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Allelopathic potential of root endophytic bacterial metabolites on seeds germination of *Casuarina equisetifolia*

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ABSTRACT

We determined the role of endophytic bacteria of *C. equietifolia*, involved in synthesis of allelochemicals. The root endophytic bacteria were isolated and their biological activity was studied to determine their allelopathic potential in fermentation broth. Twenty one species of endophytic bacteria were isolated from the *C. equisetifolia* roots, of which *Bacillus* were the dominant genus, *Acinetobacter* and *Staphylococcus* were other dominant genera. These 21 endophytic bacteria inhibited the seeds germination of *C. equisetifolia*, among which *Bacillus amyloliquefaciens* was most inhibitory. Main allelopathic components of fermentation broth of 3-endophytic bacteria (*Bacillus amyloliquefaciens*, *Bacillus aryabhattai* and *Paenibacillus glycanilyticus*) with strongest allelopathic potential were rich in phenols, esters, organic acids, aldehydes, alcohols, ketones etc. Among them, two allelopathic substances [2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-Phenol and 1,2,3,4-Butanetetrol] were present in both endophytic bacteria fermentation broth and in root and soil extracts. These results confirmed that the secondary metabolites of *C. equisetifolia* root endophytic bacteria were allelopathic.

Keywords: *Acinetobacter,* allelochemicals, allelopathic potential, *Bacillus, Casuarina equisetifolia,* inhibitory effects, metabolites, root endophytic bacteria, seeds germination, *Staphylococcus.*

INTRODUCTION

Casuarina equisetifolia L. is the main tree specie of coastal shelter belt in Hainan Province and had been planted >60,000 hm^2. It plays an important role in restoring, improving and protecting the coastal ecosystems. This shelter Belt also acts as wind break, prevents sand erosion and improves the soil fertility etc. (30). However, the *C. equisetifolia* forests face self-sustaining problems with increasing age due to senescence, difficulty in regeneration and the prevalence of serious diseases, all these decreases its efficiency. One of the primary causes of these problems is allelopathy (3,10).

Allelopathy is defined as "the direct or indirect harmful or beneficial effects of a plant (including microorganisms) on another plant (including microorganisms) by releasing chemicals into the environment"(16). Previous studies have shown that the formation of allelopathic substances and allelopathy are closely related to microorganisms. The allelopathic substance are not only toxic to the plant roots, but also changed the community structure of soil (27,32). Our previous studies have shown that rhizosphere microorganisms are one of the sources of *C. equisetifolia*'s allelochemicals (7,11,25). The endophytes in plants form the symbiotic relationship with the host and mediate the physiological processes of the host. Thus, we speculate that endophytes may be involved in the synthesis of allelochemicals in *C. equisetifolia.*

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We did preliminary study on the relationship between endophytic fungi and allelopathy of *C. equisetifolia*. Four endophytic fungi [*Aspergillus aculeatus* Izuka, *Penicillium melinii* Thom, *Neosartorya fisccheri* (Wehmer) Malloch & Cain BGA and *Penicillium solitum* WestlingBGB) were isolated from *C.equisetifolia* roots of different ages(7). The 2,4-di-tert-butylphenol, stearic acid and palmitic acid were detected in the fermentation broth of these fungi. These substances were also detected in the extracts of *C. equisetifolia* soil, roots and litter, which proved that endophytic fungi were involved in the synthesis of these allelochemicals. However, there is no research to suggest that endophytic bacteria of *C. equisetifolia* are also involved in the synthesis of allelochemicals.

In this study, we used traditional standard methods to isolate the endophytic bacteria of *C. equisetifolia*. The biosensitivity method was used to determine the allelopathic potential of different endophytic bacterial fermentation broths on the seeds germination of *C. equisetifolia*. Besides, the components of 3-endophytic bacteria (*Bacillus amyloliquefaciens*, *Bacillus aryabhattai* and *Paenibacillus glycanilyticus*) fermentation broth with the strongest inhibitory allelopathic potential were identified by GC-MS, and compared with those in *C. equisetifolia* root and soil extract, to explore their common substances. The results could provide theoretical basis to prove the involvement of endophytic bacteria in allelopathy.

MATERIALS AND METHODS

C. equisetifolia roots (about 1.5 cm dia) were collected from the coast of Guilinyang, Haikou, Hainan Province in October 2017（N20°01′02″, E110°31′20″, Mean annual temperature: 24.1°C, mean annual rainfall: 1760 mm). After collection, the samples were transported to the laboratory in ice boxes with ice bags and stored at -20 °C. *C. equisetifolia* seeds were collected from its forest in Dongao Town, Wanning, Hainan Province in October 2018.

Figure 1. The sampling sites of *Casuarina equisetifolia* and its single plant

1.2 Isolation and identification of endophytic bacteria

The skin of fresh root samples was peeled and rinsed thrice with sterile water. The root surfaces were disinfected by soaking in 75 % ethanol for 30 s, rinsed thrice with sterile water, rinsed in 5 % NaClO for 5 min, and then rinsed again thrice with sterile water (20). The sterile water of last flush material was added in Nutrient AgarNA, Luria-Bertani (LB) and Gauze's Synthetic Agar（G） culture medium as surface disinfection control (13,21). The roots were shredded and grinded fully with electric grinder. Fifty µl of was coated on the petri plate on NA, LB and G culture medium and incubated at 30 ℃ for 3-5 days. After development of bacterial colonies on the plate, the endophytic bacteria strains were isolated and purified repeatedly by plate streaking method until a single pure colony was obtained, and the bacteria were numbered and preserved. The purified single colony was cultured for 48 h and the colony morphology was recorded. The colonies were taken for dilution, smear, Gram staining, morphology and staining of colonies were observed under a microscope.

