Tissue-Specific Endophytic Fungal Community of Wild Sophora tonkinensis and Its In Vitro Antimicrobial Activity Against Pathogenic Bacteria: A Postprint

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Abstract

Beneficial microbiomes can assist host plants in defending against diseases. The roots, stems, and seeds of wild Vietnamese Sophora germinate and grow healthily under natural conditions, whereas tissues of cultivated Vietnamese Sophora are highly susceptible to diseases. To investigate the potential of utilizing beneficial endophytic fungal communities from wild Vietnamese Sophora for disease control in the host, endophytic fungi were isolated from healthy wild Vietnamese Sophora roots, stems, and seeds, identified through a combination of morphological and ITS sequence characteristics, and analyzed for phylogenetic evolution, diversity, and similarity using phylogenetic trees, alpha diversity indices, and beta diversity indices, respectively. The in vitro anti-pathogen functions of the endophytic fungal communities were tested using the agar block method and plate confrontation method. The results demonstrated: (1) From the endophytic fungal communities of Vietnamese Sophora roots, stems, and seeds, 131 isolates (23 taxa), 108 isolates (23 taxa), and 64 isolates (11 taxa) were isolated and identified, respectively; (2) The abundance of unique genera and the fact that all species were endemic indicated tissue specificity in the genus-species evolution of root, stem, and seed endophytic fungal communities; (3) Extremely low beta diversity between root-stem/root-seed/stem-seed pairs revealed minimal species similarity among the endophytic fungal communities of roots, stems, and seeds; (4) High alpha diversity indicated rich and diverse endophytic fungal communities in Vietnamese Sophora roots, stems, and seeds; (5) More than one-third of the taxa from each endophytic fungal community could antagonize tested pathogens in vitro, with root and stem endophytic fungal communities exhibiting strong broad-spectrum anti-pathogenic bacterial/fungal functions, and seed endophytic fungal communities exhibiting strong broad-spectrum anti-pathogenic fungal functions. These results indicate that healthy wild Vietnamese Sophora roots, stems, and seeds harbor beneficial endophytic fungal communities possessing biodiversity, tissue specificity, and strong, broad-spectrum, and abundant in vitro anti-pathogen functions, which may play important roles in disease resistance of host tissues. This study provides materials and an experimental foundation for further utilization of beneficial fungal communities to control diseases in various tissues of cultivated Vietnamese Sophora.

Full Text

Tissue-Specificity and Pathogen-Resistant Function in vitro of Endophytic Fungal Microbiome Harbored in Wild Sophora tonkinensis

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Abstract: Beneficial microbiomes assist host plants in disease defense. The roots, stems, and seeds of wild Sophora tonkinensis germinate and grow healthily in natural environments, whereas cultivated S. tonkinensis tissues are highly susceptible to diseases. To explore the potential of utilizing beneficial endophytic fungal microbiomes from wild S. tonkinensis for disease control, we isolated endophytic fungi from healthy wild S. tonkinensis roots, stems, and seeds, identified them using morphological and ITS sequence characteristics, and analyzed their phylogenetic evolution, diversity, and similarity through phylogenetic trees, α-diversity indices, and β-diversity indices. The pathogen-resistant function in vitro was tested using agar plug and plate confrontation methods. The results showed: (1) A total of 131 strains belonging to 23 taxa, 108 strains belonging to 23 taxa, and 64 strains belonging to 11 taxa were isolated and identified from the roots, stems, and seeds of S. tonkinensis, respectively; (2) The presence of numerous endemic genera and all species being endemic indicated tissue specificity in the genus and species evolution of the endophytic fungal microbiomes in roots, stems, and seeds; (3) Extremely low β-diversity between root-stem/root-seed/stem-seed pairs revealed very low species similarity among different endophytic fungal microbiomes; (4) High α-diversity demonstrated that S. tonkinensis roots, stems, and seeds harbor rich and diverse endophytic fungal microbiomes; (5) More than one-third of the taxa in each microbiome antagonized tested pathogens in vitro, with root and stem microbiomes showing strong broad-spectrum activity against both bacterial and fungal pathogens, while seed microbiomes showed strong broad-spectrum activity against fungal pathogens. These findings indicate that healthy wild S. tonkinensis roots, stems, and seeds harbor beneficial endophytic fungal microbiomes characterized by biodiversity, tissue specificity, and strong, broad-spectrum, abundant pathogen-resistant functions in vitro, which likely play important roles in disease resistance across host tissues. This study provides materials and an experimental foundation for further utilization of beneficial fungal microbiomes to control diseases in cultivated S. tonkinensis tissues.

