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Taxonomic Diversity, Functional Guilds, and Spatial Distribution of Endophytic Fungi in Healthy Spinach via Amplicon Sequencing

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ABSTRACT

Endophytic fungi, residing within plant tissues without causing disease, play critical roles in plant health and ecosystem dynamics. This study characterized the taxonomic composition, diversity, functional guilds, and spatial distribution of endophytic fungal communities in healthy spinach (Spinacia oleracea L.) leaves using ITS1 amplicon sequencing. Surface-sterilized leaf samples were processed for DNA extraction, followed by Illumina MiSeq sequencing. Sequences were clustered into 24 operational taxonomic units (OTUs) using QIIME2 and the UNITE database. Fusarium spp. dominated (77.23% of reads), followed by Thanatephorus cucumeris (19.90%) and Plectosphaerella spp. (2.73%). Alpha diversity analysis revealed moderate richness (24 OTUs) but low evenness (Shannon index: 2.097, Simpson index: 0.702). FUNGuild annotation assigned ecological roles to 21 OTUs, with saprotrophs comprising 94.82% of abundance, symbiotrophs 0.04%, and other guilds 5.14%. Multifunctional taxa, such as Fusarium (saprotroph-pathotroph-endophyte), were prevalent. Dispersion analysis revealed a highly aggregated community (VMR: 53,647.54, Morisita's Index: 6.76), characterized by the dominance of specific genera. Rank-abundance curves and guild distribution visualizations, generated using R (ggplot2), highlighted Fusarium dominance and saprotrophic prevalence. Most taxa exhibited filamentous morphology, aligning with saprophytic strategies. These findings suggest a low-diversity, highly aggregated fungal community with saprotrophic dominance, which may potentially influence spinach health. This study provides a foundation for understanding endophytic fungal ecology in agricultural systems, with implications for crop management.

Keywords: *Endophytic fungi, Spinach, Fungal diversity, ITS amplicon sequencing, FUNGuild, Alpha diversity*

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INTRODUCTION

Endophytic fungi are microorganisms that colonize internal plant tissues without causing visible harm, forming mutualistic associations that enhance host growth, increase stress tolerance, and improve resistance to pathogens (Rodriguez *et al*., 2009; Belfiore *et al*., 2024; Diakaki *et al*., 2025; Riccioni *et al*., 2025). As integral members of the plant microbiome, they contribute significantly to plant resilience, productivity, and ecological adaptation.

In leafy vegetables such as spinach (*Spinacia oleracea* L.), core fungal genera such as *Alternaria*, *Vishniacozyma*, *Mycosphaerella*, *Stemphylium*, and *Cladosporium* have been identified, with *Alternaria* and *Vishniacozyma* often dominating seed endophyte communities (Liu D., *et al*., 2022; Liu M., *et al*., 2022; Diakaki *et al*., 2025). These communities can vary based on factors such as seed lot, environment, and plant health status. The dominant phyla associated with spinach and other leafy greens are typically Ascomycota and Basidiomycota (Thazha *et al*., 2023; Wei *et al*., 2023; Shen *et al*., 2024; Eslami *et al*., 2025). Traditional studies of fungal endophytes have relied on culture-dependent methods and microscopic classification. Media such as Potato Dextrose Agar, Sabouraud Dextrose Agar, Malt Extract Agar, and Czapek-Dox Agar are commonly used for fungal isolation, with morphological features helping to classify isolates into morphotypes prior to molecular identification (Makhoahle & Gaseitsiwe, 2022; Demeni *et al*., 2025). However, these methods often overlook non-sporulating or slow-growing taxa.

To overcome these limitations and comprehensively characterize the endophytic fungal community in spinach, we employed high-throughput amplicon sequencing of the ITS region. Root and leaf tissues from healthy spinach plants were pooled for DNA isolation, followed by amplicon sequencing. Quality-filtered reads were processed using bioinformatics pipelines (e.g., DADA2), and taxonomic assignments were made using the UNITE database (Abarenkov *et al*., 2020; Dhanasekar *et al*., 2022; Shen & Bao, 2025). Community diversity was assessed through alpha diversity indices (Shannon, Simpson, Chao1, Observed OTUs), and functional ecological roles were assigned using the FUNGuild tool.

Fungal guilds—categorized as saprotrophs (decomposers), pathotrophs (pathogens), and symbiotrophs (mutualists)—help clarify ecological functions. Many fungi exhibit multitrophic strategies, contributing simultaneously to multiple roles (Rodriguez *et al*., 2009; Aamir *et al*., 2020; Graefen *et al*., 2023). Understanding these functional dynamics helps distinguish beneficial endophytes from latent pathogens, which has practical implications for developing biocontrol agents and improving sustainable cropping systems (Baron & Rigobelo, 2022; Wilhelmy *et al*., 2022; Kwatra *et al*., 2024; Nazir *et al*.,

2024).

Research gap and justification

While bacterial endophytes have been extensively studied, fungal endophytes—especially in open-field crops like spinach—remain underexplored. There is a significant lack of data regarding their taxonomic diversity, community structure, and ecological functions. Specifically, the diversity (in terms of richness and evenness), dominance patterns, and guild-level contributions of endophytic fungi in spinach have not been quantified. The functional ambiguity of dominant genera such as *Fusarium* limits their biotechnological applications. Moreover, multitrophic potential and functional redundancy within the fungal community remain poorly understood.

Objectives of the Study

To address these gaps, this study aimed to:

- Isolate and identify endophytic fungi from the roots and leaves of healthy spinach.
- Analyze their diversity using amplicon sequencing of the ITS region.
- Assign ecological guilds to the fungal taxa to evaluate their functional roles within the plant.