1.3 DNA extraction

Total genomic DNA was extracted with an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions. The integrity of DNA was validated by agarose gel electrophoresis. The quality of DNA samples was checked with spectrophotometer (Nanob Drop, ND2000, Thermo Scientific, Wilmington, DE, USA) after extraction.

1.4 PCR amplification

The universal 16S rRNA gene primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were chosen for the amplification. Each 50-µl PCR mixture contained 25 µl Green Taq Mix, 1.0 µl Forward primer (10µM), 1.0 µl Reverse primer (10 µM), 1.0 µl PCR template, 22µl ddH_2O. PCR was performed as follows: 95 ℃ for 5 min, then 30 cycles of 95 ℃ for 30 s, 57 ℃ for 30 s and 72 ℃ for 2 min; with a final extension at 72℃ for 8 min. Products were purified and recovered by agarose gel electrophoresis. The recovered products were quantified with Pico Green using a QuantiFluorTM -ST, and equimolar concentrations of PCR products for each sample were pooled.

1.5 DNA sequencing and Construction of phylogenetic Tree

Sequencing was conducted by Guangzhou Saizhe Biotechnology Co., Ltd. Blast was used to compare the sequencing results with the known sequences in Genbank to find the strains with the highest similarity. To further determine the evolutionary status of the strains, the phylogenetic tree was constructed by Neighbor-Joining method of MEGA 6 software (1,15).

1.6 Preparation of endophytic bacterial fermentation broth

Twenty-five mL bacterial liquid culture was added separately to 500ml nutrient broth [Nutrient Agar (NA): peptone 10 g, beef extract 3 g, NaCl 5 g, AGAR 20 g, distilled water 1000 ml, pH7.2 ~ 7.4] respectively, and then fermented under aeration on shaker (150r/min, 28 °C) for 30-38h. In preliminary observations, the growth of bacteria entered a stationary stage after 30 h. The fermentation broth was centrifuged at 6000 rpm for 10 min at room temperature and then the supernatant was filtered through 0.22 µm microporous membrane. The filtrate was divided into two parts, one was used to determine the allelopathic potential and the other one was freeze dried by rotating for 30min at 50℃. The resulting samples were extracted by (i) 2 mL methanol and (ii) 2 mL n-hexane and the extract was filtered through 0.22 um microporous membrane.

1.7 Allelopathic potential

C. equisetifolia seed germination was determined using the petri dish filter paper method. The seeds were soaked in gibberellin solution (400mg/L) for 24 h and then washed with tap water 5 times. One hundred seeds of uniform size were selected and placed evenly in petri dish (15 cm dia), lined with two layers of filter paper. Ten mL fermentation broth of endophytic bacteria was added and the control group was distilled water. All treatments were replicated thrice in complete randomised design. The Petri plants were kept in incubator (32 ºC, 75 % humidity, 12 h light and 12 h dark). Then 2-3 Ml bacterial fermentation broth was added daily to keep the filter paper moist. The seeds germinated were recorded per day until the number of seeds germinated did not change. The germination rate and allelopathy effect index (Williamson, 1988) were calculated as under:

Germination rate (%) = number of germinated seeds / total number of seeds tested×100%.

Allelopathic effect index was assessed as Response Index (RI) as under:

$$RI = 1-C/T,\ T \geq C \text{ or } RI = T/C-1,\ T < C$$

Where, C: Control T: Treatment when RI > 0: Indicates promoting effect; RI < 0: Indicates inhibition effect.

1.8 Identification of secondary metabolites of endophytic bacteria

Methanol and n-hexane extracts of three endophytic bacteria (*Bacillus amyloliquefaciens*, *Bacillus aryabhattai* and *Paenibacillus glycanilyticus*) allelopathic fermentation broths against *C. equisetifolia* were identified by gas chromatography-mass spectrometry (GC-MS) (Thermo Finnigan 120150-T230L).

GC-MS: The chromatographic column was HP-5MS capillary column and the stationary phase was (5%-phenyl)-methylpolysiloxane. It was bombarded with electron bombardment source, the voltage was 70eV, the scanning speed was 0.4s in the scanning range of m/z 30-450 amu, the scanning process was carried out, and the temperature of ion source was 250 ºC. Capillary column specification 30 m×0.01 mm×0.25 mm, injection port temperature 280 ºC, column temperature 120 ºC (3 min, at 15 ºC / min program to 250 ºC, maintain 3 min); The carrier gas was Helium, flow rate 1 mL/min, injection volume 1ul. The identified samples were analyzed by area normalization method, and the retrieval database was NIST 08 MS Library and AMDIS.

1.9 Statistical Analyses

SPSS16.0 was used for variance analysis of data, and Microsoft Excel was used for drawing analysis. The chromatographic peaks in total ion chromatography diagrams of different endophytic bacterial fermentation broth were searched automatically in Willey mass spectrometry database. The components in the mixture were identified and the peak area was normalized, and the relative percentage content of each component was calculated. The chemical dictionary (http://cheman.chemnet.com/dict/zd.htm) was used to query the CAS number.

RESULTS AND DISCUSSION

Isolation and identification of endophytic bacteria

A large number of colonies with clear size and different colony morphology were visible on the culture medium plates inoculated with root extracts of experimental group.