Keywords: Wild Sophora tonkinensis; beneficial endophytic fungal microbiome; biodiversity; tissue specificity; pathogen-resistant function

Sophora tonkinensis is the source plant of Shandougen, a renowned genuine medicinal material from Guangxi. Shandougen possesses heat-clearing, detoxifying, and anti-inflammatory properties, and is commonly used to treat damp-heat jaundice, arrhythmia, and bladder cancer. It contains various chemical components including alkaloids, flavonoids, and saponins, with matrine and oxymatrine as its main active ingredients. Pharmacological studies have demonstrated anti-tumor, antiviral, anti-inflammatory, antioxidant, antibacterial, hepatoprotective, immunomodulatory, and blood pressure-lowering effects. With the development of the health industry and rural revitalization, S. tonkinensis has been cultivated in Baise, Jingxi, and Hechi regions of Guangxi. However, our research group observed that cultivated S. tonkinensis is prone to various diseases: seeds frequently mold and show poor germination, while roots and stems often rot, develop anthracnose ulcers, and exhibit stripe and black spot diseases that affect medicinal material growth. Extensive chemical pesticide use leads to excessive pesticide and heavy metal residues, seriously compromising medicinal safety. Therefore, developing environmentally friendly disease control strategies is crucial for the sustainable utilization and development of Shandougen.

Plants possess innate immune systems that respond to molecular mechanisms and effectors from pathogenic microorganisms. Beneficial plant microbiomes interact with plant innate immune systems to induce systemic acquired resistance, playing vital roles in plant health. Studies on tomato rhizosphere microbiomes revealed significant differences in structure and antimicrobial functions between disease-resistant and susceptible plants. The microbiome from resistant plants, when transferred to susceptible ones, could activate disease resistance. Plant microbiomes include both fungal and bacterial communities. Rhizosphere microbiomes primarily originate from highly diverse soil organisms and are influenced by soil type, plant genotype, and plant age, while stem and seed microbiomes mainly derive from aerosols, insects, and root migration. In plant-beneficial endophytic fungal microbiomes, various endophytic fungi activate plant innate immune systems through complex and delicate interactions with the host, conferring resistance against pests and pathogens. Some endophytic fungi can even secrete toxic metabolites to directly antagonize pathogen invasion. Thus, beneficial endophytic fungal microbiomes contribute to host disease defense.

Wild S. tonkinensis grows in limestone mountain crevices at 500–800 m altitude, surrounded by diverse vegetation and complex local microclimates, yet maintains healthy germination, growth, and unique medicinal properties. Our previous research found that S. tonkinensis tissues harbor endogenous fungi. We hypothesize that this unique and complex wild habitat fosters distinctive and diverse beneficial endophytic fungal microbiomes in S. tonkinensis roots, stems, and seeds, which likely play important roles in pest and disease resistance. This study examined wild S. tonkinensis roots, stems, and seeds collected at different developmental stages but with the same growth period. Using tissue block methods for fungal isolation, combined morphological and ITS sequence identification, maximum likelihood phylogenetic tree construction, and R software for α- and β-diversity index calculations, we tested the antimicrobial activity of each microbiome against pathogens using agar plug and plate confrontation methods. By analyzing the phylogenetic relationships, α-diversity, β-diversity, and in vitro pathogen resistance of endophytic fungal microbiomes from wild S. tonkinensis roots, stems, and seeds, we addressed three questions: (1) Do beneficial endophytic fungal microbiomes from wild S. tonkinensis roots, stems, and seeds differ in genus and species phylogeny? (2) What are the diversity and similarity of these microbiomes? (3) Do they exhibit disease resistance functions in vitro? The results provide materials and an experimental basis for exploring the use of beneficial fungal microbiomes from wild S. tonkinensis to control diseases in cultivated S. tonkinensis tissues.