These efforts offer a comprehensive understanding of fungal endophyte diversity and ecological functionality in spinach, contributing to the development of microbial-based strategies for sustainable agriculture.

MATERIALS AND METHODS

Sample collection

To investigate the endophytic fungal communities, healthy (asymptomatic) spinach plants (*Spinacia oleracea* L.) were cultivated in a controlled greenhouse environment. The soil used for cultivation was characterized by low bulk density (0.825 g/cc), high porosity (68.87%), neutral pH (7.2), and elevated nutrient levels, including nitrogen (1120 mg/kg), phosphorus (106 mg/kg), and potassium (143 mg/kg). Under these conditions (soil humidity maintained at 60% and temperature at 30 ± 2 °C), spinach plants were grown to maturity. From ten randomly selected plants, one healthy leaf and the corresponding root from the same plant were sampled, totalling ten leaf and ten root samples. Surface sterilization of plant tissues followed the protocol of Petrini (1986), which involved immersion in 70% ethanol for 60 seconds, followed by immersion in 1% sodium hypochlorite for 30 seconds. Sterilized leaf and root fragments were stored at 4 °C for 24–48 hours to preserve DNA integrity for downstream analyses.

DNA extraction and amplicon sequencing

Total genomic DNA was extracted from approximately 0.25 g of surface-sterilized spinach leaf tissue (sample FP) using the DNeasy Plant Mini Kit (Qiagen, catalogue #69104, 2023 version), following the manufacturer's instructions. This kit was selected for its effectiveness in isolating high-quality DNA from plant tissues with minimal contamination by polysaccharides and polyphenols, ensuring compatibility with downstream PCR and sequencing applications. For samples with high polysaccharide content, a CTAB-based protocol (Doyle & Doyle, 1987) was used to enhance DNA yield. DNA quality and quantity were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA) and 1% agarose gel electrophoresis. The fungal internal transcribed spacer (ITS1) region was amplified using primers ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′), targeting the 18S rRNA gene, and a reverse primer (5′-GCTGCGTTCTTCATCGATGC-3′), targeting the 5.8S rRNA gene. PCR was performed in a Bio-Rad T100 thermal cycler with the following conditions: initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds, followed by a final extension at 72°C for 5 minutes. Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced on an Illumina MiSeq platform. Sequencing generated 250 bp paired-end reads with a depth of 204,332 raw reads (191,063 effective tags, ~45,383,772 bases), sufficient to capture the fungal community diversity.

Sequence processing and taxonomic assignment

Raw sequence reads from the Illumina MiSeq platform (250 bp paired-end) were processed to ensure high-quality data for taxonomic analysis. Paired-end reads were demultiplexed based on unique barcodes and merged using FLASH (v1.2.7) to generate raw tags. Quality filtering was performed using QIIME (v1.7.0), with a Q20 quality score threshold (sequencing error rate <1%) applied to remove low-quality reads, resulting in clean tags. Chimeric sequences were detected and removed using the UCHIME algorithm (Edgar *et al*., 2011) against the SILVA database (Quast *et al*., 2013).

Sequences were clustered into Operational Taxonomic Units (OTUs) at a 97% similarity threshold using UPARSE (v7.0.1090) (Edgar, 2013). Taxonomic assignments were made at the genus or species level by comparing representative OTU sequences against the UNITE database (v8.3) (Abarenkov *et al*., 2020) using QIIME's Mothur method with a confidence threshold of 0.8–1. The UNITE database was selected for its comprehensive and curated fungal ITS reference sequences, optimized for fungal taxonomy.

Alpha diversity analysis

Fungal alpha diversity was assessed to evaluate the richness, evenness, and phylogenetic diversity of endophytic communities in sample FP. Observed Species, Shannon, Simpson, Chao1, ACE, Good's coverage, and PD Whole Tree indices were calculated using QIIME (v1.7.0) (Caporaso *et al*., 2010; Zhao *et al*., 2025) and visualized in R (v 4.4.0). OTU tables, generated at a 97% similarity threshold, were rarefied to 89,207 sequences (the normalized effective tag count) to standardize sequencing depth. In QIIME, the alpha_diversity.py script computed Shannon (entropy-based diversity, reflecting richness and evenness), Simpson (dominance-based diversity), Chao1 (richness estimator accounting for rare taxa), and ACE (abundance-based coverage estimator) (Chao, 1984; Chao & Lee, 1992). Good's coverage ('Good average') estimated sampling completeness, with a value of 1.000 indicating full community capture (Good, 1953). PD Whole Tree quantified phylogenetic diversity, measuring evolutionary breadth across OTUs (Faith *et al*., 1992), calculated using QIIME's phylogenetic_diversity.py. These indices were selected for their complementary insights: Chao1 and ACE estimate total species richness, Shannon and Simpson assess community diversity and evenness, and PD Whole Tree captures phylogenetic variation.

Rarefaction curves for Observed Species, Shannon, Simpson, Chao1, and PD Whole Tree confirmed sufficient sequencing depth, flattening at 89,207 sequences.

Functional annotation with FUNGuild

Ecological functions of fungal operational taxonomic units (OTUs) were annotated using the FUNGuild tool (Nguyen *et al*., 2016). The FUNGuild repository was cloned from GitHub [https://github.com/UMNFuN/FUNGuild] and executed in a Linux environment. An input OTU table, containing absolute abundance values for 21 OTUs (3 unclassified OTUs excluded from analyses) from sample FP (surface-sterilized spinach tissue), was processed using the FUNGuild Python script (v1.1) configured with the fungal database (FUNGuild v1.1, 2023). OTUs were assigned to ecological guilds, including saprotrophs, symbiotrophs, pathotrophs, and multifunctional guilds (e.g., saprotroph-pathotroph-endophyte). Only guild assignments with 'probable' or 'highly probable' confidence levels were retained to ensure reliability. Three unclassified OTUs, representing <0.1% of total sequence reads, were excluded from guild proportion analyses due to insufficient taxonomic resolution or lack of matching entries in the database.