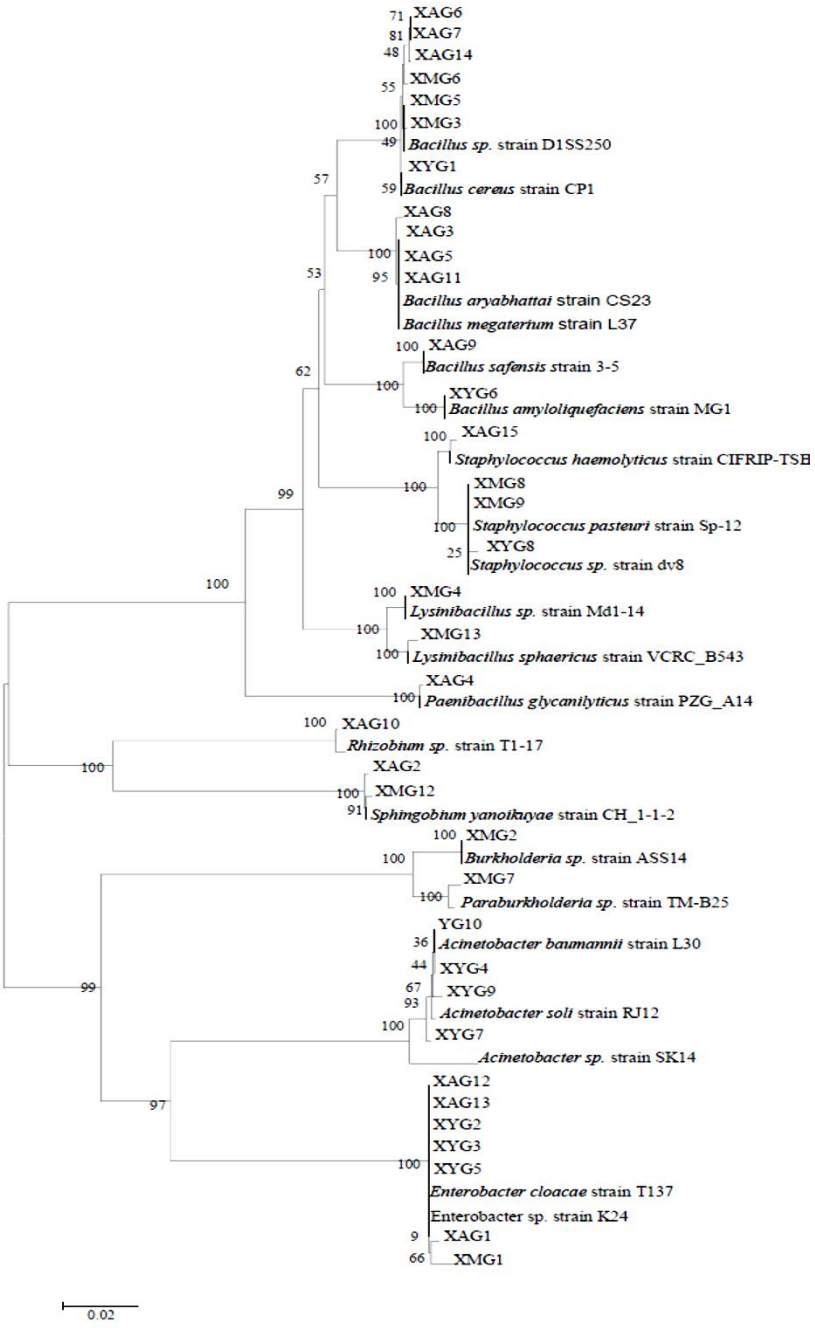


Figure 2. Phylogenetic Tree of 16S rDNA Sequence of Endophytic Bacteria.

After 3 to 4 times isolation and purification, 36 strains of endophytic bacteria were isolated from the *C. equisetifolia* roots. The morphology of purified colony was observed after Gram staining. Among them, 17 strains were Gram-negative bacteria and the other 19 strains were Gram-positive bacteria. On the other hand, the control did not show any growth indicating that the test material was sterilized.

The 16s rDNA sequences of 36 strains of endophytic bacteria were compared with those in NCBI (National Center for Biotechnology Information) by Blast. The results showed that the 16s rDNA sequences of all endophytic bacteria had > 98 % homology with the known sequences in NCBI and then built the phylogenetic tree (Fig. 2).

According to the phylogenetic tree, 13 strains of endophytic bacteria had high homology with different species of Bacillus; 7 strains had high homology with different species of *Enterobacter*; 4 strains had high homology with different species of *Acinetobacter*; 4 strains had high homology with different species of *Staphylococcus*; 2 strains had high homology with different species of *Lysinibacillus*, 2 strains had high homology with different species of *Sphingobium*; 2 strains had high homology with *Burkholderia*; One strain had high homology with *Paenibacillus* and *Rhizobium*, respectively. We found that these 36 strains of endophytic bacteria belonged to 9 genera and 21 species. Among them, 6 species were of *Bacillus*, *Acinetobacter* and *Staphylococcus* each had 3 species, *Enterobacter*, *Lysinibacillus* and *Burkholderia* each had 2 species, *Sphingobium*, *Paenibacillus* and *Rhizobium* each had one specie, respectively.

Seeds germination of *C. equisetifolia* seeds

The effects of different endophytic bacteria fermentation broth on the seeds germination of *C. equisetifolia* are shown in Fig. 3. The various endophytic bacteria fermentation broths were inhibitory to seeds germination than control. In addition, the germination was also delayed. The seeds in control, germinated on third day, while the seeds treated with fermentation broth of *Burkholderia sp.*, *Sphingobium yanoikuyae* and *Acinetobacter baumannii* germinated on fourth day, the other experimental group germinated on fifth day i.e. 2 days later than control. The treatment with endophytic bacteria fermentation broth also inhibited the germination to varying degrees. The germination peak of control group was on 3rd day, while in experimental groups it was on 7th day, i.e. 4 days later than control group.

