

Culture Separation, Identification and Unique Anti-pathogenic Fungi Capacity of Endophytic Fungi from Gucheng *Salvia Miltiorrhiza*

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Abstract. *Background:* *Salvia miltiorrhiza* is a medical herb for human disorders including cardiovascular diseases and cancer. However, the interactions between *Salvia miltiorrhiza* and its endophytes are largely unknown. The current study aimed at identifying its endophytic fungi and examining their inhibitory effects on anti-pathogenic fungus. *Materials and Methods:* Distinct species of endophytic fungi were isolated from the roots of *Salvia miltiorrhiza*, cultured, sequenced, aiming to predict their taxonomical structures. Meanwhile, extracts from each endophytic fungus fermentations were isolated, compared and evaluated on the inhibitory efficacies on five pathological fungi, *Cercospora nicotianae*, *Phoma arachnidicola*, *Staphylococcus*, *Phytophthora eggplant*, and *Rhizoctonia cerealis*. *Results:* A total of 34 strains of endophytic fungi were obtained from *Salvia miltiorrhiza*. Among them, SX19 and *C. Gloeosporioids* exhibited the most effective inhibitions on five pathogenic fungi. *Conclusion:* The anti-fungal activities of the endophytic fungus from *Salvia miltiorrhiza* were confirmed for the first time, and this may benefit crop quality and production in the future.

Endophytic fungi refer to those fungi which colonize inside the tissues of the host plants, typically causing no apparent symptoms to the hosts. They play mutually beneficial roles to medical plants, balance the plant micro-ecosystems, and have the capacity to produce some natural bioactive products such as secondary metabolites in the host plants (1-4). Noticeably, there are also some pathogenic fungi which are parasitic on plants and may cause various plant diseases. Up to 70~80% of crop diseases are caused by these pathogenic fungi, and there are several or even dozens of fungal diseases in every crop. From the era of 2000s, mounting evidence for endophytic fungi serving as protectors for host plants against these pathogenic fungi have been accumulated at a relatively slow speed and the literature is extremely limited (5-18). The screening methodology for endophytic fungi as potent anti-pathogenic fungicides is also developed (19-21), and very exciting, the subtle alterations are also getting started to be revealed at not only signaling interaction network but DNA and RNA levels systematically (22, 23).

For more than one thousand years, *Salvia miltiorrhiza* (also have the names of *Salvia miltiorrhiza* Bge, *Salvia miltiorrhiza* Bunge and Danshen, hereon abbreviated as SM) has been applied as a traditional Chinese herbs with several physiological effects such as activating blood circulation, easing the stasis pain, and strengthening the capacity of kidney as diuresis (24). With the development of Chinese medical science, the evidence between SM components and their efficacies to human diseases have been revealed in recent years. In brief, the active medical ingredients of SM are mainly divided into two groups, the hydrophilic phenolic acids and lipid-soluble tanshinones. The former group include salvianolic acid A (Sal A), salvianolic acid B (Sal B), lithospermic acid, rosmarinic acid, and danshensu (25, 26), while the latter group include tanshinone I, tanshinone IIA,

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tanshinone IIB, cryptotanshinone, and dihydrotanshinone I (27). Take Salvianolic acids, which are found to be most abundant SM compounds, for example, they are reported to have beneficial capacities including anti-inflammatory (28), antioxidant (29), anti-thrombotic (30), and cardio-protective (31, 32) efficacies. Many laboratories report that extracts of SM dried roots may have therapeutic effects for a panel of human diseases, including several types of cancers (33, 34), cardiovascular diseases (34-37), neuronal disorders (34, 38), Alzheimer's disease (39) and liver diseases (34, 40). A state-of-the-art summary about the signaling network of SM in its therapeutic affects was proposed by Jung and his colleagues in 2020 (34). However, the knowledge on endophytic fungi is relatively limited. In 2015, Li and his colleagues firstly isolated endophytic fungi from SM and examined their individual antioxidant capacity (41). They concluded that the antioxidant potential positively correlated to the phenolic and flavonoid compounds present in the ethanol extracts of *A. alternata* SaF-2 and *F. proliferatum* SaR-2 from SM (41). In 2016, another group found that a specific endophytic fungi of SM, *Phoma glomerata* D14, is capable of producing salvianolic acid C (42).

