578426 –
	pstC	Phosphate ABC transporter permease	2580239–2579310 –
	pstS	Phosphate ABC transporter substrate-binding protein	2581210–2580308 –
Siderophore production	*entB*	Isochorismatase	552212–552754 + 3288208–3287270 –
	entC	Isochorismate synthase	3153313–3151898 – 3153500–3153375 – 3291891–3291106 –
	entE	2,3-dihydroxybenzoate-AMP ligase	3289855–3288236 –
	entA	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	3291080–3289884 –

Genome annotation revealed several genes that contributed directly or indirectly to PGP abilities, i.e., IAA production, phosphate solubilization, and siderophore production (Table II).

Discussion

Endohyphal bacteria (EHBs) exist in a variety of fungi which species and number are also variable within the same genus or family (Hoffman and Arnold 2010; Shaffer et al. 2016). EHBs in *Fusarium* have been studied and isolated, and the effect of EHB presence on its host fungi has already been recognized. EHB *Chitinophaga* sp. can promote the utilization of carbon sources in *Fusariun keratoplasticum* from *Fusarium solani* species complex (Shaffer et al. 2017). *Enterobacter* sp. increases *Fusarium fujikuroi* virulence (Obasa et al. 2020), and *Klebsiella aerogenes* enhances the growth-promoting ability of *F. oxysporum* (Cheng et al. 2022). In this study, EHB was isolated from an endophytic *F. acuminatum* YZU 172038 of *S. sinensis* and exhibited the plant growth-promoting (PGP) ability. This EHB was identified as *B. subtilis* based on morphological, phylogenetic, and genomic analyses. To our knowledge, it is the first time that *B. subtilis* has been found in *F. acuminatum*. The results also enrich the EHB resources of *Fusarium* fungal endophytes.

Bacillus is a worldwide bacterium that exists in various environments and can produce different compounds involved in the biological control of plant pathogens and the promotion of plant growth (Miljaković et al. 2020). Furthermore, many reports describe EHB *Bacillus* helping their host fungi in many aspects. EHB *Bacillus pumilus* can aid the maize pathogen *Ustilago maydis* to fix nitrogen (Ruiz-Herrera et al. 2015). EHBs *B. pumilus* and *B. subtilis* can improve fungal competitiveness by inhibiting other endophytes in plants (Pakvaz et al. 2016). EHBs *Bacillus* spp. (*Bacillus anthracis*, *Bacillus thuringiensis*, and *Bacillus mycoides*) in wild mushrooms exhibit biological control potential (Aslani et al. 2018). In this study, EHB *B. subtilis* YZSR384 had significant PGP effects on rice seeds to improve their development and growth of rice root and shoot. It could help improve the PGP ability of its host *F. acuminatum* YZU 172038. More work shall be conducted in the future on this topic.

Bacillus can promote plant growth in numerous ways, such as IAA production, phosphate solubilization, siderophore production, and nitrogen fixation (Kashyap et al. 2019). IAA production was found in EHB *B. subtilis* YZSR384 broth as it was determined by the Salkowski colorimetric technique. Since tryptophan is the precursor of auxin synthesis, the comprehensive genomic analysis identified the presence of the *trpABCDEFS* genes in EHB YZSR384 (Table II); tryptophan-linked genes in the genome might implicate the IAA biosynthesis (Singh et al. 2021). Furthermore, *B. subtilis* uses the phosphate-specific transport (*pst*) system for transporting free inorganic phosphate. The *pst* operon of *B. subtilis* contains the *pstS*, *pstC*, pstA, *pstB* (*pstB*1 and *pstB*2) genes (Xie et al. 2016). Besides, phosphate (Pho) regulon genes are expressed in response to phosphate starvation and regulated by the PhoP-PhoR two-component regulators (Santos-Beneit 2015). In the present study, the genome of EHB YZSR384 revealed the existence of the *pstABCS* and *phoABDHPR* genes.

The siderophores synthesized by *Bacillus* are diverse. Catecholate siderophores are synthesized in the cytoplasm when iron starvation occurs, and their biosynthesis requires seven enzymes encoding EntA-F and H. (Pakarian and Pawelek 2016). A siderophore-associated transport cluster (*entABCE*) was found in the genome of YZSR384. This genomic information underlined the PGP potential of EHB *B. subtilis* YZSR384 suggesting its role as a plant growth promoter.

EHB can assist the host fungi in surviving in unfavorable environments (Ghignone et al. 2012; Salvioli et al. 2016) and perform specific biological functions living outside the fungus (Ruiz-Herrera et al. 2015; Pakvaz et al. 2016; Aslani et al. 2018). It indicates that EHB is also a potential biological rich resource, which is worthy of further exploration and development. The present EHB could be used for the development of PGP candidates.

Acknowledgments

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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