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Antifungal Activity of Endophytic *Nigrospora* Species Isolated from *Pluchea* Plants against Some Fungal Phytopathogens

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***Abstract*— Endophytic fungi *represent promising biocontrol agents due to their ecological compatibility and production of diverse bioactive metabolites. In this study, two endophytic* Nigrospora *species* N. sphaerica *and* N. osmanthi *were isolated from healthy leaves of* Pluchea dioscoridis *and evaluated for their antagonistic activity against eight phytopathogenic fungi, including foliar pathogens (*Alternaria *sp.,* Stemphylium sp. *and* Myrothecium verrucaria*) and soilborne pathogens (*Fusarium oxysporum, F. solani, Rhizoctonia solani, Macrophomina phaseolina, Sclerotium rolfsii*). Dual culture assays revealed that* N. sphaerica *exhibited superior inhibitory effects, achieving up to 60% inhibition against* Alternaria *sp. and 53% against* M. verrucaria, *alongside moderate suppression of soilborne pathogens. Correspondingly, filtrate assays showed significant biomass reduction, particularly in foliar fungi, suggesting the presence of potent diffusible antifungal metabolites. GC–MS profiling of Nigrospora sphaerica and N. osmanthi culture extracts revealed the predominance of phenol, 2,4-bis (1,1-dimethylethyl)-, along with several key antifungal metabolites. N. sphaerica, in particular, exhibited a broader chemical spectrum, producing α-linolenic acid, arachidonic acid, lactic acid, citric acid, 2-butenedioic acid, glycerol, and aromatic hydrocarbons compounds with well-documented antifungal properties. This highlights the value of isolating safe endophytic fungi capable of naturally synthesizing potent bioactive metabolites for sustainable fungal disease management. These findings highlight* N. sphaerica *as a strong candidate for further development in sustainable plant disease management. However, additional in vivo and field-based investigations are essential to validate the practical application of endophytic* Nigrospora *spp. as biological control agents against fungal phytopathogens.*

Keywords*— *Pluchea dioscoridis, Nigrospora sphaerica, biocontrol, GC–MS, antifungal activity, foliar pathogens, endophytic fungi, culture filtrates.

I. INTRODUCTION

Pluchea dioscoridis (L.) DC., commonly known as "Barnûf," is a perennial wild shrub in the family *Asteraceae*, naturally distributed across arid and semi-arid regions of North Africa and the Middle East, including the Nile Valley, Delta, and desert peripheries of Egypt. In addition to its extensive use in traditional medicine for treating ailments such as colds, rheumatism, gastric ulcers, and epilepsy, it holds notable ecological value. This species contributes to soil stabilization, desertification control, and phytoremediation of heavy metal contaminated soils, making it important for both biodiversity conservation and environmental restoration (Youssef and Diatta, 2023). Its adaptability to harsh climates and ability to host diverse microbial endophytes further enhance its potential as a model for plant microbe interaction studies under stress conditions.

significant threat to agricultural productivity, especially in tropical and subtropical regions. Among foliar pathogens, *Stemphylium* spp., *Alternaria* spp., and *Myrothecium* *verrucaria* are well-known for causing leaf spots and blight symptoms, which compromise photosynthetic efficiency and reduce overall plant growth and yield (*Fravel* et al., 2003). Meanwhile, soilborne fungal pathogens continue to jeopardize root health in many economically important crops. Notably, species of Fusarium, such as F. *oxysporum* and *F. solani*, are well-established causal agents of vascular wilt, characterized by xylem colonization and systemic blockage that ultimately leads to plant death. *Macrophomina phaseolina* is a common cause of charcoal rot, particularly under heat and drought stress conditions, while *Rhizoctonia solani* and *Sclerotium rolfsii* are responsible for damping-off and stem/collar rots in a wide host range (*Fontana* et al., 2021).

Although chemical fungicides have long been the mainstay of disease management, their excessive use health risks to humans and non-target organisms. These challenges have spurred the development of sustainable alternatives, with biological control emerging as a promising strategy (Fontana et al., 2021).

In this context, endophytic fungi microorganisms that colonize internal plant tissues without causing visible disease to have gained increasing attention for their ability to produce diverse bioactive secondary metabolites with antifungal properties (Manganyi, 2020). These fungi can suppress pathogens via competition for nutrients and space, mycoparasitism, production of inhibitory metabolites, or by triggering systemic resistance in host plants. Among the most promising endophytes, species of the genus Nigrospora have attracted attention due to their biochemical diversity and antagonistic potential. For example, Nigrospora sp. produces phomalactone, a broad-spectrum antifungal compound active against several major phytopathogens (Ramesha *et al*., 2020). More recently, other metabolites such as nigrosphaeritriol and nigrosphaerilactol have been identified from *N. sphaerica*, showing potent antifungal activity and potential for development as eco-friendly fungicides (Salvatore *et al*., 2024). Importantly, endophytes isolated from the same host plant are often ecologically pre-adapted, enhancing their compatibility and persistence, which supports their utility as long-term biological control agents (Manganyi, 2020).

Given these considerations, the present study aims to isolate and characterize endophytic *Nigrospora* species from healthy *P. dioscoridis* plants and evaluate their antagonistic activity against a spectrum of fungal phytopathogens isolated from diseased *P. dioscoridis*, soil, and other crops. The goal is to identify ecologically compatible endophytes with strong biocontrol potential, contributing to sustainable crop protection while reducing dependence on synthetic fungicides and supporting environmentally sound agricultural practices.

II. MATERIALS AND METHODS

1. Plant Material Collection and Taxonomic Verification

Field surveys were conducted during the active growing season of *Pluchea dioscoridis* (L.) DC. Both symptomatic plants showing typical foliar disease symptoms (necrotic spots, chlorotic halos, and irregular lesions) and asymptomatic, apparently healthy plants were targeted. From each site, representative samples including leaves, stems, and roots were collected in sterile polyethylene bags and transported to the laboratory under cooled conditions. Plant specimen was identified by Dr. Omran Ghaly, Herbarium of Desert Research Center (CAIH) with Identification Code: CAIH-1350-R.

2. Isolation of Fungal Pathogens from Diseased Plants and Soil

Isolation procedures aimed to recover the full spectrum of fungal pathogens associated with *P. dioscoridis*. Diseased tissues were first washed under running tap water to remove debris, surface-sterilized in 1% sodium hypochlorite for 2 minutes, rinsed thrice with sterile distilled water, and blotted dry on sterile filter paper. Tissue segments (5 mm^2) from lesion margins were plated onto Potato Dextrose Agar (PDA; Difco, USA) supplemented with streptomycin (100 mg/L) to suppress bacterial contamination. Plates were incubated at 25 ± 2°C in darkness for 5–7 days, and emerging fungal colonies were purified by hyphal-tip or single-spore isolation (Leslie and Summerell, 2006).

To broaden the pathogen panel, additional isolates were obtained from rhizosphere soils and infected tissues of other crops grown in proximity to *P. dioscoridis*, including faba bean, citrus, peanut, and alfalfa. Soil samples were processed using soil dilution plating, while plant tissues followed the same surface sterilization and plating protocol.