1.1.1 Plant Material

Eight healthy wild S. tonkinensis plants (4-year-old, approximately 2 m tall, with root diameter ~1 cm and stem diameter ~1.5 cm) were collected from Chengliang Village, Jingxi City (23°08 49 N, 106°25 26 E). Uniform, plump seeds (0.5 × 0.3–1 × 0.8 cm) were also collected. Root and stem samples were collected in March, August, and November 2020, while seeds at different maturity stages were collected in July, August, and September 2020. All samples were bagged, labeled, and transported to the Biochemistry and Molecular Biology Laboratory at Guangxi University of Science and Technology for processing within 24 hours.

1.1.2 Pathogenic Test Organisms

Bacterial pathogens: *Pectobacterium carotovorum* (bacterial soft rot), *Xanthomonas oryzae* pv. *oryzicola* (bacterial leaf streak), and *Xanthomonas campestris* pv. *citri* (bacterial canker). Fungal pathogens: *Fusarium solani* (root rot), *Colletotrichum gloeosporioides* (anthracnose), and *Alternaria panax* (black spot). All strains were preserved in the Department of Basic Medicine at Guangxi University of Science and Technology.

1.1.3 Culture Media, Reagents, and Sequencing

PDA medium, corn medium, and LA medium were purchased from Guangdong Huankai Microbial Technology. All media were sterilized by autoclaving at 121 °C and 1×10^5 Pa for 20 minutes. Sodium hypochlorite, glycerol, and ethanol were purchased from Chengdu Jinshan Chemical Reagents. 2×Taq MasterMix was purchased from Tiangen Biotech. Primer synthesis (ITS1/ITS4) and PCR product sequencing were completed by Nanjing Zhongding Biotechnology.

1.1.4 Instruments and Equipment

Biological safety cabinet (Thermo 1376), PCR amplifier (BIO-RAD C1000), gel imaging system (Bio-Best 200E), biochemical incubator (SPX-250), ultra-low temperature freezer (Thermo 902), centrifuge (Sigma 3K15), microscope (Olympus IX83), drying oven (DHG-9030), and autoclave (Tomy XS500).

1.2.1 Isolation and Purification of Endophytic Fungi from Roots, Stems, and Seeds

Fresh, healthy root segments, stem segments, and seeds of S. tonkinensis were thoroughly rinsed under running water and air-dried before surface sterilization in a laminar flow hood. Sterilization involved treatment with 75% ethanol for 1 minute, three rinses with sterile water, 5% sodium hypochlorite for 1-6 minutes (1-2 minutes for root/stem segments; 5-6 minutes for seeds), and three additional sterile water rinses. After drying on sterile absorbent paper, root and stem segments had their ends and epidermis removed before being cut into 1 cm × 0.5 cm pieces. Seeds were separated into seed coats and embryos. All tissues were transferred to 1/2 cornmeal medium (containing chloramphenicol and ampicillin) and incubated in darkness at 25 °C. Emerging hyphae were promptly transferred to potato dextrose agar (PDA) plates. If colony morphologies were inconsistent, single hyphae were repeatedly transferred until uniform colonies were obtained.

1.2.2 Taxonomic Identification of Endophytic Fungi

Based on tissue origin, culture time, and colony morphology, strains isolated from roots, stems, and seeds were grouped into morphotypes. Representative strains from each morphotype were identified using DNA molecular markers. The universal primer pair ITS1/ITS4 was used to amplify ITS sequences (ITS1-5.8S rDNA-ITS2) from representative strain genomic DNA. Sequences were analyzed using ClustalX 1.81 and BioEdit v7.0, compared against GenBank, and the most similar (preferentially formally published) sequences were downloaded. Phylogenetic trees were constructed using the neighbor-joining method in MEGA 6.0 with 1,000 bootstrap replicates to assess reliability. Fungal identification followed established similarity thresholds: <95% as unidentified, $ $95% to genus level, $ $97% to species level. Within-genus comparisons showing significantly higher similarity to one species than others were identified as the same species.