Statistical analysis and visualization in R

Data processing and statistical analyses were performed using R (version 4.4.0). The tidyverse package suite (v2.0.0), including dplyr (v1.1.4), tidyr (v1.3.1), and readr (v2.1.5), was used for data wrangling and transformation. Visualizations, including rank-abundance curves and trophic mode distributions, were generated using ggplot2 (v3.5.2). Rank-abundance curves were plotted to assess the dominance and rarity of fungal genera, with abundance data log-transformed as needed to enhance visualization clarity. To infer ecological strategies (e.g., endophyte, pathogen, saprophyte) of dominant fungal genera, OTU guild assignments from FUNGuild were cross-referenced with literature-based annotations and disease association data. Growth morphology (e.g., filamentous, yeast) was integrated with abundance and guild data to explore correlations with ecological function, using dplyr for data manipulation and ggplot2 for visualization. Multifunctional taxa (OTUs/genera with multiple guild assignments) were identified by parsing FUNGuild outputs to detect guild co-occurrence.

Dispersion and aggregation analysis

To assess the spatial distribution patterns of endophytic fungal communities in spinach endosphere (sample FP), a suite of dispersion and aggregation indices was applied, following methodologies from ecological studies (Tirkey & Saxena, 2016). Sample units were defined as the fungal sequence read counts per operational taxonomic unit (OTU) obtained from ITS1 amplicon sequencing of surface-sterilized leaf tissues, resulting in 24 OTUs that represented the endophytic community. The Variance-to-Mean Ratio (VMR) was calculated as VMR = variance / mean, where a value of 1 indicates a random (Poisson) distribution, <1 denotes a regular distribution, and >1 suggests aggregation (negative binomial). Additional indices included Lloyd's Index of Mean Crowding ($X^* = \bar{x} + [s^2/\bar{x}] - 1$), which accounts for density and clumping, and the derived Patchiness Index ($X^*/\bar{x}$), where values <1, =1, and >1 indicate dispersed, random, and aggregated distributions, respectively. David and Moore's Index of Clumping was calculated as IDM = VMR − 1, with values >0 reflecting contagious patterns, 0 for random, and <0 for regular distribution. Cole's Index ($I = [X^* - \bar{x}]/\bar{x}$) measures aggregation intensity, while Morisita's Index ($Im = n \Sigma [xi (xi - 1)] / N [N - 1]$) tests for unevenness in distribution, where *n* is the number of sample units, *xi* the count per unit, and *N* the total count. Formulas and interpretations followed sources: Cole (1946), David and Moore (1954), Morisita (1962), and Lloyd (1967). All calculations were performed in R (v4.4.0) using custom scripts, with the *vegan* package (v2.6-10) supporting OTU table processing and ecological analysis (Oksanen *et al*., 2013). Different formula of Aggregation Indices and Their Interpretation mentioned in **Table 1**.

Table 1. Different formula of Aggregation Indices and Their Interpretation.

Index	Purpose	Formula & Interpretation	
Variance Mean Ratio (VMR)	Indicates the nature and pattern in space and time.	**Formula:** VMR = Variance / Mean **Interpretation:** VMR = 1 → Random (Poisson) VMR < 1 → Regular VMR > 1 → Aggregated (clumped)	…. (1)
Lloyd's Mean Crowding (1967)	Indicates mean crowding, considering clumping and population density.	**Formula:** $X^* = \bar{x} + (s^2 / \bar{x}) - 1$	… (2)
Patchiness Index	Expresses dispersion of the distribution.	**Formula:** Patchiness = $X^* / \bar{x}$ **Interpretation:** < 1 → Dispersed = 1 → Random > 1 → Aggregated	… (3)
David & Moore's Clumping Index (DMI) (1954)	Measures clumping of populations.	**Formula:** IDM = $(s^2 / \bar{x})$ - 1 **Interpretation:** 0 → Random > 0 → Contagious < 0 → Regular	… (4)
Cole's Index (1946)	Measures the aggregative nature of distribution.	**Formula:** $I = (\Sigma x)^2 / (\Sigma x^2)$ **Interpretation:** Higher values indicate greater aggregation	… (5)
Morisita's Index (1962)	Tests for uneven distribution among sample	**Formula:** $Im = n \Sigma[xi(xi - 1)] / N(N - 1)$	…(6)

units.	Where: n = No. of samples, x = count/unit, N = total count

RESULTS AND DISCUSSION

Sequence processing

A total of 191,063 effective tags were obtained for sample FP after quality filtering and chimera removal, with an average sequence length of 237.53 nucleotides. Clustering at 97% sequence similarity produced 24 OTUs. The relative abundance of each OTU was calculated based on effective tag counts, enabling quantitative representation of fungal community structure in the FP sample.