Up to now, there is no study identifying the components of MS endophytic fungi and examining their anti-fungal capacities on pathogenic fungi. Geographically, SM is mainly produced in Anhui and Jiangsu provinces of China, while the SM produced in Gucheng (the county area named as "ancient city") characterized with red-colored skin, purple and grainy meat, dry and loose quality, bid head without reed, is uniquely famous for its therapeutic application. Based on the above information, we aimed to systematically isolate the endophytic fungi from Gucheng SM roots, identify and classify them from a genetic point of view, and investigate their overall anti-fungal capacities in a panel of pathogenic fungi.

Materials and Methods

Isolation and morphological identification of endophytic fungi from Gucheng SM. Gucheng SM was collected from Anhui Hanshan Zhongda Shennong Co., Ltd, in July of 2017, and identified by Nian-Jun Yu of the Anhui University of Chinese Medicine. Briefly, fresh and healthy SM was surface sterilized as follows: samples were washed in 75% ethanol for 3 min, followed by rinsing with autoclaved distilled water, then immersed in 3% sodium hypochlorite for 3 min, rinsed with autoclaved distilled water, and finally rinsed with 75% ethanol again for 30 s. The effective surface sterilization was ensured via inoculating the final wash onto potato dextrose agar (PDA, Beijing Aobox Biotechnology, PR China) and LB plates at 25°C and 37°C, respectively. The results showed that all the washes had no growth. The sterile filter paper is blotted dry and the sterile blade was cut into small pieces. The tissue pieces were inoculated on the medium and cultured in a constant temperature incubator for 5-15 d. The grown hyphae were transferred onto new PDA plates with tip hyphae picking, grown to

almost full plate, and repeat the processes of tip hyphae picking and re-grown for thrice for the pure single colony. Then, three full plates with individual fungus were ready for fungus inhibitory tests.

Genetic and taxonomic identification of endophytic fungi. Genomic DNA from cultured fungal endophytes was extracted using the plant/fungal genome DNA Mini-Preps Kit (Tiangen Biotech Co. Ltd., Beijing, PR China) and immediately quantified using a Nanodrop machine (Thermo Scientific, Waltham, MA, USA). The extracted DNA served as template for PCR amplify of Internal Transcribed Spacer (ITS) with the forward primer rDNA-ITS (5'-TCCGTAGGTGAACCTGCGG-3') together with primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3' and primer ITS1 (5'-TCCGGTGAACCTGCGG-3'), respectively. The PCR amplification conditions were set as: initial denaturation for 5 min at 95°C, followed by 35 amplification cycles (95°C for 30 s, 49°C for 30 s, 72°C for 90 s), and a final extension at 72°C for 7 min, using a Biometra PCR thermal cycler. The PCR products were separated with a 2% agarose gel electrophoresis at 100 Volts; the bands were excised and eluted using a gel purification kit (Illustra GFX96 PCR Purification kit, GE Healthcare, Chicago, IL, USA). The purified DNA was sequenced and subject to the sequence comparisons using BLAST searches to Genbank on NCBI website. The phylogenetic trees were constructed according to the Kimura2-Parameter model with Neighbor-Joining cluster analysis using MEGA ver. 5.0. System evolutionary matrix estimation, and each train was identified by the stability of the tree topology with 1,000 cycles.

Preparation of endophytic fungal fermentation products. The endophytic fungi of Gucheng SM were inoculated into PDA medium and cultured at 28°C for 5-7 days in the dark, and it was punched with a puncher of about 6 mm. Water was added in the potato dextrose broth medium powder, mixed well, sterilized for 20 min, cooled, and picked up of 2 or 3 pieces for perforated endophytic fungus tablets and place them in a liquid medium. The shaker was set as 180 rpm, and the fermentation was carried out at 28°C for 7 d. After filtration, the fermentation broth was extracted with ethyl acetate, concentrated and evaporated to dryness. A total of 1 mg/ml solution was prepared in sterile water, filtered, and set aside.