The allelopathic effect index of different endophytic bacteria fermentation broth on *C.equisetifolia* seeds was shown in Fig 3. The different endophytic bacterial fermentation broth has different degrees of allelopathic effects on *C. equisetifolia* seeds. The allelopathic effect index of *Bacillus amyloliquefaciens* fermentation broth was the strongest, which was -1. However, the allelopathic effect index of *Bacillus aryabhattai* and *Paenibacillus glycanilyticus* fermentation broth was the second highest, with -0.99 and -0.94, respectively, which significantly differed from control other experimental group. In addition, *Bacillus cereus* and *Burkholderia sp.* fermentation broth showed the weakest allelopathic effect index on *C. equisetifolia* seeds and there were significant differences with other experimental groups, and their allelopathic effect index was -0.008 and -0.041, respectively.

Function prediction of endophytic bacteria

The above experiments results showed that 3-kinds of bacteria (*Bacillus amyloliquefaciens*, *Bacillus aryabhattai* and *Paenibacillus glycanilyticus*) have strong allelopathic effects, including *Bacillus amyloliquefaciens*, *Bacillus aryabhattai* and *Paenibacillus glycanilyticus*, so based on the KEGG Database (Kyoto Encyclopedia of genes and genomes), we selected the dominant genus of endophytic bacteria (*Bacillus*) to predict and analyze the functions of dominant bacteria. The results of metabolic pathway

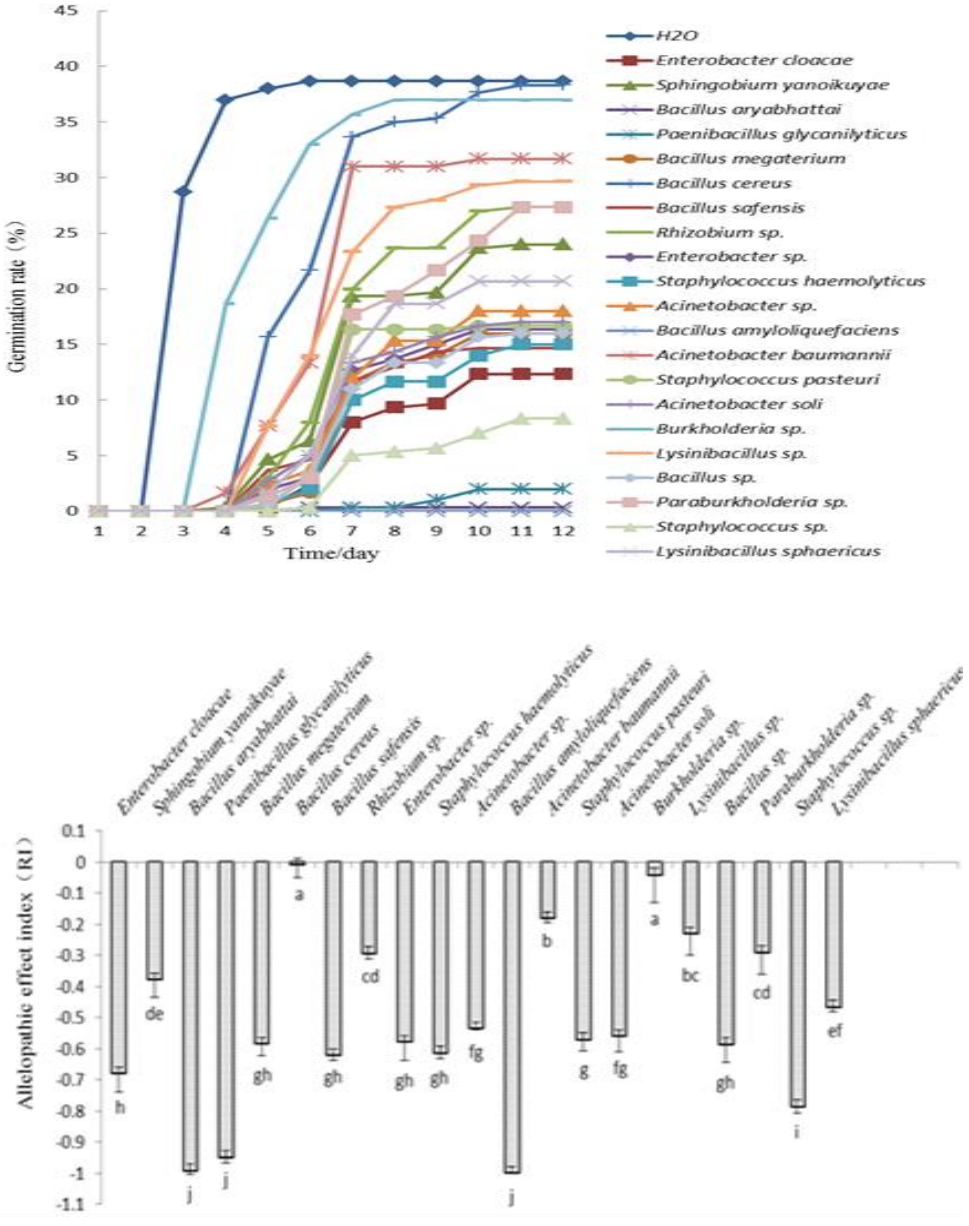


Figure 3. Effect of endophytic bacterial fermentation broth on seed germination of *C. equisetifolia* and allelopathy index.

of samples in KEGG Database were shown in the Fig 4. Metabolism is the primary function at the first level. Thus the results of Annotation Data at the second level, showed that the main annotation in the sample is related to carbohydrate metabolism, amino acid metabolism and lipid metabolism. Then we predicted the Tertiary Function based on the secondary function.