1.2.3 Phylogenetic Analysis of Root, Stem, and Seed Microbiomes

Phylogenetic trees for each endophytic fungal microbiome were constructed using maximum likelihood methods in MEGA 6.0 based on ITS sequences of fungal taxa to analyze genus and species evolution.

1.2.4 Diversity and Similarity of Root, Stem, and Seed Microbiomes

α-diversity indices (Shannon-Wiener index, Simpson' s diversity index) and β-diversity indices (Sorensen similarity index, Jaccard index) were calculated based on taxa and isolate numbers for each microbiome. SPSS 28.0 was used for variance analysis and multiple comparisons to assess diversity differences among root, stem, and seed microbiomes.

1.2.5 In Vitro Pathogen Resistance of Root, Stem, and Seed Microbiomes

Representative strains from each taxon were screened for antimicrobial activity. The agar plug method was used to test antagonistic activity against bacterial pathogens: fresh bacterial suspensions were adjusted to 0.5 McFarland turbidity and spread on LA plates within 15 minutes. Six-millimeter fungal plugs were placed on dried LA plates (5 plugs per plate) and incubated at 35$±$2 °C for 16–18 hours before measuring inhibition zone diameters. The plate confrontation method tested activity against fungal pathogens: 6-mm plugs of endophytic fungi and pathogens were prepared, with one fungal plug placed at the center of a PDA plate and three pathogen plugs placed equidistantly around it. Controls lacked endophytic fungi. After incubation at 28 °C, pathogen growth was measured from the pathogen plug center to the plate center when controls reached the plate center, and inhibition rates were calculated.

1.3 Statistical Analysis

Relative frequency (RF), α-diversity indices (Shannon-Wiener, Simpson' s), and β-diversity indices (Sorensen, Jaccard) were calculated based on taxa and isolates. SPSS 28.0 was used for variance analysis and multiple comparisons to assess diversity differences between wild and cultivated S. tonkinensis microbiomes.

2.1 Isolation and Identification of Endophytic Fungi from Roots, Stems, and Seeds

A total of 131 endophytic fungal strains were isolated from 60 root tissue blocks [Figure 1: see original paper], 108 strains from 60 stem tissue blocks [Figure 2: see original paper], and 64 strains from 40 seeds [Figure 3: see original paper]. Strains were first separated by tissue origin, then cultured. Those with consistent spore morphology during the same culture period were grouped as morphotypes. ITS sequence analysis identified 23 taxa from 42 root morphotype representatives, 23 taxa from 38 stem morphotype representatives, and 11 taxa (all from seed coats) from 20 seed morphotype representatives. Among the 23 root taxa, 11 were identified to genus level, 10 to species level, and 2 remained unidentified. For stems, 9 taxa were identified to genus, 11 to species, and 3 were unidentified. For seeds, 1 taxon was identified to genus, 9 to species, and 1 was unidentified. Sequences were deposited in GenBank under accession numbers KP204264-KP204442, KT935174, and KR611926.

2.2 Phylogenetic Analysis of Root, Stem, and Seed Microbiomes

Maximum likelihood phylogenetic trees were constructed based on ITS sequences of 23 root taxa, 23 stem taxa, and 11 seed taxa [FIGURE:1, FIGURE:2, FIGURE:3]. In root microbiomes, 12 genera and 10 species were identified, with four dominant genera: *Fusarium* (most dominant), followed by *Phoma*, *Penicillium*, and *Rhexocercosporidium*. Stem microbiomes contained 15 genera and 11 species, with four dominant genera: *Arthrinium* (most dominant), *Penicillium*, *Fusarium*, and *Cladosporium*. Seed microbiomes comprised 6 genera and 9 species, with three dominant genera: *Colletotrichum* (most dominant), *Cladosporium*, and *Pseudocercospora*. Only two genera (*Penicillium* and *Cladosporium*) were shared among all three tissues, while unique genera numbered 10, 13, and 4 for roots, stems, and seeds, respectively—approximately 5, 7, and 2 times the number of shared genera. No species were shared among tissues; all were endemic (10, 11, and 9 unique species for roots, stems, and seeds, respectively). These results demonstrate that endophytic fungal microbiomes in wild S. tonkinensis roots, stems, and seeds exhibit tissue specificity at both genus and species levels.