3. Morphological Identification of Pathogens

Purified isolates were transferred to fresh PDA and incubated under controlled conditions to induce sporulation. Colony morphology, pigmentation, and growth rate were documented, while microscopic examination of conidia, hyphae, and reproductive structures was performed using a compound microscope (Olympus BX51). Identification to genus level relied on morphological keys and descriptions (Barnett and Hunter, 1998), and representative isolates were retained for molecular identification.

4. Pathogenicity Testing

Pathogenicity of each isolate was confirmed using detached leaf assays (Bashyal *et al*., 2022). Healthy leaves of *P. dioscoridis* were surface-sterilized and placed on moist filter paper in sterile Petri dishes. Mycelial plugs (5 mm diameter) from actively growing cultures were placed on the leaf surfaces; controls received sterile PDA plugs. Plates were incubated at 25 °C under a 12 h photoperiod, and lesion development was assessed over 5–7 days. Pathogens were re-isolated from symptomatic leaves and compared morphologically to the original cultures to satisfy Koch's postulates.

5. Isolation of Endophytic Fungi from Healthy Plants

Endophytic fungi were isolated from symptom-free tissues of *P. dioscoridis* collected from the same sites as diseased plants. Leaves and stems were surface sterilized by immersion in 1% sodium hypochlorite for 2 minutes, followed by 70% ethanol for 30 seconds, then rinsed in sterile distilled water. After blotting dry, tissue segments (5 mm^2) were plated on PDA supplemented with streptomycin. Plates were incubated at 25 °C for up to 10 days, with daily monitoring for emerging fungal colonies from internal tissues. Colonization frequency (%) was calculated as the proportion of tissue segments yielding fungal growth relative to the total plated segments (Pavithra *et al*., 2020).

6. Molecular Identification of Endophytes and Selected Pathogens

Genomic DNA was extracted from pure fungal cultures using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). The internal transcribed spacer (ITS) region of rDNA was amplified by PCR using primers ITS1 and ITS4. PCR reactions were performed in a 25 µL mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 1 U Taq DNA polymerase, and 50 ng template DNA. Thermal cycling conditions included initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min.

PCR products were purified and sequenced commercially. Sequences were aligned and analyzed using BLASTn against the NCBI GenBank database. Phylogenetic trees were constructed in MEGA X to confirm species-level identification.

7. *In Vitro* Antagonistic Activity Assays

Two complementary approaches were used to evaluate the antagonistic potential of endophytes against the isolated phytopathogens:

a) Dual culture assay following Skidmore and Dickinson (1976), 5 mm mycelial plugs of the pathogen and endophyte were placed 6 cm apart on PDA plates. Plates were incubated at 25 °C, and radial growth was measured after 7 days. Percentage inhibition was calculated relative to pathogen growth on control plates.

b) Culture filtrate assay endophytes were grown in Potato Dextrose Broth (PDB) at 120 rpm and 25 °C for 14 days. Culture broth was filtered through Whatman No.1 paper, then sterilized using a 0.22 µm membrane filter. The sterile filtrate was incorporated into PDA at final concentrations of 5%, 10%, and 15% (v/v). Pathogen mycelial plugs were placed centrally, and radial growth was measured after incubation.

8. Extraction and GC–MS Analysis of Bioactive Metabolites

The ethyl acetate extracts of the culture filtrates obtained from liquid-grown endophytic fungi were subjected to GC–MS analysis. Specifically, sterile culture filtrates of *Nigrospora osmanthi* and *Nigrospora sphaerica* grown in potato dextrose broth (PDB) were extracted with ethyl acetate in a 1:1 (v/v) ratio under vigorous shaking for 30 minutes. The organic layers were separated, dried over anhydrous sodium sulphate to remove residual moisture, and subsequently concentrated under reduced pressure using a rotary evaporator to yield the crude extract containing secondary metabolites.

The extracts were analysed using gas chromatography mass spectrometry (GC–MS) to identify volatile and semi-volatile components. Metabolites were tentatively identified by comparing their mass spectral fragmentation patterns and retention times with reference spectra from the NIST (National Institute of Standards and Technology) Mass Spectral Library. This method is widely accepted for metabolite profiling of fungal endophytes due to its sensitivity and reproducibility (Devi *et al*., 2010; Kaur and Arora, 2009; Strobel and Daisy, 2003).

Such an approach enables the characterization of complex metabolite mixtures and facilitates the identification of bioactive compounds that may be involved in antifungal or antimicrobial activities (Saranraj and Sivasakthi, 2014; Narvaez-Barragan *et al*., 2020).

9. Statistical Analysis

All experiments were conducted in triplicate following a completely randomized design. Data were analyzed by one-way ANOVA using SPSS software. Mean comparisons were performed using Tukey's HSD test at a significant level of $p < 0.05$.

III. RESULTS AND DISCUSSION

1. Isolation and Identification of Pathogenic Fungi

Field surveys of *Pluchea dioscoridis* plants showing foliar and stem lesions resulted in the isolation of two consistent pathogenic fungi directly associated with symptomatic tissues: *Alternaria* sp., producing olive-green to black colonies with beaked conidia, and *Stemphylium* sp., which exhibited slow-growing, dark brown to black colonies with multicellular muriform conidia. Both isolates successfully reproduced characteristic leaf spot and blight symptoms on detached leaves under controlled pathogenicity assays, confirming their pathogenic role on *P. dioscoridis*.

To extend the relevance of the antifungal screening, a collection of agriculturally important soilborne fungi was established from rhizospheric soils or infected plant debris in nearby fields cultivated with crops such as alfalfa, peanut, citrus, and faba bean. The isolated pathogens included *Fusarium oxysporum, F. solani, Macrophomina phaseolina, Rhizoctonia solani, Sclerotium rolfsii,* and all of which are well-documented causal agents of root rots, vascular wilts, charcoal rot, and damping-off diseases, also *Myrothecium verrucaria* as Foliar/stem blight causal agent in a wide range of crops.

This pathogen panel, comprising host-specific foliar pathogens and ecologically relevant soilborne fungi, was selected to support a comprehensive assessment of potential biocontrol agents, focusing on their antagonistic capabilities against diverse phytopathogens of both medicinal and economic significance.

Fig. 1: Morphological appearance of Pluchea dioscoridis plants collected during field survey. (A, B) Healthy plant showing normal foliage and stem development. (C–F) Naturally infected plants exhibiting foliar and stem symptoms caused by fungal pathogens isolated in this study.