Taxonomic composition of endophytic fungal communities

High-throughput sequencing of the fungal ITS1 region from healthy spinach leaves yielded 24 operational taxonomic units (OTUs), representing a diverse array of fungal genera. *Fusarium* spp. dominated the community, comprising 77.23% of total abundance (136,749 reads), with *Fusarium sp.* (101922 reads, 57.56%) and *Fusarium sublunatum* (34827 reads, 19.67%) being the most prevalent. *Thanatephorus cucumeris* was the second most abundant genus (35,227 reads, 19.90%), followed by *Plectosphaerella* spp. (4,834 reads, 2.73%). Other genera, including *Curvularia* (0.11%), *Leptospora* (0.009%), *Ceratobasidiaceae* (0.006%), *Alternaria* (0.006%), *Acremonium* (0.004%), *Cladosporium* (0.002%), *Nigrospora* (0.002%), *Phaeosphaeria* (0.002%), *Sarocladium* (0.001%), and *Aspergillus* (0.001%), were present at low abundances. Several genera, notably *Fusarium* and *Alternaria*, were associated with soft rot disease, though no disease symptoms were observed in sampled leaves. **Table 2** presents the proportional abundance (%) of the top 10 identified endophytic fungal taxa.

Table 2. Proportional Abundance (%) of Identified Endophytic Fungal Taxa (Top 10). *

Species name	% of composition
***Fusarium* sp**	50.36739166
Thanatephorus cucumeris	19.89562801
Fusarium sublunatum	19.66971461
***Fusarium* sp**	7.196471233
***Plectosphaerella* sp**	2.649399353
***Plectosphaerella* sp**	0.077940122
Curvularia verruculosa	0.070033153
Curvularia verruciformis	0.02259134
Curvularia lunata	0.012425237
***Leptospora* sp**	0.009036536

* Note: Multiple entries for the same genus represent distinct OTUs classified under the same taxonomic label.

The endophytic fungal community in the spinach endosphere is dominated by *Fusarium* (136,749 reads, ≈77.25%) and *Thanatephorus* (35,227 reads, ≈19.89%), together comprising over 97% of known abundances, with *Plectosphaerella* contributing a minor 4,834 reads (≈2.73%). Rare taxa, each with ≤10 reads, include *Acremonium* (8), *Cladosporium* (4), *Nigrospora* (3), *Phaeosphaeria* (3), *Sarocladium* (2), and *Aspergillus* (2), indicating low-abundance genera with minimal community impact.

Abundance distribution of fungal genera

The fungal community exhibited a highly uneven abundance distribution, with *Fusarium* and *Thanatephorus* accounting for over 97% of total reads. Rare taxa, defined as genera with ≤10 reads, included *Acremonium* (8 reads), *Cladosporium* (4 reads), *Nigrospora* (3 reads), *Phaeosphaeria* (3 reads), *Sarocladium* (2 reads), and *Aspergillus* (2 reads). Rank-abundance analysis revealed a steep curve, indicating low evenness and dominance by a few genera **(Figure 1)**.

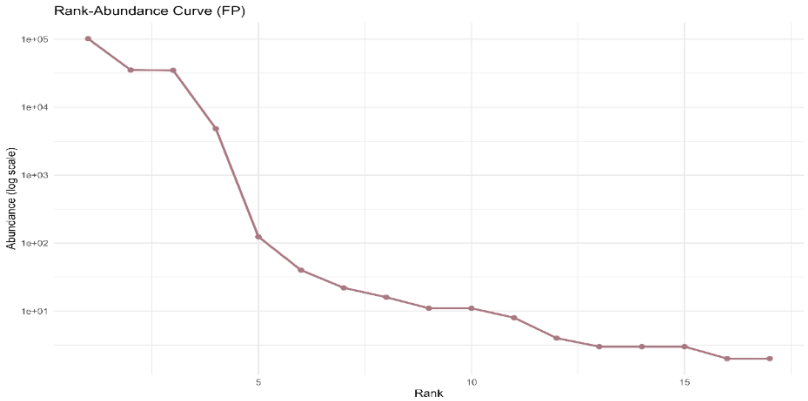

Figure 1. Rank-abundance curve of endophytic fungal genera. The steep slope indicates low evenness and strong dominance by a few genera, suggesting an aggregated community structure with limited taxonomic diversity.

Alpha diversity of endophytic fungal communities

Alpha diversity analysis of the endophytic fungal community in the spinach endosphere revealed a community characterized by moderate diversity and complete sampling coverage. The analysis, based on 24 operational taxonomic units (OTUs) derived from ITS1 amplicon sequencing, yielded the following metrics: Observed Species was 24, indicating the total number of distinct fungal OTUs detected. The Shannon index (2.097) reflected moderate diversity, accounting for both richness and evenness, while the Simpson index (0.702) suggested a community with notable dominance by a few taxa. Both Chao1 and ACE indices, which estimate total species richness, were 24.000, aligning with the observed species count and indicating no undetected rare taxa**.** Good's coverage reached 1.000, confirming that the sequencing depth (191,063 effective tags) fully captured the community. The PD Whole Tree index (3.400) indicated moderate phylogenetic diversity, reflecting the evolutionary breadth across OTUs. These metrics collectively highlight a fungal community dominated by a few abundant taxa, such as *Fusarium* (77.25%) and *Thanatephorus* (19.89%), with comprehensive sampling of all present OTUs. Alpha diversity indices of endophytic fungal communities in spinach (FP sample) are presented in **Table 3**.

Table 3. Alpha Diversity Indices of Endophytic Fungal Communities in Spinach (FP Sample)

Sample	Observed Species	Shannon	Simpson	Chao1	ACE	Good average	PD Whole Tree
FP	24	2.097	0.702	24.000	24.000	1.000	3.400

Dominant FUNGuilds of spinach endophytic fungi

The endophytic fungal community associated with the FP treatment was characterized by sequencing 20 operational taxonomic units (OTUs), revealing a diverse assemblage of microfungi dominated by Ascomycota (18 OTUs) and Basidiomycota (2 OTUs).