Anti-pathologic fungi screening procedure. *Cercospora nicotianae*, *Phoma arachnidicola*, *Staphylococcus*, *Phytophthora eggplant*, and *Rhizoctonia cerealis* were kindly offered by Dr. Bin-Ji Ma of Henan Agricultural University. The five species of pathogenic fungi are the components of our anti-fungal screening platform.

Each pathogenic fungus was inoculated into PDA medium, and then transferred to a new medium, and cultured at 26°C for 5 days at constant temperature along the edge of the colony. These were captured into 6 mm pieces by a puncher. It contained 1 ml of fermentation broth for each endophyte in each medium plate with 10 ml medium. The pathogenic fungi were inoculated into them, and the rate of anti-pathogenic fungi capacity is determined by a cross method. The agar plates were incubated at 30°C for 48 h in darkness. The radius of each zone of inhibition was measured. The combined fungicides Amphotericin B (Catalog#A2942, Sigma Aldrich, St. Louis, Mo, USA) and Nystatin (Catalog#N6261, Sigma Aldrich) were used as the positive control, and distilled water was used as a negative control. Each endophyte was screened in three independent replicates in the first turn, and Each endophyte were overall re-screened for activity against the five pathogenic fungi

Table I. Compositions and diversities of endophytic fungi in Gucheng *Salvia miltiorrhiza*.

| Genus | Species | No. | GenBank accession number | Similarity | No. of strains |
|-----------------------|------------------------|-------|--------------------------|------------|----------------|
| <i>Aspergillus</i> | <i>Flavus</i> | DS-5 | LN482516.1 | 99 | 2 |
| | <i>Ochrceus</i> | DS-6 | KU743890.1 | 99 | 4 |
| | <i>Niger</i> | DS-7 | KU904480.1 | 99 | 3 |
| | <i>Udagawae</i> | DS-12 | KY808744.1 | 99 | 2 |
| <i>Fusarium</i> | <i>Proliferatum</i> | DS-3 | MG543763.11 | 100 | 2 |
| | <i>Sp. R24</i> | DS-9 | KM979804.1 | 99 | 2 |
| | <i>Verticillioides</i> | DS-10 | KC684889.1 | 99 | 1 |
| <i>Epicoccum</i> | <i>Sp. SX19</i> | DS-4 | KC329632.1 | 99 | 1 |
| | <i>Nigrum</i> | DS-13 | MF800916.1 | 99 | 2 |
| <i>Arthrinium</i> | <i>Sp. LH11</i> | DS-1 | HQ832842.1 | 99 | 3 |
| <i>Coprinellus</i> | <i>Radians</i> | DS-2 | FJ462761.1 | 99 | 2 |
| <i>Dictyosporium</i> | <i>Aquaticum</i> | DS-8 | KM610236.1 | 95 | 3 |
| <i>Colletotrichum</i> | <i>Gloeosporioides</i> | DS-11 | KP900290.1 | 100 | 3 |
| <i>Rhizoctonia</i> | <i>Bataticola</i> | DS-14 | HQ392782.1 | 99 | 2 |
| <i>Phomopsis</i> | <i>TP-41</i> | DS-15 | DQ780435.1 | 98 | 1 |
| <i>Pithomyces</i> | <i>Chartarum</i> | DS-16 | GU073119.1 | 99 | 1 |

again in triplicate to confirm the antifungal activity spectrum for each extract.

Statistical and evaluation system. Inhibitory rate (%) is calculated with the formula: (control plate colony diameter - fermentation broth colony diameter)/control plate colony diameter $\times 100\%$. All statistical analysis had been conducted using Excel. All data shown in the Table II represent the average of six replicates.

Results

Morphological identification results of endophytic fungi. A total of 34 endophytic fungi were obtained from SM, belonging to 10 genera and 16 species (named as DS-1 to DS-16), mainly distributed in *Fusarium*, *Epicoccum*, and *Aspergillus* (Table I). The characteristics of the endophytic fungal colonies obtained were isolated according to the “Handbook of identification of fungi”, such as edge shape, hypha color, transparency, surface wet and dry state, shape, size and other characteristics (43). According to the typical classification of fungal morphology (44), each endophytic fungus was microscopically determined. Figure 1 shows each endophytic fungus in 1 \times and 40 \times under microscope.