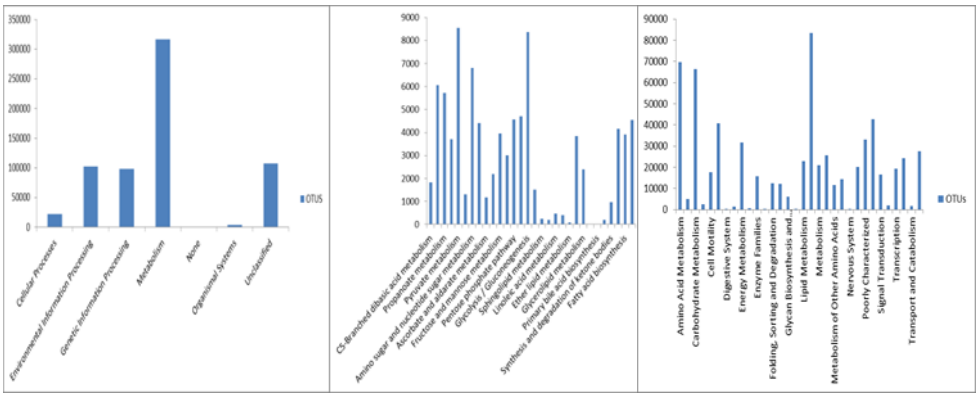


Figure 4. The metabolic pathways of KEGG

The results of "lipid metabolism" metabolic pathway annotation of samples in KEGG Database (at the third level). The results showed that lipid biosynthesis, fatty acid metabolism, fatty acid biosynthesis, glycerophospholipid metabolisms were mainly annotated in the samples of this experiment. On the other hand, the annotation results of "carbohydrate Metabolism" in KEGG database (at the third level). The results showed that butanoate metabolism, pyruvate metabolism, amino sugar and nucleotide sugar metabolism and glycolysis/gluconeogenesis were mainly annotated in the samples.

Identification of secondary metabolites of endophytic bacteria by GC-MS

The fermentation broths of 3-endophytic bacteria (*Bacillus amyloliquefaciens*, *Bacillus aryabhattai* and *Paenibacillus glycanilyticus*) were extracted with methanol and n-hexane and their components were identified by GC-MS. The total ion flow chromatography results are presented in Fig.5-Fig.7.The analysis of extracts of various endophytic bacteria fermentation broths differed. Their components were identified by GC-MS standard mass spectrometry database, and their relative contents were determined by Area Normalization Method (Table 1 and Table 2). The results of GC-MS showed that the metabolites of endophytic bacteria mainly included phenols, esters, organic acids, aldehydes, alcohols, ketones etc.

I. *Bacillus amyloliquefaciens*: After the removal of some alkanes, a total of 29 compounds with relative content > 0.6 % were identified in its fermentation broth (Table 1 and 2). Among the 22 identified compounds in methanol phase, 7 compounds were identified by n-hexane phase.

(i). Methanol Phase: The higher contents in methanol phase were d-Proline-n-butoxycarbonyl-undecyl ester (9.65 %), hexahydro-pyrrole[1,2] pyrazine -1,4-dione (9.546 %), heptanal (4.278 %), hexanoic acid-2-methylphenyl ester (3.254 %), DL-alanylglycylglycine (3.089 %), 1,2,3,4-tetrahydro-6-methoxy-Naphthalene (2.641 %), N-methyl-N-(1-methylethyl)-1-Pentylamine (2.107 %), and N,N-dimethyl -N'-(3-nitrophenyl)-Methylaminamide (2.028 %).

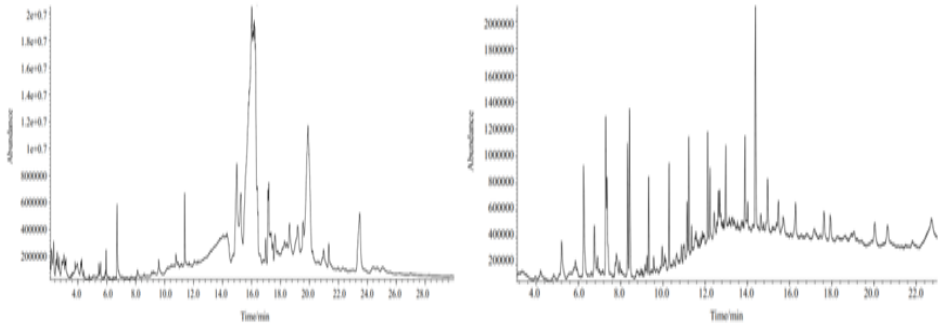


Figure 5. Total ion flow chromatogram of methanol and n-hexane extraction phase of *Bacillus amyloliquefaciens* fermentation broth

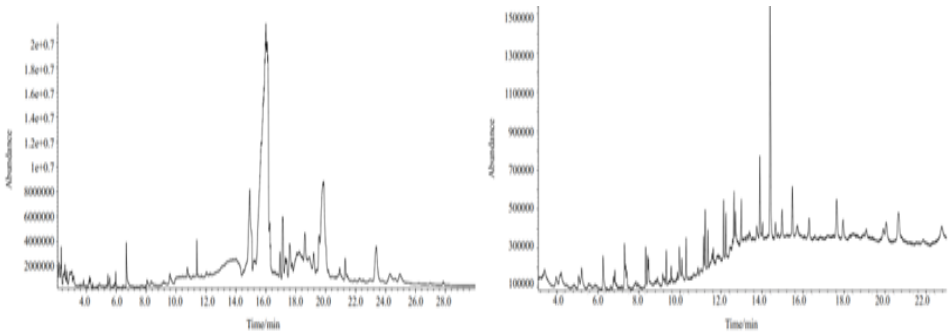


Figure 6.Total ion flow chromatogram of methanol and n-hexane extraction phase of *Bacillus aryabhattai* fermentation broth.