Table 1. Pathogenic fungi isolated from P. dioscoridis, their source, morphology, and growth rate on PDA

Pathogen	**Source of Isolation**	**Colony Morphology on PDA**	**Key Microscopic Features**	**Growth Rate (mm/day) ± SE**	**Disease Type**
Alternaria sp.	Diseased leaves	Olive green to black	Muriform, beaked conidia	5.6 ± 0.3	Foliar (leaf spot)
Stemphylium sp.	Diseased leaves	Greyish, compact	Branched conidiophores, clusters of conidia	3.3 ± 0.2	Foliar (leaf spot)
Myrothecium verrucaria	Diseased stems	Whitish with green sporodochia	Cylindrical conidia in sporodochia	5.8 ± 0.2	Foliar/stem blight
Fusarium oxysporum	Diseased roots	Cottony white, pinkish reverse	Sickle-shaped macroconidia, microconidia in false heads	6.9 ± 0.2	Vascular wilt

Fusarium solani	Soil (peanut field)	Cream to pale brown	Elliptical macroconidia, abundant chlamydospores	6.5 ± 0.3	Root rot, stem canker
Macrophomina phaseolina	Alfalfa roots	Dark grey, fluffy	Numerous black microsclerotia	7.0 ± 0.2	Charcoal rot
Rhizoctonia solani	Soil (citrus orchard)	Brown, web-like mycelium	Right-angled branching hyphae	8.2 ± 0.3	Root rot, damping-off
Sclerotium rolfsii	Peanut stem base	White mycelium, abundant sclerotia	Tan to brown sclerotia	7.7 ± 0.2	Stem base rot, wilt

2. Isolation and Preliminary Identification of Endophytic Fungi

Endophytic fungal isolates were successfully recovered from surface-sterilized tissues of *Pluchea dioscoridis* plants that appeared asymptomatic in the field. Initial morphological and microscopic examinations revealed that the dominant isolates belonged to the genus *Nigrospora*. The colonies exhibited rapid growth with a characteristic cottony to fluffy aerial mycelium, initially white turning grayish-black with age. Microscopically, the isolates were identified by their dark, globose to subglobose, single-celled conidia, typically borne laterally on short, hyaline conidiophores (Fig. 2). The conidia measured approximately 10–15 µm in diameter, consistent with taxonomic descriptions of *Nigrospora* spp. (Ellis, 1971; Kirk, P.M., 1991; Barnett and Hunter, 1998,).

The vegetative mycelium consisted of branched, septate, and hyaline hyphae that later turned pigmented, a common feature of dematiaceous fungi. No signs of pathogenicity were observed on host tissues at the time of sampling, supporting their endophytic nature.

To confirm their identity and differentiate between closely related taxa, molecular characterization was subsequently performed using ITS-rDNA sequencing. This molecular approach provided accurate species-level identification, confirming the isolates as *Nigrospora osmanthi* and *Nigrospora sphaerica*. The molecular data were further supported by phylogenetic analyses aligning the isolates within established *Nigrospora* clades.

These results underscore the importance of integrating morphological and molecular tools for the accurate identification of endophytic fungi and support the potential of *Nigrospora* spp. as candidates for biocontrol applications.

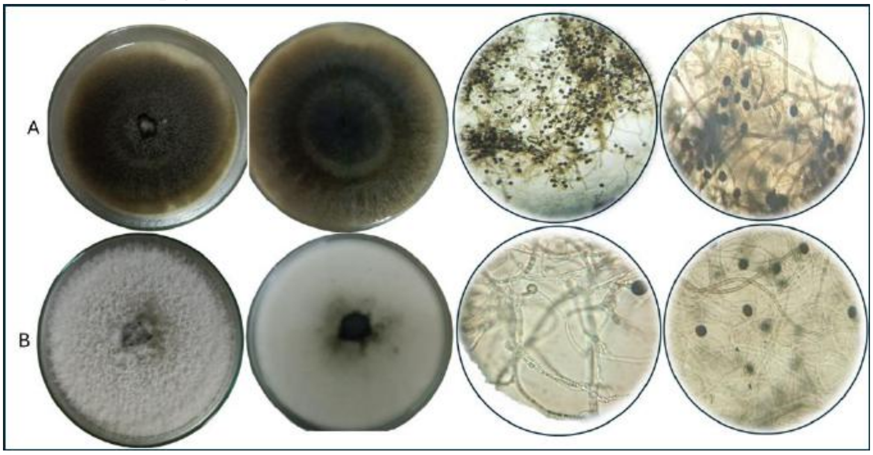

Fig. 2: Morphological and cultural characteristics of endophytic Nigrospora isolates from Pluchea dioscoridis; (A) Colony morphology and microscopic features of Nigrospora osmanthi, showing septate hyphae and typical black, single-celled conidia; (B) Colony morphology and microscopic features of Nigrospora sphaerica, displaying branched hyphae and globose to ellipsoid conidia.

3. Pathogenicity Testing

Given the biological and ecological divergence between foliar and soilborne pathogens, pathogenicity assessment was selectively applied only to the isolates suspected of causing foliar diseases in *Pluchea dioscoridis*. These included *Alternaria* sp. and *Stemphylium sp.*, all of which were originally recovered from symptomatic leaves and stems of naturally infected *P. dioscoridis* plants.

***3.1. Detached Leaf Assay for Foliar Pathogens*:** Pathogenicity of the three foliar isolates was tested using the detached leaf assay. Healthy, surface-sterilized leaves of *P. dioscoridis* were placed on moist filter paper in sterile Petri dishes and inoculated with mycelial plugs from 7-day-old cultures of each fungal isolate. Control leaves received sterile PDA plugs. Inoculated leaves were incubated at 25 ± 2°C under a 12-hour photoperiod for 10 days, during which symptoms were monitored and lesion development recorded. The results, summarized in Table 2, showed clear variation in virulence among the tested isolates. *Alternaria* sp. caused the largest mean lesion diameter (15.1 ± 0.8 mm), followed closely by *Myrothecium verrucaria* (13.6 ± 0.7 mm), both producing lesions within six days post-inoculation. *Stemphylium sp.*. induced significantly smaller lesions (9.6 ± 0.5 mm) and exhibited a slightly longer incubation period of seven days. Based on these observations, the pathogenicity ranking among foliar isolates was (*Alternaria* sp.; *Myrothecium verrucaria; Stemphylium sp.*). This ranking aligns with known pathogenic behaviour of these fungi in various host plants, where *Alternaria* species are typically aggressive necrotrophs (Kusaba and Tsuge, 1995; Shoemaker and Babcock, 1992).

***3.2. Rationale for Excluding Root Pathogens from Pathogenicity Assay*:** In contrast, the five soilborne fungi isolated from rhizospheric soil and nearby crops (*Fusarium oxysporum*, *F. solani*, *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Sclerotium rolfsii*) were not subjected to detached-leaf assays. This was due to their fundamentally different infection biology, which involves root colonization and systemic vascular invasion, rendering leaf-based assays ineffective for evaluating their virulence. Moreover, the pathogenicity of these fungi is well-established in the literature, with multiple studies confirming their role in causing wilting, root rot, and damping-off symptoms in various economically important crops (Leslie and Summerell, 2006; Kaur *et al.*, 2012).