In detail, the DS-5, DS-6, DS-7, DS-12 are of the common characters including conidia, spherical, elliptical or pear-shaped, light yellow or yellow colored, easy to scatter, spores brownish green in microscopic, irregular aggregation distribution, all belong to the genus *Aspergillus* (Table I, top genus). The DS-3, DS-9, and DS-10 have the following characteristics: white, relatively dense and flocculent surface hyphae, conidia existed in large numbers, wedge-shaped or fusiform file type, with the obvious diaphragm, 3 to 6 membranes (most of them are 3 and several are 4-6). They are all identified as the genus *Fusarium* (Table I, second genus).

The sporodochium of DS-4 are dark-colored and cushion-shaped, while their conidiophores are short and dark-colored. Their spores are globose and dark-colored and stored in irregularly brick-wall-like structures. Like those of DS-4, the spores of DS-13 are also globose and dark-colored. However, the spores of DS-13 are characterized with verrucous protrusions and wrinkled rugoses, and relatively lower infection activities. The DS-1 is of branched filamentous body, no septum, a split fruit production type, and a thick spore formed in the hyphae, and identified as the family *Arthrinium*. The DS-2 is produced on the pleats or in the pores. The fruiting body has a stalk and was born in the center of the cap. It is identified as *Coprinellus* fungus. The spores of DS-8 are stored in brick-wall-like structures and are almost colorless. The DS-11 that has a surface that is hyphae gray-brown, dense, blister-like, conidial stalk single, unbranched, bristles black, sparse, perennial, microscopic hyphae, radial distribution, was identified as *Colletotrichum*. The diameters of sclerotium for DS-14 can vary from 5 to 100 μm , while their conidiophores are cylindrical with elliptical or clavarielike micro-bending spores. The DS-15 has spherical of conidia, oval or stick-shaped, leathery, dark brown, cracked by small holes, epigenetic, conidial single cavity, born on the surface of the sub-seat, identified as *Phomopsis* Fungus. The conidiophore and spores of DS-16 are colorless, while spores are multi-membraned with various shapes including fusiform, elliptical, cylindrical and globose.

Genetic identification and phylogenetic tree of Gucheng SM endophytic fungi. The DNA of endophytic fungi were amplified by rDNA-ITS methodology, and the PCR amplicons for ITS domain of each strain purified, was sequenced and

Table II. Inhibitory effects for 14 endophytic fungi of seven dominant species in Gucheng *Salvia miltiorrhiza* on 5 plant pathogenic fungi.