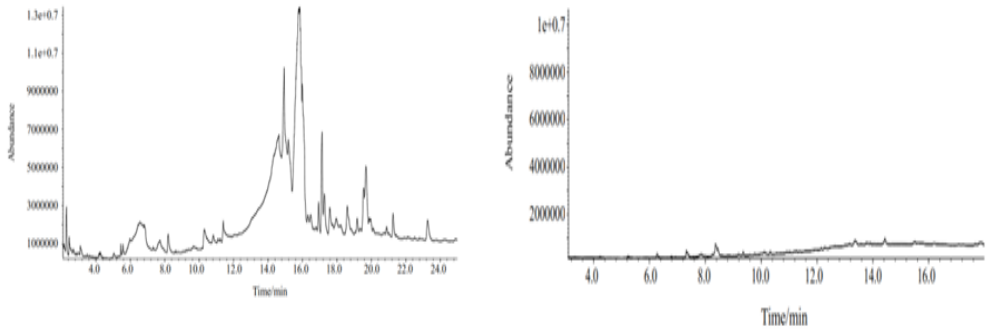


Figure 7. Total ion flow chromatogram of methanol and n-hexane extraction phase of fermentation broth of *Paenibacillus glycanilyticus*

(ii). n-Hexane Phase: While the higher content of n-hexane phase were 2,2'-methylenebis[6-(1,1-dimethylethyl) -4-methyl-Phenol (8.373 %) and N,N-dimethyl-Octanamide (2.074 %) and the relative contents of other components < than 2 %.

Table 1. Chemical components of methanol extraction phase of endophytic bacteria fermentation broth

No.	Compounds	Relative content (%)		
		Bacillus amyloliquefaciens	*Bacillus aryabhattai*	*Paenibacillus glycanilyticus*
1	Acetamide, 2-amino-	0.657	0.772	1.752
2	D-Leucine	1.405	1.24	-
3	1-Pentanamine, N-methyl-N-(1-methylethyl)-	2.107	1.265	-
4	Heptanal	4.278	6.244	-
5	d-Proline, n-butoxycarbonyl-, undecyl ester	9.65	3.835	-
6	Naphthalene, 1,2,3,4-tetrahydro-6-methoxy-	2.641	2.049	-
7	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	-	10.783	14.86
8	Nitro-L-arginine	-	0.875	0.992
9	Leucine	1.874	-	-
10	dl-Alanylglycylglycine	3.089	-	-
11	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	9.546	-	-
`12	4-Methoxy-3,5-dihydroxybenzoic acid	1.082	-	-
13	Methanimidamide, N,N-dimethyl-N'-(3-nitrophenyl)-	2.028	-	-
14	Hexanoic acid, 2-methylphenyl ester	3.254	-	-
15	2-Hydrazino-4-methyl-6-methylthio pyrimidine	1.379	-	-
16	Piperidine, 3,3-dimethyl-	-	2.069	-
17	2-Piperidinone, 1-methyl-	-	1.523	-
18	Methanimidamide, N,N-dimethyl-N'-(4-nitrophenyl)-	-	1.997	-
19	3-Methyl-2-pyrrolidinone	-	1.724	-
20	1,4-Benzenediamine	-	1.343	-
21	Propan-1-one,1-[3-fluoro-4-(4-methyl -1-piperidyl)phenyl]-	-	1.62	-
22	Glycerine	-	-	2.164
23	Valeraldehyde, dimethylhydrazone	-	-	13.161
24	L-Prolinamide	-	-	9.665
25	1-Butanamine, N-methyl-N-(1-methylethyl)-	-	-	5.399
26	2-Undecenal	-	-	6.118
27	Pyrrolo[1,2-a]pyrazine-1,4-dione,he xahydro-3-(2-methylpropyl)-	-	-	2.311
28	Iminostilbene	-	-	1.307
29	Benzoic acid, 3,4,5-trihydroxy-	-	-	1.346
30	Cyclobutanecarboxamide, N,N-dibutyl-	-	-	2.844

-: Absent

II. *Bacillus aryabhattai*: After removing some alkanes, a total of 36 compounds with relative contents of > 0.6% were identified in its fermentation broth.

Table 2. Chemical components of n-hexane extraction phase of endophytic bacteria fermentation broth

No.	Compounds	Relative content (%)		
		Bacillus amyloliquefaciens	*Bacillus aryabhattai*	*Paenibacillus glycanilyticus*
1	Phenol,2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl-	8.373	17.466	13.927
2	Octanamide, N,N-dimethyl-	2.074	3.49	-
3	Cyclopropanecarboxylic acid, heptadecyl ester	1.74	1.359	-
4	Methoxyacetic acid, 2-tetradecyl ester	0.939	-	-
5	Benzenemethanamine, N-(phenylmethyl)-	1.059	-	-
6	Octatriacontyl pentafluoropropionate	0.657	-	-
7	tert-Hexadecanethiol	1.197	-	-
8	Dibutyl phthalate	-	1.826	-
9	Sulfurous acid, butyl tetradecyl ester	-	0.944	-
10	N-[[2-p-Tolylsulfonyl]ethyl]phthalimide	-	0.9	-
11	E-15-Heptadecenal	-	2.227	-
12	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	-	-	27.17

(i). Methanol Phase: 29 compounds were identified by methanol phase and 7 by n-hexane phase. The contents in methanol phase were: hexahydro-Pyrrolo[1,2-a]pyrazine-1,4-dione (10.783 %), heptanal (6.244 %), D-proline N-butoxycarbonyl-undecyl ester (3.835 %), 3,3-dimethyl-Piperidine (2.069 %), 1,2,3,4-tetrahydro-6-methoxy-Naphthalene (2.049 %).