The genus *Fusarium* was the most abundant, contributing 77.24% of the total abundance (136,749 reads across OTU_1, OTU_2, and OTU_5), classified as mixotroph (Pathotroph-Saprotroph-Symbiotroph). In FUNGuild these OTUs function as foliar endophytes, litter saprotrophs, and plant pathogens, capable of causing damping-off disease and decaying leaves, fruits, seeds, roots, soil, and animal material.

The second most abundant is the *Thanatephorus* (19.9%, 35,227 reads) characterized as a Pathotroph-Saprotroph with a corticioid growth morphology. It functions as a plant pathogen and plant saprotroph, primarily decaying leaves, fruits, and seeds. Third abundant -*Plectosphaerella* (2.73%, 4,834 reads, OTU_6, OTU_10, OTU_18), displayed a Pathotroph-Saprotroph-Symbiotroph trophic mode, with guilds encompassing Endophyte-Plant Pathogen-Plant Saprotroph. These microfungi are known for their ability to decay leaves, fruits, seeds, and animal material.

Curvularia (0.11%, 189 reads across OTU_7, OTU_8, OTU_9, and OTU_22), all classified as Pathotroph-Saprotroph with a guild of Plant Pathogen-Plant Saprotroph. These microfungi decay wood, leaves, fruits, seeds, roots, and algal material.

Other genera, such as *Alternaria* (OTU_13), *Cladosporium* (OTU_24), and *Aspergillus* (OTU_21), shared the Pathotroph-Saprotroph-Symbiotroph trophic mode. *Acremonium* (OTU_14) also acts as a fungal parasite, while *Leptospora* (OTU_11) and *Nigrospora* (OTU_17) are primarily saprotrophs. *Phaeosphaeria* (OTU_23) and *Sarocladium* (OTU_20) contribute as endophytes and pathogens, respectively. Ceratobasidiaceae (OTU_12) was notable for its endomycorrhizal associations with plant roots (Cevallos *et al*., 2022).

A striking feature of this endophytic guild is the prevalence of the Pathotroph-Saprotroph-Symbiotroph trophic mode across multiple genera, suggesting a highly adaptable fungal community. The functional guilds and trophic modes of spinach endophytic fungal genera are summarized in **Table 4**.

Table 4. Functional Guilds and Trophic Modes of Spinach Endophytic Fungal Genera**

Genus	Guild	Growth Morphology	Trait	Confidence Ranking	Trophic Mode	OTUs	Reads	Percentage %
Fusarium	Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Plant Saprotroph-Undefined Saprotroph-Wood Saprotroph	Microfungus	Soft Rot	Possible	Pathotroph-Saprotroph-Symbiotroph	OTU_1, OTU_2, OTU_5	136,749	77.23%
Thanatephorus cucumeris	Plant Pathogen-Plant Saprotroph	Corticioid	None	Probable	Pathotroph-Saprotroph	OTU_3	35,227	19. 9%
***Plectosphaerella* sp.**	Endophyte-Plant Pathogen-Plant Saprotroph	Microfungus	None	Probable	Pathotroph-Saprotroph-Symbiotroph	OTU_6, OTU_10, OTU_18	4,834	2.73%
Curvularia	Plant Pathogen-Plant Saprotroph	Microfungus	None	Probable	Pathotroph-Saprotroph	OTU_7, OTU_8, OTU_9, OTU_22	189	0.11%

Leptospora sp.	Plant Saprotroph	Microfungus	None	Probable	Saprotroph (Single)	OTU_11	16	0.009%
Ceratobasidiaceae	Endomycorrhizal-Plant Pathogen-Undefined Saprotroph	Microfungus	None	Possible	Pathotroph-Saprotroph-Symbiotroph	OTU_12	11	0.006%
Alternaria destruens	Animal Pathogen-Endophyte-Plant Pathogen-Plant Saprotroph-Wood Saprotroph	Microfungus	Soft Rot	Possible	Pathotroph-Saprotroph-Symbiotroph	OTU_13	11	0.006%
Acremonium sclerotigenum	Animal Pathogen-Endophyte-Fungal Parasite-Plant Pathogen-Undefined Saprotroph-Wood Saprotroph	Microfungus	Soft Rot	Possible	Pathotroph-Saprotroph-Symbiotroph	OTU_14	8	0.005%
***Cladosporium* sp.**	Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Plant Saprotroph-Wood Saprotroph	Microfungus	None	Possible	Pathotroph-Saprotroph-Symbiotroph	OTU_24	4	0.002%
Nigrospora vesicularifera	Plant Saprotroph-Undefined Saprotroph	Microfungus	None	Probable	Saprotroph (Single)	OTU_17	3	0.002%
Phaeosphaeria oryzae	Endophyte-Plant Pathogen-Plant Saprotroph	Microfungus	None	Probable	Pathotroph-Saprotroph-Symbiotroph	OTU_23	3	0.002%
Sarocladium strictum	Plant Pathogen-Plant Saprotroph-Undefined Saprotroph	Microfungus	None	Probable	Pathotroph-Saprotroph	OTU_20	2	0.001%
Aspergillus penicillioides	Animal Pathogen-Endophyte-Plant Saprotroph-Undefined Saprotroph-Wood Saprotroph	Microfungus	None	Probable	Pathotroph-Saprotroph-Symbiotroph	OTU_21	2	0.001%
Unclassified	Not assigned	Not assigned	None	Not assigned	Unclassified	3 OTUs	19	~0.11%

**Note: Functional guilds and trophic modes were assigned using the FUNGuild database, and all information was obtained directly from FUNGuild (Tedersoo *et al*., 2014; Põlme *et al*., 2021; Kulkarni *et al*., 2023; Pavlova, 2024).