| Endophytic fungus | Inhibitory rate for individual pathogenic fungus (%) | | | | |
|-------------------------|--|----------------------------|-----------------------|------------------------------|-----------------------------|
| | <i>Cercospora nicotianae</i> | <i>Phoma arachnidicola</i> | <i>Staphylococcus</i> | <i>Phytophthora eggplant</i> | <i>Rhizoctonia cerealis</i> |
| <i>E.sp. SX19</i> | 60.3±2.2% | 57.6±8.1% | 58.6±6.6% | 68.6±6.6% | 38.5±3.7% |
| <i>A.Ochrceu</i> | 38.0±8.2% | 26.1±9.7% | 54.1±3.8% | 46.5±5.7% | 45.9±7.8% |
| <i>A.niger</i> | 21.8±3.5% | 2.5±1.4% | 44.5±3.0% | 54.5±7.1% | 53.7±2.0% |
| <i>F.sp. R24</i> | 18.9±2.9% | 7.5±5.0% | 34.8±9.9% | 55.5±4.1% | 44.5±1.0% |
| <i>F.verticillioide</i> | 21.2±9.7% | 24.4±1.3% | 41.1±7.6% | 48.4±9.5% | 53.2±4.0% |
| <i>A.udagawae</i> | 23.3±10.8% | 10.1±1.3% | 41.9±8.0% | 57.2±4.9% | 48.5±7.0% |
| <i>E.nigrum</i> | 15.7±5.6% | 5.7±3.1% | 18.4±3.8% | 54.5±4.3% | 48.0±8.5% |
| <i>A.sp. LH11</i> | 23.1±8.2% | 20.5±5.1% | 36.0±5.5% | 25.7±2.9% | 17.3±3.8% |
| <i>C.radians</i> | 25.1±4.2% | 27.2±3.8% | 34.1±1.1% | 30.7±3.9% | 27.1±2.3% |
| <i>F.proliferatum</i> | 25.1±6.6% | 26.2±10.3% | 38.3±5.0% | 29.3±2.7% | 25.4±2.1% |
| <i>D.aquaticum</i> | 23.5±3.2% | 27.7±6.5% | 30.0±5.9% | 23.1±5.8% | 24.2±2.5% |
| <i>C.Gloeosporioids</i> | 49.6±5.0% | 62.1±5.2% | 61.9±3.5% | 51.1±6.2% | 54.5±4.2% |
| <i>P.TP-41</i> | 33.8±7.9% | 31.9±2.0% | 33.1±2.5% | 26.4±4.7% | 23.1±0.8% |
| <i>P.chartarum</i> | 26.8±3.6% | 25.3±3.6% | 35.4±3.1% | 25.7±5.1% | 22.6±1.6% |

E.sp. SX19: *Epicoccum Sp. SX19*; *A.Ochrceu*: *Aspergillus Ochrceus*; *A.niger*: *Aspergillus Niger*; *F.sp. R24*: *Fusarium Sp. R24*; *F.verticillioide*: *Fusarium Verticillioides*; *A.udagawae*: *Aspergillus Udagawae*; *E.nigrum*: *Epicoccum Nigrum*; *A.sp. LH11*: *Arthrinium Sp. LH11*; *C.radians*: *Coprinellus Radians*; *F.proliferatum*: *Fusarium Proliferatum*; *D.aquaticum*: *Dictyosporium Aquaticum*; *C.Gloeosporioids*: *Colletotrichum Gloeosporioids*; *P.TP-41*: *Phomopsis TP-41*; *P.chartarum*: *Pithomyces Chartarum*.

compared with each other for similarity. Finally, the phylogenetic trees for the Gucheng SM endophytic fungi are firstly constructed and shown in Figure 2. Noticeably, the diversities of Gucheng SM endophytic fungi are very high.

In vitro measurements of anti-pathogenic fungi activity. The inhibitory capacity against *Cercospora nicotianae*, *Phoma arachnidicola*, *Staphylococcus*, *Phytophthora eggplant* and *Rhizoctonia cerealis* for fermentation products of from the 14 endophytic fungi of seven dominant species in Gucheng *Salvia miltiorrhiza* were measured and summarized in Table II. Among the most effective endophytic fungi against pathogenic *Cercospora nicotianae*, *Phoma arachnidicola* and *Staphylococcus*, *E.sp. SX19* and *C. Gloeosporioids* were the top two. As for *Phytophthora eggplant*, *E.sp. SX19* was the top one and *C. Gloeosporioids* was the sixth with an inhibitory rate of 51.1%. As for *Rhizoctonia cerealis*, *C. Gloeosporioids* was the top one and *E.sp. SX19* was the eighth with an inhibitory rate of 38.5%. Overall, the average inhibition rate of the fermentation products of *C. Gloeosporioids* against five crop pathogenic fungi were 56.72%, and the average inhibition rate of *E.sp. SX19* was 55.84% (Figure 3). The overall results show that these two endophytic fungi of Gucheng SM have good antibacterial effects on five pathogenic fungi.

Discussion

SM is a medicinal herb that has been applied in therapeutic treatments of various diseases such as cancer, cardiovascular

diseases, liver diseases, nervous system diseases in East Asia. The endophytic fungi from SM may benefit the SM with antioxidant capacity (41), and elevating the production of salvianolic acid (42). However, the knowledge about their diverse species, taxonomic relationships, and anti-fungal capacities are extremely limited. In the current study, we have systematically cultured, isolated, and examined the endophytic fungi of Gucheng SM of their antifungal capacities against five crop pathogenic fungi, *Cercospora nicotianae*, *Phoma arachnidicola*, *Staphylococcus*, *Phytophthora eggplant*, and *Rhizoctonia cerealis*.