(i) n-Hexane Phase: The higher content of n-hexane phase were: 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-Phenol (17.466 %), N,N-dimethyl-Octanamide (3.49 %) and E-15-Heptadecenal (2.227 %). The relative contents of other components were < 2 %.

III. *Paenibacillus glycanilyticus*: After removing some alkanes, a total of 21 compounds with relative content of > 0.6% were identified in its fermentation broth. Among them, 19 compounds were identified by methanol phase and 2 compounds by n-hexane phase.

(i). Methanol Phase: The higher content in methanol phase were: hexahydro-Pyrrolo[1,2-a]pyrazine-1,4-dione (14.86 %), dimethylhydrazone-Valeraldehyde (13.161 %), L-Prolinamide (9.665 %), 2-Undecenal (6.118 %), N,N-dibutyl-Cyclobutanecarboxamide (2.844 %), hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione (2.311 %) and glycerin (2.164 %).

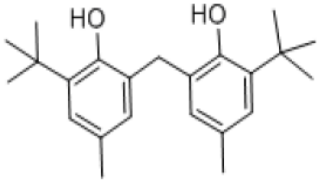
(ii). n-Hexane Phase: The higher contents in n-hexane phase were: hexahydro-Pyrrolo[1,2-a]pyrazine-1,4-dione (27.17 %) and 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl-Phenol (13.927 %). The relative contents of other components were < 2 %.

Comparison between endophytic bacterial fermentation broth, root and soil extract

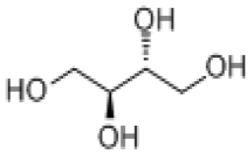
By comparing the GC-MS results of fermentation broth from three endophytic bacteria (*Bacillus amyloliquefaciens*, *Bacillus aryabhattai* and *Paenibacillus glycanilyticus*) with the corresponding forest-age root and soil extraction liquid (28) we found that common products were 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-Phenol and 1,2,3,4-Butanetetrol. It was suggested that these substances may be synthesized by endophytic bacteria, or by endophytic bacteria and plants and accumulated in soil.

2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-Phenol (%): Its molecular formula is$C_{23}H_{32}O_2$ and molecular weight is 340.5 (Fig. 8). It is white powder, long-term exposure to air slightly makes it yellow-pink and slightly phenolic odour. It was present in n-hexane phase of *Bacillus amyloliquefaciens* fermentation broth, and its relative contentwas8.373%, methanol and n-hexane phase of *Bacillus aryabhattai* fermentation broth (0.338 % and 17.466%), n-hexane phase of *Paenibacillus glycanilyticus* fermentation broth (13.927%）, n-hexane phase of young-aged forest root extract（0.33%）, n-hexane phase of mature-aged forest root extract (1.16 %, methanol and n-hexane phase of mature-aged forest soil extract (0.019 % and 0.59 %).This substance was present in soil, plant and root endophytic bacteria and the content in endophytic bacteria was higher than in root and soil, indicating that the substance may be produced directly by root endophytic bacteria and entered the soil through infiltration, or secreted by the roots into the soil.

1,2,3,4-Butanetetrol (%): Its molecular formula is $C_4H_{10}O_4$ and molecular weight is 122.12 (Fig. 8). Itis soluble in water and pyridine, slightly soluble in alcohol, almost insoluble in ether, and its colour was white tetragonal prism crystal. It was detected in the methanol phase of *Paenibacillus glycanilyticus* fermentation broth (0.691 %) and methanol phase of mature-aged forest root (0.034 %). It was suggested that it may be produced by endophytic bacteria and transferred to the roots.



2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-Phenol



1,2,3,4-Butanetetrol

Figure 8. Structural diagram of compounds of endophytic bacteria fermentation broth, root and soil extract.

Isolation and identification of endophytic bacteria from *C. equisetifolia* roots

Endophytic bacteria were found in all studied plants (*Dendrobium officinale*, Watermelon, Tomato, Banana, Pepper, Cucumber) (8,18). The endophytic bacteria promotes the host resistance ability to stress by secreting the secondary metabolite and is affected by tissue, host, environment and other factors (14,31).

In this study, 36 strains of endophytic bacteria were isolated from *C. equisetifolia* root, the dominant bacteria was *Bacillus*. According to the functional prediction of its dominant genera, it participates in the biological process of flavonoids and also has volatile terpenes (limonene and pinene). It is speculated that the endophytic bacteria of *C. equisetifolia* participate in processes related to allelopathy. The flavonoids are widely distributed in plants and have important chemical ecological functions, including microbial information exchange, pest prevention, allelopathy, etc. (6). In plant communities, terpenoids also play a role in controlling weeds, preventing diseases, attracting pollen by aroma, and causing toxicity to other plants, especially highly volatile terpenoids, among which limonene and pinene are common volatile terpenoids (22). The study on the diversity of endophytic bacteria in *C. equisetifolia* root not only found the sources of its endophytic bacteria, but also provided theoretical basis for their role allelopathy.

Allelopathic potential of endophytic bacterial fermentation broth

Our results showed that fermentation brothsof different endophytic bacteria of *C. equisetifolia* roots inhibited its seeds germination. Among them, *Bacillus amyloliquefaciens* had the strongest allelopathic effect, followed by *Bacillus aryabhattai* and *Bacillus cereus*, indicating that these endophytic bacterial fermentation broths had allelopathic potential. The previous research on these bacteria mainly focused on growth promotion, bacteriostasis, drug resistance, phosphorus hydrolysis etc. He *et al.* (9) reported that *Bacillus amyloliquefaciens* B10-26 promoted the growth, prevent disease and colonizethe sesame plants. Deng *et al*. (4) found that *Bacillus aryabhattai* WN-F promotedthe biological nitrogen fixation and there by reduced the nitrogen fertilizer dose in corn. Guan *et al.* (5) found that *Bacillus cereus* degraded the organophosphorus pesticides such as methamidophos. However, the research on the allelopathy of these bacteria has not been reported.