Table 2. Pathogenicity of foliar pathogens on detached P. dioscoridis leaves

Pathogen	Mean Lesion Diameter (mm) ± SE	Incubation Period (days)	Pathogenicity Rank
Alternaria sp.	15.1 ± 0.8	6	2
Myrothecium verrucaria	13.6 ± 0.7	6	2
Stemphylium sp.	9.6 ± 0.5	7	3

4. Antagonistic Activity of Endophytic Nigrospora Isolates

4.1 Dual Culture Assay

To assess the antagonistic efficacy of endophytic *Nigrospora* isolates against phytopathogenic fungi, a dual culture assay was employed. The test included three foliar pathogens (*Alternaria* sp., *Stemphylium* sp. *and Myrothecium verrucaria*) and soilborne pathogens (*Fusarium oxysporum*, *F. solani*, *Sclerotium rolfsii*, *Macrophomina phaseolina*, and *Rhizoctonia solani*). Each isolate of *N. sphaerica* and *N. osmanthi* was evaluated for its ability to suppress the radial growth of these pathogens. The results revealed that *Nigrospora sphaerica* exhibited superior antagonistic activity compared to *N. osmanthi* against all tested pathogens, indicating a species dependent difference in biocontrol efficacy. Notably, foliar pathogens showed higher susceptibility to both endophytes, particularly to *N. sphaerica,* which achieved inhibition rates exceeding 60% in *Alternaria* sp. and 53.9% in *Myrothecium verrucaria*. This supports earlier findings that foliar endophytes are more competitive in the phyllosphere due to niche overlaps with foliar pathogens (Strobel and Daisy, 2003; Mousa and Raizada, 2013; Deshmukh *et al.*, 2018). In contrast, root infecting fungi such as *Sclerotium rolfsii. Macrophomina phaseolina* and *Rhizoctonia solani* exhibited markedly lower inhibition 32.3%, 24.2%, and 25.3% (Table 3), which could be due to their aggressive colonization strategies and physical resilience, such as sclerotia formation (Dean *et al.*, 2012). The intermediate response observed in Fusarium species suggests that endophyte-derived metabolites may partially limit their growth. Although initial inhibition zones were observed around the endophytic isolates, further incubation revealed that the endophytes continued to grow, progressively occupying the space and suppressing pathogen development through competitive exclusion. This suggests that inhibition was not only due to static antagonism but also involved active overgrowth and space competition (Fig. 3).

These findings underscore the ecological relevance of endophyte-host interactions and support the use of *N. sphaerica* as a potential biocontrol candidate, particularly for foliar disease management in *Pluchea dioscoridis* and possibly other hosts.

Table 3. Radial Growth Inhibition of Pathogenic Fungi by Nigrospora spp. in Dual Culture Assays

Pathogen	**Growth Alone (mm/day)**	**Growth with *N. osmanthi***	**Inhibition %**	**Growth with *N. sphaerica***	**Inhibition %**
Alternaria sp.	9.6 ± 0.3	6.3 ± 0.2	34.4%	4.2 ± 0.3	56.3%
Myrothecium verrucaria	8.9 ± 0.4	5.8 ± 0.2	34.8%	4.1 ± 0.3	53.9%
Stemphylium sp.	7.8 ± 0.4	5.9 ± 0.2	24.4%	3.8 ± 0.3	51.3%
Fusarium oxysporum	10.1 ± 0.4	7.6 ± 0.3	24.8%	5.7 ± 0.4	43.6%
Fusarium solani	9.8 ± 0.3	7.3 ± 0.2	25.5%	5.6 ± 0.3	42.9%
Sclerotium rolfsii	9.3 ± 0.3	7.7 ± 0.2	17.2%	6.3 ± 0.4	32.3%
Macrophomina phaseolina	9.5 ± 0.4	8.0 ± 0.3	15.8%	7.2 ± 0.2	24.2%
Rhizoctonia solani	8.7 ± 0.3	7.4 ± 0.3	14.9%	6.5 ± 0.3	25.3%

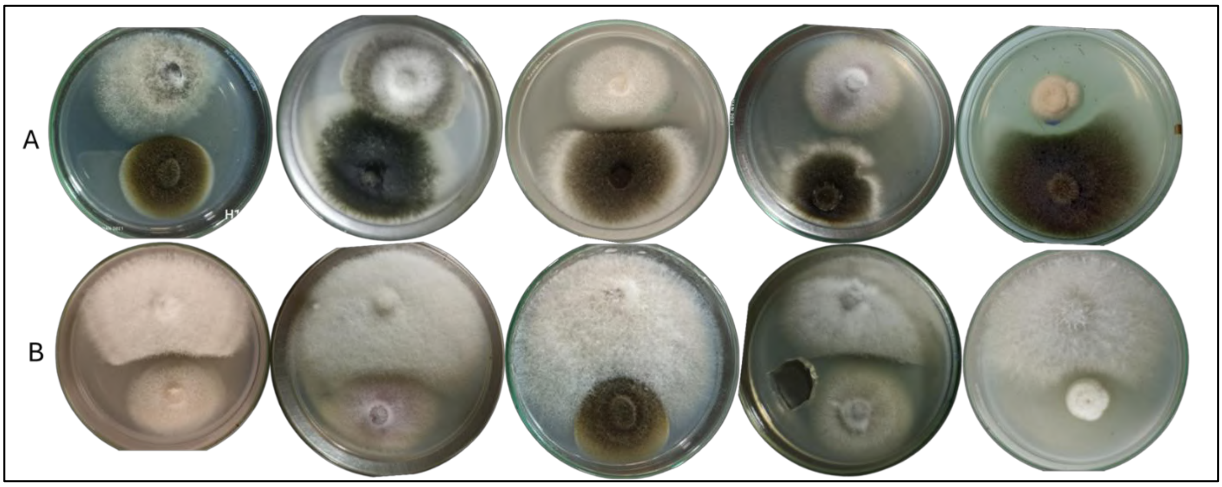

Fig. 3: Dual culture assay showing antifungal activity of endophytic Nigrospora isolates against phytopathogenic fungi. (A) Nigrospora osmanthi; (B) Nigrospora sphaerica. Although initial inhibition zones were observed around the endophytic isolates, further incubation revealed that the endophytes continued to grow, progressively occupying the space and suppressing pathogen development through competitive exclusion. This suggests that inhibition was not only due to static antagonism but also involved active overgrowth and space competition.

4.2 Effect of Culture Filtrates of *Nigrospora* spp. on Growth of Pathogens

To further assess the antagonistic efficacy of *Nigrospora osmanthi* and *Nigrospora sphaerica*, culture filtrate assays were conducted to evaluate the extracellular inhibitory potential of these endophytes against a range of phytopathogens. Filtrates obtained from liquid cultures of each isolate were incorporated into PDA medium at standardized concentrations. The radial growth of each pathogen was then recorded and compared to controls after 7 days of incubation. Table 4 summarizes the mean radial growth rates and percentage inhibition values for each pathogenic fungus in response to the culture filtrates, along with Duncan's multiple range test letters indicating statistically significant differences ($P \leq 0.05$).

The results presented in Table 4 show clear differences in the antifungal activity of culture filtrates between *N. sphaerica* and *N. osmanthi.* In all tested pathogens, filtrates from N. sphaerica demonstrated significantly stronger inhibitory effects compared to *N. osmanthi*, suggesting a superior production of bioactive metabolites by this isolate. This intra-genus variability in antagonistic capacity is consistent with earlier studies, which highlighted that secondary metabolite production among endophytes can vary markedly depending on strain and environmental adaptation (Kharwar *et al.*, 2011; Strobel, 2003).