We identified up to 34 endophytic fungi from Gucheng GM, which belong to 10 genera and 16 species that are mainly distributed in *Fusarium*, *Epicoccum*, and *Aspergillus* (Table I). One of the highlight findings in the current study is that two of the strains of Gucheng SM endophytic fungi, *E.sp. SX19* and *C. Gloeosporioids*, are of the high-inhibitory and broad-spectrum anti-fungal characters to effectively kill the fungus (Table II). In detail, the inhibitory capacities of *E.sp. SX19* against *Cercospora nicotianae*, *Phoma arachnidicola*, *Staphylococcus*, *Phytophthora eggplant*, and *Rhizoctonia cerealis* are 60.3% (top of 14 examined), 57.6% (2/14), 58.6% (2/14), 68.6% (1/14) and 38.5% (8/14), respectively. Those for *C. Gloeosporioids* are 49.6% (2/14), 62.1% (1/14), 61.9% (1/14), 51.1 (6/14) and 54.5% (1/14), respectively. It is of great agricultural value to elevate the overall yield and quality of Gucheng SM via application of endophytic fungi such as *E.sp. SX19* plus *C. Gloeosporioids* from lab to field.

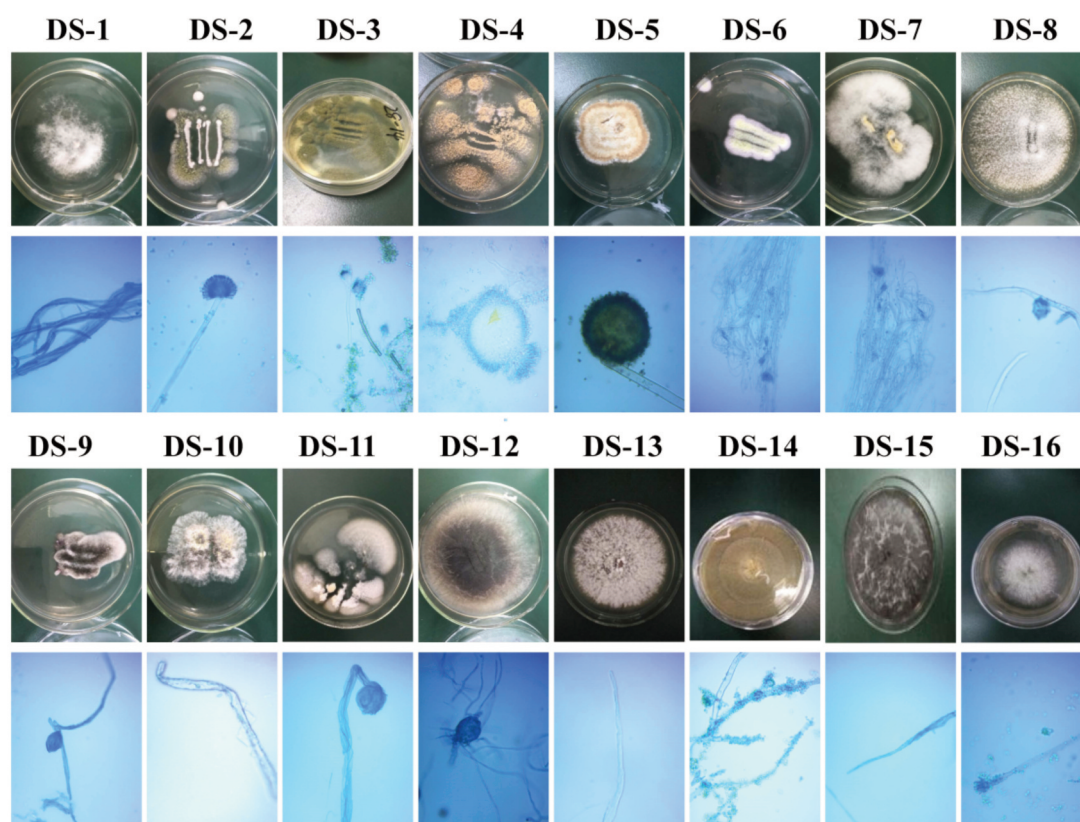


Figure 1. Colony and microstructure of DS1~16 endophytic fungi. Magnification $\times 1$ (upper panel) and $\times 40$ (lower panel) pictures of DS1~16 endophytic fungi taken under microscopy observation.