Dispersion and aggregation patterns of endophytic fungi in spinach

Dispersion analysis of fungal communities isolated from spinach endosphere revealed a highly aggregated spatial distribution. The following indices were calculated: To assess the spatial distribution patterns of endophytic fungal communities in spinach, multiple aggregation indices were calculated and subsequently log-transformed to facilitate normalization and comparative interpretation. The VMR and DMI exhibited high values of 53,647.54 and 53,646.54, respectively, both with identical $\log_{10}$-transformed values of 4.73, indicating strong aggregation. Similarly, Lloyd's Mean Crowding had the highest original value of 62,499.49, corresponding to a $\log_{10}$ value of 4.80, reinforcing the tendency toward clustered fungal populations. Moderate aggregation was observed for the Patchiness Index and Morisita's Index, with original values of 7.06 and 6.76, and corresponding $\log_{10}$ values of 0.85 and 0.83, respectively. In contrast, Cole's Index reflected comparatively lower aggregation, with a raw value of 2.96 and a $\log_{10}$ value of 0.47. These results collectively highlight a non-random, spatially aggregated distribution of endophytic fungi in the spinach endosphere. The visual summary of these indices is presented in **Figure 2**. Different Aggregation Indices (Log-transformed values) indicating strong spatial clustering of endophytic fungi (FP) in spinach.

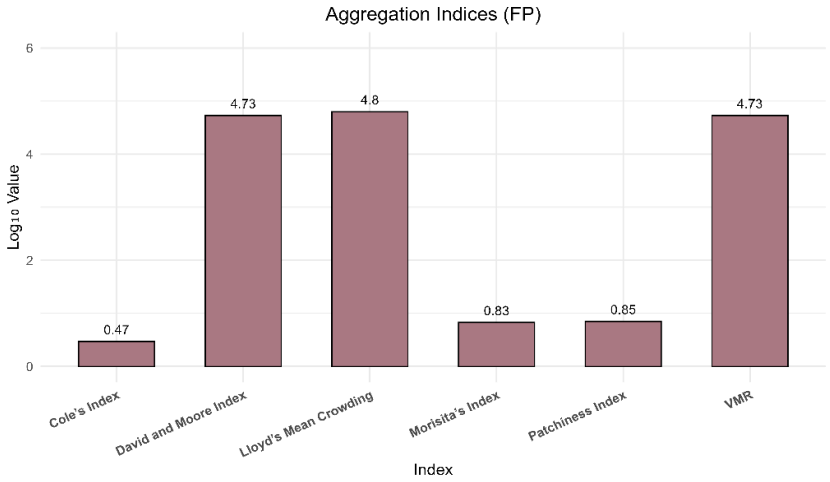

Figure 2. Different Aggregation Indices (Log-transformed values) indicating strong spatial clustering of endophytic fungi (FP) in spinach.

High-throughput sequencing revealed that *Fusarium* spp. and *Thanatephorus cucumeris* dominate the endophytic fungal community in healthy spinach leaves, comprising over 97% of the total abundance. The overwhelming presence of *Fusarium* spp. and *T. cucumeris* in the spinach endosphere suggests these fungi may be functionally significant in maintaining plant health or enhancing resilience, despite *Fusarium*'s common association with pathogenicity. Their prevalence in asymptomatic tissues implies a potential shift from a pathogenic to an endophytic or mutualistic lifestyle under certain environmental or physiological conditions.

Comparable patterns of *Fusarium* dominance have been observed in endophytic communities of other crops. Imazaki and Kadota (2015) reported that the majority of endophytic *Fusarium* isolates from tomato stems belonged to the *F. oxysporum*, *F. fujikuroi*, and *F. solani* species complexes, which were also prevalent in surrounding soils. Similarly, Mamaghani *et al*. (2024) and Osman *et al*. (2025) identified *Fusarium* spp. as one of the most abundant endophytes in healthy potato and medicinal plants, respectively, across different agro-climatic regions, with several strains demonstrating mycotoxin production potential. These findings collectively indicate that *Fusarium* spp. are widespread and adaptable fungal endophytes in various plant hosts (Asim *et al*., 2022; Seema *et al*., 2023; Ravoori *et al*., 2024).

The coexistence of *Fusarium* spp. and *T. cucumeris* as dominant fungi in healthy spinach leaves suggests they may participate in complex plant–fungal interactions. Their mutualistic roles could include modulating host immune responses, facilitating nutrient acquisition, or competing with pathogenic microbes. Such interactions may suppress visible disease symptoms, thereby maintaining an asymptomatic but colonized state.

Thanatephorus cucumeris, the teleomorph of *Rhizoctonia solani*, is a well-documented soil-borne phytopathogenic basidiomycete responsible for considerable yield losses in numerous economically important crops worldwide (Fernandes *et al*., 2022; Akber *et al*., 2023; Shaheen *et al*., 2023; Jash & Sarkar, 2025). In foliar vegetables, *R. solani* causes a wide range of symptoms including sheath blight, foliar blight, leaf blight, and leaf spot. Particularly in lettuce, an economically important raw-consumed leafy vegetable, *R. solani* is known to cause severe diseases such as late sugar beet rot, damping-off, and bottom rot, with potential yield losses of up to 70% (Ohkura *et al*., 2009; Maneea *et al*., 2024; Benitez-Andrade *et al*., 2025). Based on hyphal anastomosis reactions, *Rhizoctonia* isolates are divided into 13 anastomosis groups (AGs), each having distinct host specificity (Nandeesha *et al*., 2021; AlHussain *et al*., 2022; Malcangi *et al*., 2023; Naqvi *et al*., 2024). A comprehensive study in Greece revealed multiple *Rhizoctonia solani* AGs and *Pythium* spp. associated with damping-off in baby leafy vegetables, showing varying pathogenicity and broad host range (Tziros & Karaoglanidis, 2022; Bulusu & Cleary, 2023; Bolay *et al*., 2024). Interestingly, despite this known pathogenicity, the dominant presence of *T. cucumeris* in healthy spinach tissues may reflect a latent, ecologically balanced role that differs from its aggressive behavior in other crops. Its endophytic colonization might therefore represent a shift in its ecological function—from virulence to conditional mutualism—within the spinach microbiome.