Foliar pathogens such as *Alternaria* sp*., Myrothecium verrucaria,* and *Stemphylium* sp. were among the most susceptible to culture filtrates, with inhibition rates exceeding 40% in the case of *N. sphaerica*. This is likely due to the shared ecological niche between these pathogens and the endophytes, which may facilitate competitive exclusion and higher local accumulation of antifungal compounds (Schulz and Boyle, 2005; Ghorbanpour *et al.*, 2018).

In contrast, soilborne pathogens such as *Macrophomina phaseolina* and *Rhizoctonia solani* showed the lowest levels of growth inhibition, not exceeding 16% even under *N. sphaerica* filtrate treatment. These fungi are known for their robust sclerotial structures and tolerance to environmental stress, which may reduce the efficacy of diffusible antifungal compounds (Dean *et al.*, 2012; Khaledi and Taheri, 2016).

Intermediate suppression was observed for Fusarium species, which are vascular pathogens with systemic colonization behavior. The moderate inhibition (~30–35%) suggests that while the endophytes metabolites interfere with hyphal development, they may not fully suppress more aggressive soilborne pathogens. Such partial inhibition aligns with previous findings in which *Fusarium* spp. showed variable responses to fungal filtrates, depending on the bioactive profile of the antagonistic strain (Palaniyandi *et al.*, 2013). Overall, the filtrate assay confirms the antagonistic potential of Nigrospora endophytes, particularly *N. sphaerica*, against a range of phytopathogens. These findings reinforce their biocontrol capacity and ecological compatibility with the aerial parts of *Pluchea dioscoridis*, making them promising candidates for further development as biological control agents.

Table 4. Effect of Culture Filtrates of Nigrospora spp. on Radial Growth of Pathogenic Fungi (mm/day) and Growth Inhibition (%).

Pathogenic Fungi	Control Growth (mm/day)	N. osmanthi Filtrate (mm/day)	Inhibition (%)	N. sphaerica Filtrate (mm/day)	Inhibition (%)
Alternaria sp.	8.0 a	5.2 c	35.0	4.4 d	45.0
Myrothecium verrucaria	7.6 a	5.3 c	30.3	4.5 d	40.8
Stemphylium sp.	7.4 a	5.5 c	25.7	4.8 d	35.1
Fusarium solani	8.2 a	6.1 bc	25.6	5.3 c	35.4
Fusarium oxysporum	8.1 a	6.3 bc	22.2	5.6 c	30.9
Sclerotium rolfsii	7.8 a	6.6 b	15.4	6.2 bc	20.5
Rhizoctonia solani	8.0 a	7.0 ab	12.5	6.7 b	16.3
Macrophomina phaseolina	7.9 a	7.1 ab	10.1	6.8 b	13.9

Effect of Fungal Culture Filtrates of *Nigrospora* spp. on Dry Biomass of Phytopathogenic Fungi

To explore the metabolite-based antagonistic potential of the endophytic *Nigrospora* isolates (*N. osmanthi* and *N. sphaerica*), culture filtrate assays were conducted, where the dry biomass of various phytopathogenic fungi served as a quantitative measure of growth inhibition. This approach distinguishes the chemical inhibitory effects mediated by secondary metabolites from direct mycelial competition.

As illustrated in Fig. 4, both isolates significantly reduced fungal biomass compared to the control. *N. sphaerica* consistently exhibited a greater inhibitory effect across all tested pathogens, corroborating its superior performance in dual culture assays. The most pronounced suppression was observed against foliar pathogens such as *Alternaria* sp. and *Myrothecium verrucaria*, with their dry weights dropping to approximately 0.29 g/plate and 0.26 g/plate, respectively. This supports the hypothesis that foliar-derived endophytes may biosynthesize metabolites specifically adapted to target aerial pathogens, consistent with findings by Amin *et al.* (2021) and Gakuubi *et al.* (2022).

Conversely, soilborne pathogens like *Rhizoctonia solani* and *Macrophomina phaseolina* showed more moderate reductions (dry weights 0.51–0.55 g/plate), which could be attributed to their well-documented resilience and enzymatic detoxification systems (Khaledi *et al.*, 2016; Singh and Yadav, 2020).

Intermediate responses were recorded for *Fusarium oxysporum* and *F. solani* (0.41–0.45 g/plate), indicating a partial susceptibility likely involving disruption of cellular processes such as membrane integrity or secondary metabolism (Larran *et al.,* 2016).

These results suggest that while *N. sphaerica* exhibits broader antifungal metabolite diversity and potency, its effectiveness is more apparent against foliar pathogens. The reduced biomass levels confirm that the secreted bioactive compounds contribute substantially to fungal inhibition and reinforce the potential use of these endophytes in integrated disease management strategies.

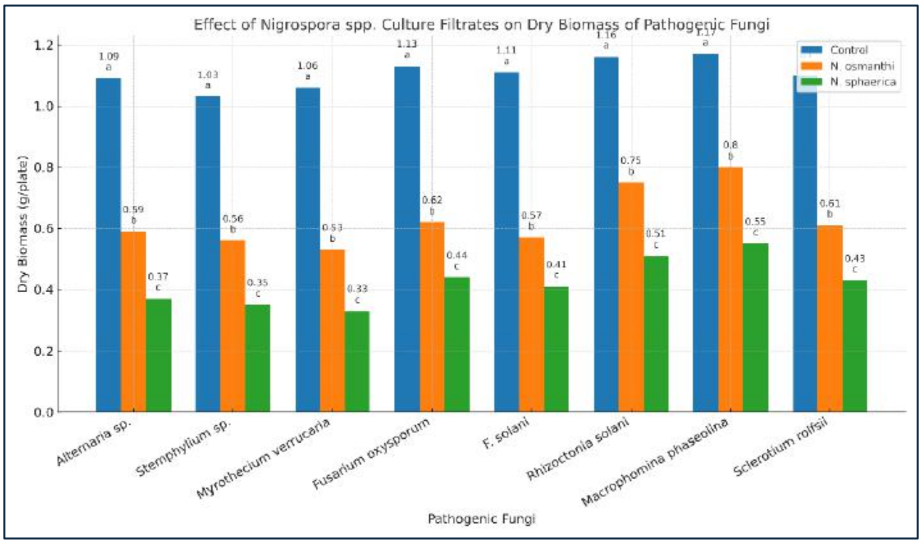

Fig. 4: Effect of Culture Filtrates of Nigrospora spp. on Dry Biomass of Phytopathogenic Fungi (g/plate)

4.3 Molecular Identification of Endophytic *Nigrospora* Isolates

To confirm the taxonomic identity of the two endophytic fungal isolates obtained from healthy *Pluchea dioscoridis* leaves, molecular identification was carried out using internal transcribed spacer (ITS) rDNA sequencing. Genomic DNA was extracted from actively growing mycelia, and amplification of the ITS region was performed using the universal primers (ITS1: 5'-TCC GTA GGT GAA CCT GCG G-3' ; ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-3'). The amplified PCR products were purified and sequenced bidirectionally. The resulting sequences were analyzed using the BLASTn algorithm against the NCBI GenBank database to determine species-level similarity (Fig. 5).