Several limitations of the study should be noted. First, the study confirmed the endophytic behaviors of fungal strains isolated to be beneficial for the host plants, further studies about the effective chemicals and detailed mechanisms are needed. Second, only ITS4 sequences were used for taxonomic identification of the putative fungal endophytes. Additional sequences such as 18S will be required for further validation. Third, it is currently impossible to purify sufficient endophytic chemicals for *in planta* validation experiments, such as on host SM itself since we do not have their mechanisms clear enough. Potentially, the crude products from candidate endophytes could be subject to further identification of pure chemicals, and large-scale synthesis and semi-synthesis.

Conclusion

This is the first systematic investigation of SM endophytes of their antifungal capacity. To sum up, these endophytes showed inhibitory capacities against diverse pathogenic fungi, including *Cercospora nicotianae*, *Phoma arachnidicola*,

Staphylococcus, *Phytophthora eggplant*, and *Rhizoctonia cerealis*. Among them, *E.sp. SX19* and *C. Gloeosporioids* have the highest potential in multiple suppression of pathogenic fungi, which will elevate the yield and quality of therapeutic medical crops, Gucheng SM. The antifungal capacities of Gucheng SM endophytes identified in this study will be of value in agricultural and clinical practice after further *in planta* experiments in the near future.

Conflicts of Interest

The Authors declare that no potential conflicts of interest exist.

Author's Contributions

Drs. Da-Tian Bau, Jai-Sing Yang and Guo-Kai Wang designed the overall research; Yu-Fei Huang and Jin-Song Liu performed the genetic and experimental research; Chia-Wen Tsai and Wen-Shin Chang contributed to the establishment and development of statistical and analyzing platform, while Guo-Kai Wang, Da-Tian Bau and Wen-Shin Chang contributed to the completing of the article. All Authors read and approved the final manuscript.