2.3 Diversity and Similarity of Root, Stem, and Seed Microbiomes

α-diversity indices (Shannon-Wiener and Simpson' s) and β-diversity indices (Sorensen similarity and Jaccard) were calculated based on taxa, isolates, and shared taxa [FIGURE:1, FIGURE:2, FIGURE:3] [TABLE:1, TABLE:2]. The α-diversity indices for root, stem, and seed microbiomes were relatively high compared to other medicinal plants, indicating rich and diverse endophytic fungal communities. Root and stem microbiomes showed significantly higher α-diversity than seed microbiomes, suggesting greater species diversity in roots and stems. β-diversity indices ranged from 0 to 0.148 . According to similarity coefficient principles, values of 0.00–0.25 indicate extreme dissimilarity, 0.25–0.50 moderate dissimilarity, 0.50–0.75 moderate similarity, and 0.75–1.00 high similarity. These results demonstrate extremely low species similarity among root, stem, and seed microbiomes.

2.4 In Vitro Pathogen Resistance of Root, Stem, and Seed Microbiomes

Three bacterial pathogens (*Pectobacterium carotovorum*, *Xanthomonas oryzae* pv. *oryzicola*, *Xanthomonas campestris* pv. *citri*) and three fungal pathogens (*Fusarium solani*, *Colletotrichum gloeosporioides*, *Alternaria panax*) that commonly cause root, stem, and seed diseases were used as targets.

Antibacterial activity screening of 23 root, 23 stem, and 11 seed taxa revealed that 3 root taxa (13%) inhibited bacterial pathogens: 2 inhibited *P. carotovorum* with inhibition zones equal to the positive control, 3 inhibited *X. oryzae* pv. *oryzicola* with zones ≥ positive control, and 3 inhibited *X. campestris* pv. *citri* (1 equal to control). Notably, *Metarhizium anisopliae* strongly inhibited all three bacterial pathogens. Two stem taxa (9%) showed inhibition, with *Bionectria* sp. inhibiting all three pathogens with zones ≥ positive control. No seed taxa exhibited antibacterial activity.

Antifungal activity screening showed that 9 root taxa (39%) inhibited fungal pathogens: 8 suppressed *F. solani* by >50%, 9 suppressed *C. gloeosporioides* by >50%, and 5 suppressed *A. panax* by >50%. Five taxa (*Fusarium solani*, *Lasiodiplodia theobromae*, *Rhexocercosporidium* sp., *Phoma* sp., *Phoma herbarum*) strongly inhibited all three fungal pathogens. Nine stem taxa (39%) showed inhibition: 4 suppressed *F. solani* by >50%, 7 suppressed *C. gloeosporioides* by >50%, and 1 suppressed *A. panax* by >50%. *Fusarium oxysporum* strongly inhibited all three pathogens. Four seed taxa (36%) exhibited inhibition: 2 suppressed *F. solani* by >50%, 3 suppressed *C. gloeosporioides* by >50%, and 1 suppressed *A. panax* by >50%. *Colletotrichum hymenocallidis* strongly inhibited all three fungal pathogens.

These results demonstrate that root and stem microbiomes possess abundant pathogen-antagonistic taxa (10 taxa each, 43%) with strong, broad-spectrum activity against all six bacterial and fungal pathogens. Notable broad-spectrum taxa include *M. anisopliae*, *F. solani*, *L. theobromae*, *Rhexocercosporidium* sp., *Phoma* sp., and *P. herbarum* from roots; *Bionectria* sp. and *F. oxysporum* from stems; and *C. hymenocallidis* from seeds. While seed microbiomes showed no antibacterial activity, they effectively antagonized fungal pathogens. These findings indicate that wild S. tonkinensis root and stem microbiomes possess strong, broad-spectrum, and abundant anti-bacterial/fungal functions, while seed microbiomes exhibit strong, broad-spectrum, and abundant anti-fungal functions in vitro.