The first isolate, designated H1, produced a high-quality sequence of 592 base pairs. BLASTn analysis revealed a 99% identity (567/571 bp) with *Nigrospora osmanthi* (GenBank accession number MH645207.1). The query coverage was 100%, confirming a high-confidence match. Phylogenetic placement aligned H1 within the *Trichosphaeriaceae* family under the order *Trichosphaeriales*, and genus *Nigrospora*. These results affirm the identification of H1 as *Nigrospora osmanthi.*

The second isolate, designated H2, yielded a 575 bp ITS sequence, which showed 99% identity (549/552 bp) with *Nigrospora sphaerica* (GenBank accession number MG669225.1), also with 100% coverage. The isolate was taxonomically assigned to *Nigrospora sphaerica* based on sequence similarity, supporting its classification within the same taxonomic lineage as H1.

Interpretation and Relevance, the high sequence identity and coverage of both isolates to their respective type strains support their accurate species-level identification. These findings align with previous studies demonstrating the utility of ITS sequencing for delimiting *Nigrospora* species (Wang *et al*., 2018; Gomes *et al*., 2013). Importantly, both isolates were recovered from asymptomatic *P. dioscoridis* tissues and did not produce symptoms under pathogenicity assays, confirming their endophytic. The confirmed identity of these isolates allows for reliable attribution of the observed biocontrol effects in subsequent antagonism and filtrate assays. Moreover, it supports future work exploring metabolite profiling or gene expression patterns related to antifungal activity (Zhao *et al*., 2011).

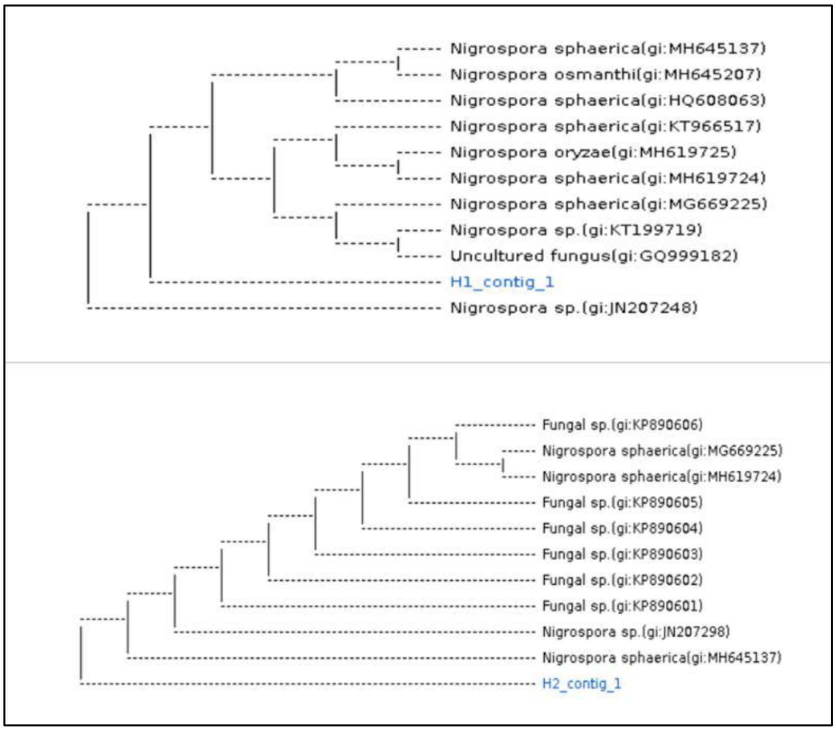

Fig. 5: Phylogenetic dendrograms illustrating the molecular identification of two endophytic fungal isolates from Pluchea dioscoridis based on ITS region sequencing. (A): Isolate H1 clustered with Nigrospora osmanthi (GenBank accession no. MH645207.1) with 99% sequence identity. (B): Isolate H2 clustered with Nigrospora sphaerica (GenBank accession no. MG669225.1) with 99% sequence identity. The trees were constructed using standard BLASTn alignment and similarity clustering NCBI database sequences. Bootstrap values indicate the confidence of clustering for each node.

4.4 GC–MS-Based Metabolomic Profiling of Endophytic Nigrospora spp. Culture Filtrates

Nigrospora species, GC–MS analysis was performed on ethyl acetate extracts of culture filtrates from *Nigrospora osmanthi* and *N. sphaerica*. The analysis enabled identification of several volatile and semi-volatile compounds, many with reported antimicrobial functions, providing a chemical basis for the observed variation in biocontrol activity.

4.4.1 Metabolite Profile of Nigrospora osmanthi

The GC–MS chromatogram of *N. osmanthi* revealed 24 peaks with retention times ranging from 8.447 to 37.687 minutes. The chemical composition was dominated by a single compound at Rt = 34.722 min (48.48%), identified as phenol, 2,4-bis(1,1-dimethylethyl)-, a compound known for its broad-spectrum antimicrobial and antioxidant properties (Kaur and Arora, 2009). Other minor compounds included 1-monopalmitin (Rt = 29.397 min, 10.09%) and citric acid (Rt = 22.874 min, 35.89%), (Fig. 6).

Although a relatively high number of peaks was detected, the predominance of a single compound indicates a metabolically biased output concentrated along a narrow biosynthetic pathway. While this major phenolic metabolite may possess antifungal potential, the overall lack of chemical diversity and the low representation of complementary bioactive compounds likely contribute to the isolate's moderate antifungal efficacy. Phenol derivatives such as 2,4-di-tert-butylphenol have been shown to inhibit fungal spore germination and growth by disrupting membrane (Devi *et al*., 2010), but their efficacy may depend on synergistic interactions with other metabolites, which appear minimal in this strain.

4.4.2 Metabolite Profile of Nigrospora sphaerica

In contrast, GC–MS profiling of *N. sphaerica* revealed a total of 15 distinct peaks, reflecting a notably richer chemical diversity and more balanced distribution of metabolite intensities. The predominant compound detected was phenol, 2,4-bis(1,1-dimethylethyl)- (Rt = 34.773 min, 100%), followed by benzene, 1,3-bis(1,1-dimethylethyl)- (Rt = 35.789 min, 49.8%), both of which are lipophilic aromatic compounds known for their membrane-disruptive properties. Beyond these major constituents, the isolate also produced a range of bioactive metabolites including lactic acid, which is recognized for its acidifying effect that creates unfavourable conditions for pathogen survival, and glycerol, a metabolite often associated with cellular stress responses and osmotic balance. Organic acids such as citric acid and 2-butenedioic acid, along with β-D-(+)-

xylopyranose, were also present and may enhance antifungal activity through mechanisms involving metal ion chelation, pH reduction, or interference with pathogen metabolism. Additionally, the detection of α-linolenic acid and arachidonic acid fatty acids known to inhibit biofilm formation and compromise fungal membranes further supports the potential of *N. sphaerica* as a multifaceted source of antifungal compounds.

The presence of multiple moderately to highly abundant compounds, spanning both polar and non-polar classes, suggests that *N. sphaerica* harbors a broad-spectrum metabolic. These compounds likely act synergistically, targeting various aspects of fungal physiology such as membrane permeability, enzymatic activit. This chemical richness is consistent with the enhanced antifungal activity observed for *N. sphaerica* in both dual culture and filtrate assays.