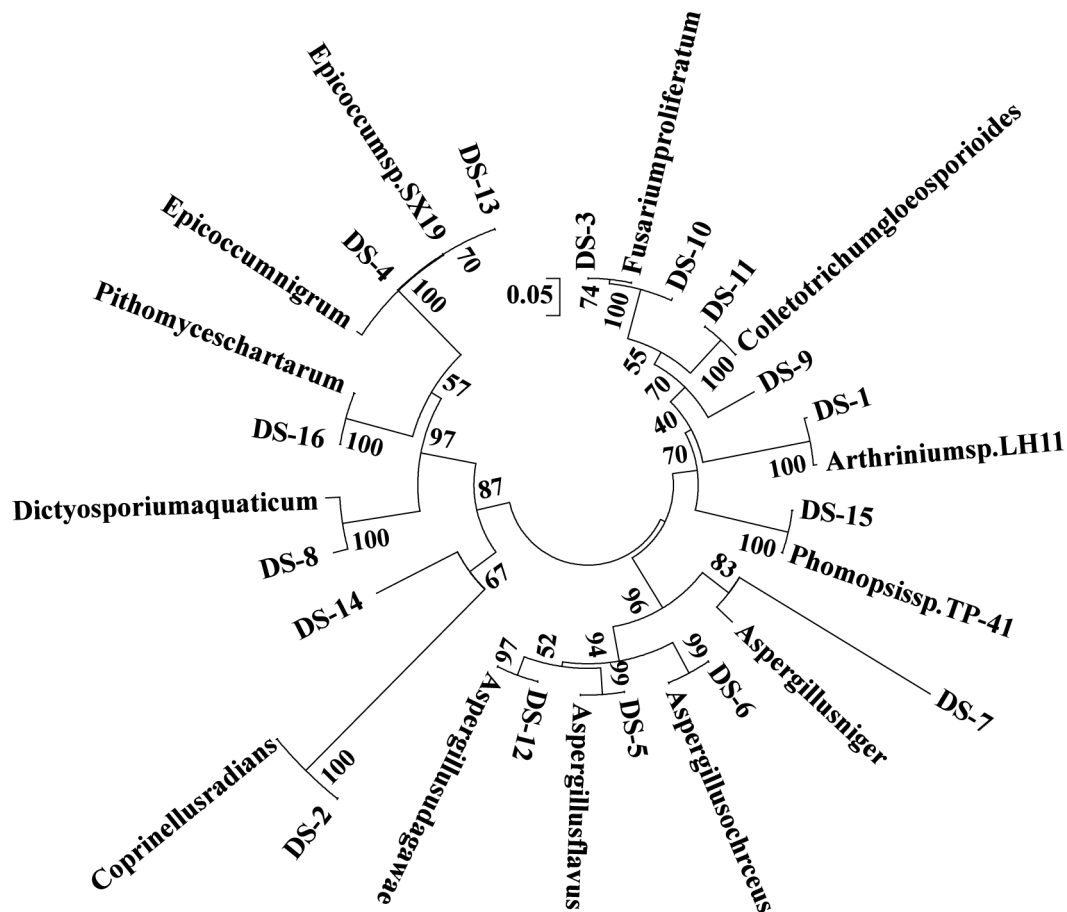


Figure 2. The phylogenetic tree of endophytic fungi based on ITS sequences. Each PCR product was excised and eluted from the agarose gel, while the purified DNA was sequenced and compared to Genbank on NCBI website. The phylogenetic trees were then constructed according to the Kimura2-Parameter model with Neighbor-Joining cluster analysis.

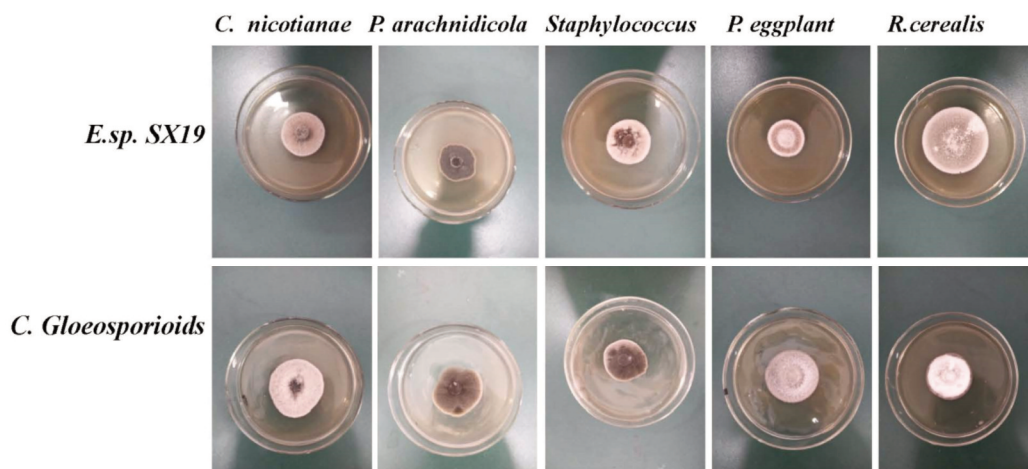


Figure 3. The inhibition rate of *E.sp. SX19* and *C. Gloeosporioids* on 5 plant pathogenic fungi in vitro. The colony diameters of *E.sp. SX19* (upper panel) and *C. Gloeosporioids* (lower panel) under the challenges of five pathogenic fungi, including *Cercospora nicotianae*, *Phoma arachnidicola*, *Staphylococcus*, *Phytophthora eggplant*, and *Rhizoctonia cerealis*, were measured. The inhibition rates for each treatment were calculated with the formula: (control plate colony diameter - fermentation broth colony diameter)/control plate colony diameter $\times 100\%$.

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