3 Discussion and Conclusion

Every plant tissue harbors distinct and diverse endophytic fungal microbiomes that co-evolve and form mutualistic relationships with their hosts. Based on "acquired immunity" theory, some endophytic fungi activate plant innate immune systems, conferring resistance against pests and pathogens. The "mosaic effect" suggests that through complex host-endophyte and endophyte-endophyte interactions, diverse chemical compounds are synthesized to protect the host. The "balanced cooperation" hypothesis proposes that some endophytes directly antagonize pathogens. Previous studies confirm that endophytic fungal microbiomes play crucial roles in plant disease resistance.

Our cultivation studies show that cultivated S. tonkinensis roots, stems, and seeds are highly disease-prone, whereas wild S. tonkinensis tissues rarely develop diseases. Previous work confirmed endophytic fungi in S. tonkinensis tissues. We hypothesized that the unique limestone mountain habitat of wild S. tonkinensis fosters distinctive beneficial endophytic fungal microbiomes that contribute significantly to disease resistance. Host age, season, environment, and tissue type strongly influence microbiome phylogeny, diversity, and function. To avoid these confounding effects and effectively utilize wild S. tonkinensis microbiomes for disease control in cultivated plants, we isolated and identified endophytic fungi from roots, stems, and seeds of the same growth period and developmental stage, analyzing their phylogeny, diversity, similarity, and antimicrobial potential.

High α-diversity indicates that wild S. tonkinensis roots, stems, and seeds harbor taxonomically rich and diverse endophytic fungal microbiomes. The significantly higher α-diversity in roots and stems compared to seeds likely reflects longer growth periods, consistent with previous studies. According to acquired immunity theory, greater taxonomic richness helps activate host innate immune systems, conferring resistance against multiple pathogens. The mosaic effect suggests that diverse microbiomes facilitate varied host-microbe and microbe-microbe interactions, synthesizing diverse chemical compounds to protect host tissues. Thus, rich and diverse wild S. tonkinensis microbiomes benefit disease defense. Low β-diversity indicates significant differences in genus and species development among root, stem, and seed microbiomes, demonstrating tissue specificity. This specificity may help host tissues antagonize specific pathogens through tailored interactions.

In vitro functional analysis revealed that over one-third of taxa in each microbiome exhibit antimicrobial activity. These antagonistic taxa, particularly broad-spectrum ones such as *M. anisopliae*, *F. solani*, *L. theobromae*, *Rhexocercosporidium* sp., *Phoma* sp., and *P. herbarum* from roots; *Bionectria* sp. and *F. oxysporum* from stems; and *C. hymenocallidis* from seeds, likely directly antagonize pathogen invasion. The two shared genera (*Penicillium* and *Cladosporium*) may help defend against common diseases across all tissues. Shared taxa between root-stem and stem-seed pairs suggest potential microbial exchange among tissues.

Studies on disease-resistant tomato plants showed that dominant rhizosphere genera, though inactive against pathogens in vitro, could activate host innate immunity and strongly suppress Fusarium wilt when inoculated into tomato. This highlights the importance of dominant genera in conferring disease resistance. Our study identified specific dominant genera in each tissue: *Fusarium*, *Phoma*, *Penicillium*, and *Rhexocercosporidium* in roots; *Arthrinium*, *Penicil-*

lium, *Fusarium*, and *Cladosporium* in stems; and *Colletotrichum*, *Cladosporium*, and *Pseudocercospora* in seeds. Notably, some dominant taxa also exhibited antimicrobial activity, suggesting they may activate host immunity and protect tissues against pathogen-induced diseases. Although seed microbiomes showed no in vitro antibacterial activity, their dominant genera may still confer antibacterial immunity upon seed colonization.

In conclusion, wild S. tonkinensis roots, stems, and seeds harbor rich, diverse, tissue-specific endophytic fungal microbiomes with strong, broad-spectrum, and abundant pathogen-resistant functions. As beneficial microbiomes, they likely play important roles in disease resistance across host tissues.

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