Comparative Interpretation: Although *N. osmanthi* exhibited a greater number of chromatographic peaks, its metabolite distribution was heavily dominated by a single phenolic compound, which accounted for over 98% of the total peak area. In contrast, the chemical profile of *N. sphaerica* was more balanced and functionally diverse, with several bioactive metabolites contributing substantially to the overall composition of the extract. This functional chemical diversity likely underpins the superior biocontrol efficacy of *N. sphaerica*, as confirmed in bioassays. The results highlight that not only the number of metabolites, but their relative abundance and functional complementarity are critical in determining antifungal potential.

The observed variation in metabolite expression between the two fungal strains may stem from inherent genetic differences in their secondary metabolic pathways, as well as disparities in host-endophyte interactions during the initial isolation process. Additionally, environmental and culture conditions are known to influence metabolic flux and can significantly shape the profile of secondary metabolites produced (Strobel, 2003; Kusari *et al*., 2012). These findings underscore the importance of integrating metabolomic profiling with antifungal bioassays to guide the selection of promising fungal isolates for further development in biological control programs. The detection of known antifungal compounds in *N. sphaerica* highlights its strong potential for incorporation into integrated pest management (IPM) strategies.

Bioactive Metabolites and Antifungal Potential: The GC–MS profiles of both *Nigrospora osmanthi* and *N. sphaerica* revealed the presence of several volatile and semi-volatile metabolites with reported antifungal properties. Most notably, 2,4-di-tert-butylphenol (also known as Phenol, 2,4-bis(1,1-dimethylethyl)-) was identified as the major compound in both extracts, with a relative abundance exceeding 98% in *N. osmanthi* and 100% in *N. sphaerica*. This phenolic compound has been widely documented for its antimicrobial and antifungal properties, attributed to its membrane-disrupting effects and interference with ergosterol synthesis in fungal cells (Devi *et al.,* 2010; Huang *et al*. 2007; Kaur and Arora, 2009; Ghorbanpour, *et al*., 2018).

Additional metabolites detected exclusively or at higher intensity in *N. sphaerica* include Benzene, 1,3-bis(1,1-dimethylethyl)-, which has been associated with antifungal activity via disruption of lipid bilayers (Saranraj and Sivasakthi, 2014), and lactic acid, a known inhibitory agent that lowers extracellular pH and impairs fungal respiration (Magnusson and Schnürer, 2001). The detection of citric acid and glycerol in both isolates may also contribute to antifungal efficacy through osmotic stress induction and chelation of essential metal ions (Jung *et al*., 2003; Hallsworth and Magan, 1995).

Furthermore, *N. sphaerica* produced fatty acid derivatives such as α-linolenic acid and arachidonic acid, both of which are recognized for their fungitoxicity and ability to disrupt membrane integrity in phytopathogens (Walters *et al.,* 2004; Narvaez-Barragan *et al*., 2020). These metabolites, especially when present in combination, may account for the enhanced and broader-spectrum antifungal activity exhibited by *N. sphaerica* in bioassays. The presence and distribution of these metabolites not only explain the superior biocontrol potential of *N. sphaerica* but also highlight the utility of metabolomic profiling in guiding the selection of promising endophytes for antifungal applications. Future work involving compound purification and structure activity relationship (SAR) studies is warranted to confirm the specific roles of these bioactives in pathogen inhibition.

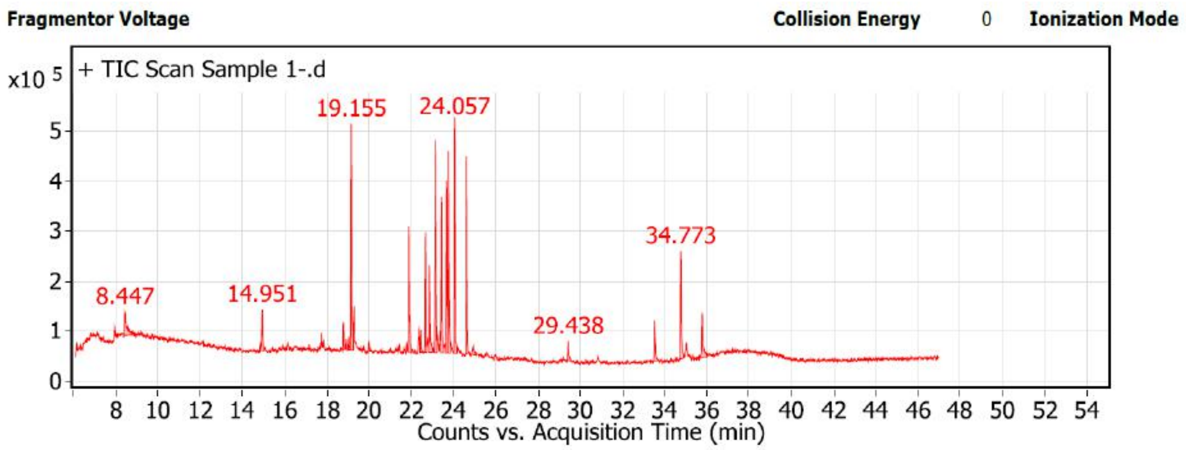

Fig. 6: GC–MS chromatograms of ethyl acetate extracts from culture filtrates of endophytic Nigrospora osmanthi showing 24 major peaks

Table 5. GC–MS Detected Peaks in Culture Filtrate of N. osmanthi

Peak	RT	Name	Formula	Area	Area Sum %
1	8.447	Lactic Acid, 2TMS derivative	$C_9H_{22}O_3Si_2$	324397.63	2.77
2	14.951	Glycerol, 3TMS derivative	$C_{12}H_{32}O_3Si_3$	257515.76	2.2
3	18.783	Propylene glycol, 2TMS derivative	$C_9H_{24}O_2Si_2$	133172.22	1.14
4	19.041	Arachidonic acid, TMS derivative	$C_{23}H_{40}O_2Si$	58312.6	0.5
5	19.155	L-5-Oxoproline,2TMS derivative	$C_{11}H_{23}NO_3Si_2$	1330542.5	11.36
6	19.997	Succinic acid (tms)	$C_{10}H_{22}O_4Si_2$	51776.68	0.44
7	21.014	2,4-Hexadien-1-ol	$C_6H_{10}O$	29310.13	0.25
8	21.895	Xylitol, 5TMS derivative	$C_{20}H_{52}O_5Si_5$	637209.12	5.44
9	22.464	Glyceric acid, 3TMS derivative	$C_{12}H_{30}O_4Si_3$	104564.7	0.89
10	22.684	.beta.-Arabinopyranose, 4MS derivative	$C_{17}H_{42}O_5Si_4$	524338.61	4.48
11	22.874	Citric acid, 4TMS derivative	$C_{18}H_{40}O_7Si_4$	477565.38	4.08
12	23.147	Methyl .alpha.-D-glucofuranoside, 4TMS derivative	$C_{19}H_{46}O_6Si_4$	1306674.3	11.16
13	23.42	2-Butenedioic acid, (Z)-, 2TMS derivative	$C_{10}H_{20}O_4Si_2$	828003.87	7.07
14	23.663	D-Galactose, 5TMS derivative	$C_{21}H_{52}O_6Si_5$	942605.46	8.05
15	23.761	.beta.-D-(+)-Xylopyranose, 4TMS derivative	$C_{17}H_{42}O_5Si_4$	1090960.2	9.32
16	24.057	Ribitol, 5TMS derivative	$C_{20}H_{52}O_5Si_5$	1121786.3	9.58
17	24.611	β-D-glucose 5-TMS derivative	$C_{21}H_{52}O_6Si_5$	825202.82	7.05
18	24.93	α-Linolenic acid, TMS derivative	$C_{21}H_{38}O_2Si$	109200.24	0.93
19	29.438	1-Monopalmitin, 2TMS derivative	$C_{25}H_{54}O_4Si_2$	134196.3	1.15
20	33.521	Phenol, 2,6-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	324724.18	2.77
21	34.773	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	$C_{42}H_{63}O_3P$	645042.4	5.51
22	35.782	Benzene, 1,3-bis(1,1-dimethylethyl)-	$C_{14}H_{22}$	395442.75	3.38
23	37.231	Silicic acid, diethyl bis(trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$	26890.61	0.23
24	37.785	1,4-Benzenediol, 2,6-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O_2$	28083.68	0.24