Furthermore, Scherwinski *et al*. (2008) demonstrated that bacterial antagonists can actively suppress *R. solani* in lettuce with negligible short-term effects on non-target microbial communities, indicating that pathogen behavior is strongly influenced by microbial interactions. This supports the possibility that *T. cucumeris*, though closely related to a known pathogen, could assume a benign or even beneficial role within a balanced endophytic community.

These findings underscore the need to understand better the dual roles of endophytic fungi, which are traditionally viewed as pathogens. Unravelling the ecological functions of their components within the plant microbiome could aid in developing microbiome-based strategies for crop protection and yield enhancement. Furthermore, assessing the environmental or host-driven triggers that facilitate a transition from endophytism to pathogenicity is critical for managing food safety risks associated with latent toxin producers and ensuring sustainable crop production.

Alpha diversity discussion

The alpha diversity analysis of the spinach endophytic fungal community revealed moderate diversity, with 24 observed operational taxonomic units (OTUs), a Shannon index of 2.097

and a Simpson index of 0.702, indicating community has several species present, but a few may be more abundant than others, alongside complete sampling coverage (Good's coverage = 1.000). These metrics suggest a community characterized by limited evenness, dominated by a small number of abundant taxa, notably *Fusarium* and *Thanatephorus*. The high Simpson index (approaching 1) indicates low evenness, a pattern consistent with endophytic fungal communities in other crops, such as the Olive plant (*Olea europaea* L.), where dominant genera similarly drive community structure despite moderate OTU richness (Costa *et al*., 2021; Pisano *et al*., 2023). The near-perfect sampling coverage further underscores that this dominance is not an artifact of under sampling but reflects true ecological dynamics.

Fungal endophytes, which asymptomatically colonize plant tissues, occupy a dynamic niche along the mutualist-pathogen continuum, often conferring increased fitness to their hosts through pathogen suppression, stress tolerance, or nutrient acquisition (Dipalma *et al*., 2022; Grabka *et al*., 2022). In this study, the high prevalence of *Fusarium* within the spinach endophytic community aligns with growing evidence that certain *Fusarium* species function as beneficial endophytes rather than pathogens. For instance, *Fusarium proliferatum* isolated from *Cissus quadrangularis* produces phenolics, terpenoids, and unsaturated alkenes that inhibit fungal pathogens like *Rhizoctonia solani* and *Fusarium oxysporum* at concentrations as low as 0.2 mg/mL, demonstrating its potential as a biocontrol agent (Singh *et al*., 2021b; Marian *et al*., 2024). Similarly, *Fusarium oxysporum* can act as a root endophyte, providing biocontrol through endophyte-mediated resistance (De Lamo & Takken, 2020; Fiodorova *et al*., 2022). These antifungal mechanisms underscore *Fusarium*'s dual ecological role: while often perceived as pathogens, their endophytic strains can enhance host health by suppressing competing pathogens or priming plant immune responses (Singh *et al*., 2021a; Dongmo & Tamesse, 2022).

The dominance of *Fusarium* in the spinach endosphere may thus reflect a mutualistic relationship where host plants selectively enrich microbial partners offering protective benefits. Such associations are increasingly recognized in agricultural contexts, where endophyte-mediated pathogen inhibition reduces reliance on synthetic fungicides (Grabka *et al*., 2022). This parallels findings in medicinal and crop plants, where *Fusarium* endophytes contribute to host fitness through secondary metabolite production, niche exclusion, or induced systemic resistance—a paradigm shift in understanding this genus' ecological versatility (Singh *et al*., 2021a, 2021b).

Trophic mode discussion

Trophic structure through FUNGuild analysis of the spinach endophytic fungal community revealed that saprotrophic fungi, especially *Fusarium* spp. and *Thanatephorus cucumeris*, unitedly dominated the community—comprising 97.75% of classified sequence reads—while pathotrophic and symbiotrophic guilds contributed minimally (0.65% and 1.47%, respectively). This striking dominance suggests that decomposition processes within the spinach endosphere are primarily mediated by these saprotrophic taxa, potentially playing a significant role in nutrient cycling despite their occasional secondary pathogenic roles. Similar patterns have been documented in rice roots, where *Fusarium* spp. were also prevalent and likely instrumental in nutrient recycling, as demonstrated by Pili *et al*. (2016), who identified *Fusarium oxysporum* and *Gibberella fujikuroi* species complexes as major components of the rice root endophytic community; notably, *Fusarium oxysporum* isolates showed clear divergence between irrigated and upland ecosystems, underscoring the influence of environmental conditions on endophyte community structure. Mechanistically, the saprotrophic predominance of *Fusarium* and *Thanatephorus* is likely to facilitate organic matter breakdown in the spinach endosphere, thereby enhancing nutrient availability. At the same time, their pathogenic traits remain suppressed in healthy plants. This mechanism is further supported by Pili *et al*. (2016), who observed the widespread adaptability and functional importance of *Fusarium* spp. across diverse rice agroecosystems. The predominance of saprotrophic fungi in the spinach endosphere underscores the potential for harnessing these taxa to enhance nutrient cycling, which could inform sustainable agricultural practices and biocontrol strategies. Moreover, as emphasized by Pili *et al*. (2016), endophytic fungi represent a valuable resource for future searches for biological control and growth-promoting agents, reinforcing their ecological and agronomic relevance.