Identification of secondary metabolites of endophytic bacteria

Endophytes live in host plants for a long time and co-evolve with them, the host provides the energy and nutrition needed for the growth of endophytic bacteria, while, the endophytic bacteria promotes plant growth and enhancesthe plant resistance to adverse environment. In the process of long-term co-evolution, gene recombination or other information exchange caused by mutation leads endophytes to the same information transmission pathway as the host. As a result, endophytic bacteria can synthesize secondary metabolites similar to host plants (22,23).

Compared with our previous GC-MS analysis of root extracts, we found that 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-Phenol existed simultaneously in bacterial fermentation broth, root and soil extract. while, 1,2,3,4-Butanetetrol existed only in bacterial fermentation broth and root extract. These substances exist in both plants and endophytic bacteria, indicating that these substances may be produced by *C. equisetifolia* plants, secreted by roots or transformed into soil by endophytic bacteria, or directly produced by endophytic bacteria and secreted into soil by roots. We found the same substances 2,4-di-tert-Butylphenol and Methyl octadecanoate in the root extract. Other substances [phenol, 6-(1,1-dimethylethyl)-4-methyl- and phenol,2-(1,1-dimethylethyl)-6-methyl-], are similar to 2,4-di-tert-butylphenoland can be converted by alkyl substitution.

Our previous study (7,25,28) showed that the metabolites of soil microorganisms and root endophytic fungi in different ages forest of *C. equisetifolia* were compared with those present in casuarina soil, root and litter extracts. It was also found that methyl stearate, 1,2,3,4-Butanetetrol were found only in soil microorganisms and in the soil extract, indicating that these substances were metabolized by soil microorganisms and the endophytic fungi and these got accumulated in soil. However, the 2,4-di-tert-Butylphenol, a metabolite of soil microorganisms and root endophytic fungi, existed simultaneously in soil, root and litter extract. Its content in soil was higher than in microbial fermentation broth, indicating that it was produced by plant and microbial metabolism and accumulated in the soil. In addition, the fermentation broth of these microorganisms had different inhibitory effects on the seeds germination of *Thespesia lampas* L. and *Calophyllum inophyllum* L. (7,11,25). These results suggested that soil microorganisms and root endophytic fungi of *C.equisetifolia* are involved in the synthesis of allelopathic allelochemicals.

The phenolic acids are important allelochemicals. Zheng *et al.* (35) found that the Autotoxicity of *Andrographis paniculata* was due to the accumulation of phenolic acids in soil released during its growth. Benzarti (24) found that the methanol extract of quince (*Cydonia oblonga* Miller) leaf contained 9-phenolic acids and flavonoids, which inhibited the formation of free radicals. Roshchina *et al.* (19) found that some phenolic compounds (Ferulic acid, vanillic acid, P-hydroxybenzoic acid) inhibited the activities of SOD and CAT enzymes in recipient plants, resulting in increase of reactive oxygen species, thereby inhibited the absorption of nutrients by plants and caused allelopathic effects. Zhou *et al.* (33)

found that in garlic root exudates, allelochemicals present were: 2,6-bis (1-methylethyl)-phenol, butylated hydroxytoluene and dially disulphide. Wang (26) found that the root exudates of *Trifolium repens* Lcontained 2,4-di-tertbutyl-phenol, butylated hydroxytoluene and the root exudates inhibited the seed germination and seedling growth of tall fescue and Kentucky bluegrass. Xu *et al.* (29) found that the seed germination and seedling growth of cucumber were stimulated by root exudates of welsh onion at low concentration but were inhibitory at high concentration. The GC-MS analysis showed in root exudates, higher contents of Phthalate esters and 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-Phenol. When the concentration of 2,4-bis(1,1-dimethylethyl)-phenol was 15mmol/m^2, it was allelopathically inhibitory to growth of *Humulus lupulus* L.

Linear alcohols and enols are allelochemicals (32). Liu (12) found that 1,2,3,4-Butanetetraol and 2-methylallyl alcohol important allelochemicals of *Phragmites communis*. Chen *et al*. (2) found that the allelochemicals of *Parthenium hysterophorus* contained 2,3-dimethyl-3-butene-2-ol and benzyl alcohol. These results suggested that the secondary metabolites of endophytic bacteria in *C. equisetifolia* root contained allelopathic inhibitory substances.

CONCLUSIONS

The endophytic bacteria of *C. equisetifolia* roots have high diversity. Among the 21 species of isolated endophytic bacteria, *Bacillus* was dominant and the *Acinetobacter* and *Staphylococcus* were subdominant. The fermentation broth of these 21 endophytic bacteria inhibited the seeds germination of *C. equisetifolia*. The main allelopathic substances in the fermentation broth of these 3- endophytic bacteria inhibitory against seed germination of *C. equisetifolia* were: phenols, esters, organic acids, aldehydes, alcohols, ketones etc. Among them1,2,3,4-Butanetetrol and 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-Phenol were present in both endophytic bacteria fermentation broth, root and soil extracts. These allelochemical proved to be allelopathic. Thus the secondary metabolites of endophytic bacteria of *C. equisetifolia* root were the source of allelochemicals. These results not only provided a theoretical basis to study the allelopathic mechanism of *C. equisetifolia*, but also provided practical guidance for the second regeneration of *C. equisetifolia* shelterbelt.

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DECLARATION

We declare that all authors of this Ms have made substantial contributions. We have not excluded any author that substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

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