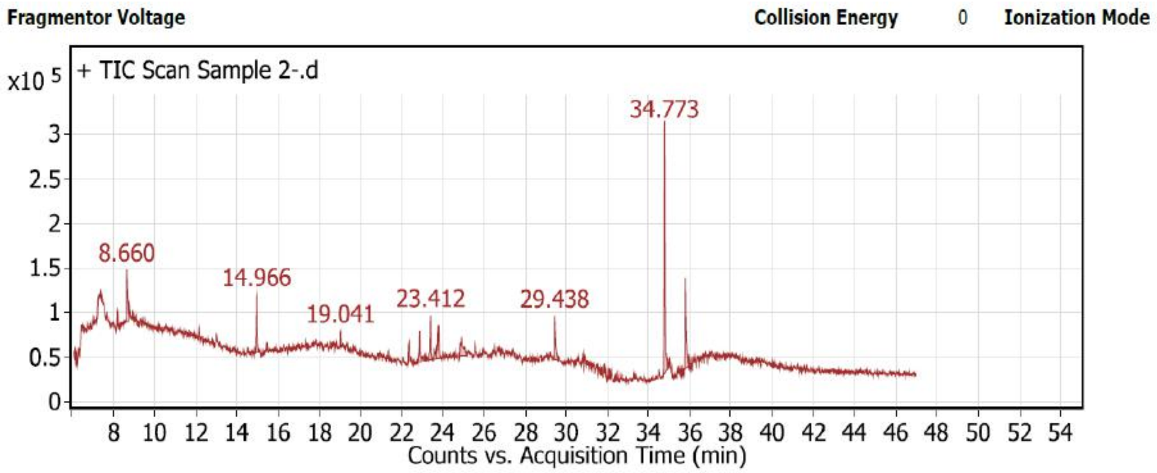

Fig. 7: GC–MS chromatograms of ethyl acetate extracts from culture filtrates of endophytic Nigrospora sphaerica displaying 15 major peaks

Table 6. GC–MS Detected Peaks in Culture Filtrate of N. sphaerica

Peak	RT	Name	Formula	Area	Area Sum %
1	8.66	Lactic Acid, 2TMS derivative	$C_9H_{22}O_3Si_2$	205574.63	7.94
2	14.966	Glycerol, 3TMS derivative	$C_{12}H_{32}O_3Si_3$	192181.08	7.43
3	19.041	Arachidonic acid, TMS derivative	$C_{23}H_{40}O_2Si$	36773.52	1.42
4	21.371	2,4-Hexadien-1-ol	$C_6H_{10}O$	25933.24	1
5	22.873	Citric acid, 4TMS derivative	$C_{18}H_{40}O_7Si_4$	110045.65	4.25
6	23.116	Methyl α-D-glucofuranoside, 4TMS derivative	$C_{19}H_{46}O_6Si_4$	22363.69	0.86
7	23.412	2-Butenedioic acid, (Z)-, 2TMS derivative	$C_{10}H_{20}O_4Si_2$	146200.52	5.65
8	23.685	D-Galactose, 5TMS derivative	$C_{21}H_{52}O_6Si_5$	68840.04	2.66
9	23.792	β-D-Xylopyranose, 4TMS derivative	$C_{17}H_{42}O_5Si_4$	149211.9	5.77
10	24.019	Ribitol, 5TMS derivative	$C_{20}H_{52}O_5Si_5$	20109.93	0.78
11	24.9	α-Linolenic acid, TMS derivative	$C_{21}H_{38}O_2Si$	184475.29	7.13
12	29.438	1-Monopalmitin, 2TMS derivative	$C_{25}H_{54}O_4Si_2$	203564.46	7.87
13	34.773	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	$C_{42}H_{63}O_3P$	798110.29	30.84
14	35.789	Benzene, 1,3-bis(1,1-dimethylethyl)-	$C_{14}H_{22}$	397456.94	15.36
15	36.23	Cyclobarbital	$C_{12}H_{16}N_2O_3$	26928.39	1.04

IV. CONCLUSION

This study provides compelling evidence for the antagonistic potential of endophytic *Nigrospora* species isolated from *Pluchea dioscoridis* against a spectrum of foliar and soilborne phytopathogens. Two isolates *Nigrospora osmanthi* and *N. sphaerica* were recovered from symptomatic leaf tissues and molecularly confirmed via ITS-rDNA sequencing. *In vitro* dual culture and culture filtrate bioassays revealed that *N. sphaerica* consistently exhibited stronger antifungal activity, particularly against foliar pathogens such as *Alternaria sp.* (inhibition rate >60%) and *Myrothecium verrucaria* (54%), whereas *N. osmanthi* showed moderate inhibition across all tested fungi.

endophytic fungi. *N. sphaerica* exhibited a chemically diverse and functionally rich profile, encompassing a broad spectrum of volatile and semi-volatile metabolites. These included phenolic compounds such as 2,4-di-tert-butylphenol, organic acids like lactic and citric acids, sugar alcohols, and polyunsaturated fatty acids including α-linolenic and arachidonic acids all of which are known for their pronounced antifungal and antimicrobial activities. In contrast, the chemical profile of *N. osmanthi* was markedly unbalanced, being dominated by a single phenolic metabolite that constituted over 98% of the total detected compounds. The dominance of a single metabolite in profile of *N. osmanthi* likely contributes to its reduced spectrum of antifungal activity relative to *N. sphaerica*.

The antifungal efficacy of *N. sphaerica* is likely attributed to the synergistic action of its chemically diverse metabolites, as opposed to reliance on a single dominant compound. These findings underscore the importance of metabolite diversity and functional complementarity in effective biocontrol, and highlight the value of combining bioassays with metabolomic data for the strategic selection of biocontrol agents.

In conclusion, *Nigrospora sphaerica* and *N. osmanthi* represent a promising candidate for development as a broad-spectrum biocontrol agent, particularly for tested pathogens. Future research should focus on purification and structure activity studies of its bioactive metabolites, alongside in plant trials, to validate its efficacy under field conditions and explore its integration into sustainable pest management strategies.

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