High-throughput sequencing (HTS)

HTS has enabled a detailed and accurate assessment of the endophytic fungal community structure, overcame the limitations of culture-dependent methods and revealed the true ecological diversity within plant tissues. In the current analysis of spinach, the functional guild composition of endophytic fungi was overwhelmingly dominated by mixotrophic modes—specifically, pathotroph-saprotroph-symbiotroph (141,622 reads, 12 OTUs) and pathotroph-saprotroph combinations (35,418 reads, 6 OTUs)—which together accounted for 177,040 reads (99.99%) across 18 OTUs. In contrast, only 19 reads (2 OTUs) were assigned exclusively to the saprotroph guild (0.01%). The key taxa contributing to this dominance were *Fusarium* spp. (136,749 reads, 77.23%) and *Thanatephorus cucumeris* (35,227 reads, 19.89%), reflecting their ecological versatility and adaptability to complex plant microenvironments. In contrast, fungi exhibiting a single trophic mode, such as saprotrophs, were exceedingly rare, contributing only 19 reads (0.01%) from *Leptospora* sp. and *Nigrospora vesicularifera*. Additionally, a small fraction of OTUs (~0.011%) remained unclassified due to insufficient taxonomic resolution, highlighting the current limitations of functional annotation databases like FUNGuild. This mixotrophic dominance aligns with findings of Wang *et al*. (2022) in case *Sophora alopecuroides*, where saprotrophs and mixotrophs were also prevalent, and supports the notion that endophytic fungi often possess flexible ecological strategies to thrive in diverse and fluctuating internal plant environments The presence of mixotrophs suggests that these fungi can shift between nutritional modes—acting as decomposers, pathogens, or mutualists—depending on the physiological state of the host tissue and environmental conditions. Such versatility may be crucial for their survival and ecological function, enabling them to contribute to nutrient cycling during plant senescence or stress, while remaining benign or even beneficial during healthy plant growth. This dynamic functional structure

underscores the importance of considering both community composition and ecological guilds when evaluating the roles of endophytic fungi in plant health, stress resilience, and potential biotechnological applications (Wang *et al*., 2022).

Aggregation patterns

The high values across all aggregation indices confirm that the endophytic fungal community in spinach is strongly clustered, with dominant taxa such as *Fusarium* and *Thanatephorus* forming localized aggregations. These fungi, known for their multi-trophic versatility, can function as saprotrophs, pathogens, or mutualists, depending on the plant's physiological conditions. This aligns with ecological theories suggesting that trophic plasticity supports niche dominance in competitive environments such as the plant endosphere.

Such functional and spatial clustering likely enhances resource capture, colonization efficiency, and resilience to environmental fluctuations. For instance, Morisita's Index (6.76) and an exceptionally high VMR (53,647.54), along with Lloyd's Index of Patchiness (62,499.49), indicate that these fungi do not distribute randomly but instead occupy specific ecological niches, possibly as a survival or competitive strategy. Similarly, the remaining three indices reflect the same trend.

This interpretation is further validated by Griffin *et al*. (2001), who used Lloyd's Index of Patchiness to assess the spatial distribution of Aspergillus flavus and *A. niger* in peanut fields with a history of aflatoxin contamination. Their values ranged from 2.32 to 6.55, representing a moderate to high aggregation of these fungi. Importantly, they linked such aggregation to pathogen persistence and environmental risk zones within agricultural fields. Compared to Griffin *et al*. (2001) the orders-of-magnitude higher Lloyd's value in the present study reflects much stronger patchiness, likely due to the compartmentalized niche structure of the endosphere and the active colonization strategies of endophytes.

This finding is also consistent with earlier studies in wheat (Gdanetz & Trail, 2017), where *Fusarium* exhibited multitrophic behavior and clustered colonization patterns. Their combined saprotrophic and pathogenic capacities enable them to persist during both healthy and senescent phases of plant growth, thereby participating in nutrient cycling while maintaining their ecological presence.

CONCLUSION

This study analysed endophytic fungal communities in healthy spinach leaves. It revealed a low-diversity, highly aggregated community. *Fusarium* spp. dominated at 77.23%, followed by *Thanatephorus cucumeris* at 19.90%. ITS1 amplicon sequencing confirmed these results. The community showed moderate species richness with 24 OTUs. Low evenness was indicated by Shannon (2.097) and Simpson (0.702) indices. These taxa exhibited mixotrophic behaviors, primarily saprotrophic. Secondary pathotrophic and symbiotrophic roles were also observed. FUNGuild analysis determined saprotrophs accounted for 94.82% of abundance. This suggested a critical role in nutrient cycling. It potentially enhanced plant resilience. The study identified a highly aggregated spatial distribution (VMR: 53,647.54, Morisita's Index: 6.76). This indicated niche-specific colonization. The ecological versatility of the dominant genera likely drove this pattern. The findings challenged the view of *Fusarium* and *Thanatephorus* as solely pathogenic, and proposed potential mutualistic roles in asymptomatic spinach. The study established a foundation for understanding trophic plasticity in mixotrophic endophytic fungi. It highlighted their role in nutrient cycling and functional shifts. It also provided a basis for exploring spatial colonization and cross-kingdom microbial interactions, specifically with bacteria present in the endosphere. These will inform advanced microbiome management and food safety strategies for leafy greens. Future studies will investigate environmental and host factors that facilitate the transition of trophic structure and optimize biotechnological applications.

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