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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION of ENDOPHYTIC FUNGI FROM PSIDIUM *Guajava and Vachellia nilotica*

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ABSTRACT

Endophytic fungi are essential components of plant ecosystems, often contributing to host health and secondary metabolites production. The present study aimed to isolate and identify fungal endophytes from the stems and leaves of *P. guajava and V. nilotica*. Morphological, Molecular and Phylogenetic Identification of were carried out using standard procedures. Fungi were isolated and categorized by host tissue using appropriate fungal atlas. Molecular identification was performed using rRNA sequence analysis (ITS1, 5.8S, ITS2), and phylogenetic relationships were established by comparing sequences against GenBank database matches. A total of 20 endophytic fungi were isolated from four host segments (*P. guajava* stem (PGS), *P. guajava leaves*, (PGL), *V. nilotica* leaves (VNL), *V. nilotica* stem (VNS) corresponding to a colonization frequency of 80.95%. The following fungal isolates were identified: *Thysanophora penicilloides, Aspergillus Niger, Aspergillus fumigatus, Aspergillus flavus, Fusarium spp, Sporangia spp, Fusarium verticillioides, Aureobasidium pullulans,* and *Gliocladium roseum. Psidium guajava* leaves (PGL) exhibited the highest colonization (26.19%), followed by its stem (23.80%), while *Vachellia nilotica* showed lower frequencies (16.66% for leaves and 14.28% for stems). Dominant species identified include: *Aspergillus fumigatus, Fusarium verticillioides, Aspergillus flavus, and Aspergillus Niger*. Molecular analysis of the ITS/rRNA regions confirmed the identity of four primary isolates (PGL, VNL, PGS, VNS) with DNA fragments ranging from 558 to 1056 bp. The DNA fragments show a sequence identity percentages range between 99.77% and 99.99% indicating a high taxonomic accuracy within the respective species clades. The study deduced that both *Psidium guajava* and *Vachellia nilotica* harbors rich microbial diversity and overlapping fungal endophytes communities, suggesting a significant potential for bio-prospecting secondary metabolites.

Keywords: Endophytic Fungi, Medicinal Plants, Morphological, Molecular and Phylogenetic Identification, Diversity

INTRODUCTION

Endophytic fungi inhabit a unique biological niche, because of their ability to asymptomatically colonize plant tissues (Sun et al., 2023). It colonizes host tissues in different organs, including leaves, stems, barks, roots, fruits, flowers, seeds, petioles, inflorescences of weeds, buds, and also dead and hollow hyaline cells of plants (Su et al., 2022). During their growth inside the living tissues of the plant, endophytic fungi establish complex relationship with their host plants, which involve mutualism, antagonism, and rarely parasitism. Generally, in the symbiotic relationship fungal endophytes receive shelter and nutrients from the host, while the host plant might benefit from an array of attributes that include: safe guarding against natural enemies such as pathogens and herbivores (Gupta et al., 2023). Trypanosomiasis, caused by Trypanosoma sp., remains a significant public health concern in sub-Saharan Africa (WHO, 2022). Current treatments face challenges such as resistance and toxicity (Kagbadouno et al., 2020 and Njoroge et al., 2020).

Recent studies have highlighted the potential of natural products, particularly endophytic fungi, as sources of anti-trypanosomal compounds (Olanrewaju et al., 2022; Singh et al., 2022). Endophytes produce bioactive metabolites with medicinal properties (Desalegn et al., 2020). *Psidium guajava* (guava) and *Acacia nilotica* (thorn tree) are traditionally used in African medicine to treat various ailments, including trypanosomiasis (Afolayan et al., 2020; Kumar et al., 2022). Studies have reported the anti-trypanosomal activity of plant extracts from *Psidium guajava* (Olajide et al., 2020) and *Vachellia nilotica* (Srivastava et al., 2022). However, the potential of their endophytic fungi remains largely unexplored. This study aimed to investigate microscopic and molecular components of endophytic fungi from *Psidium guajava* and *Vachellia nilotica*.

MATERIALS AND METHODS

Plants Sample Collection and Preparation

The healthy Stem and leaf samples of *Psidium guajava* and *Vachellia nilotica* were collected from Botanical Garden of the Department of Biological Sciences, Abubakar Tafawa Balewa University Bauchi and obtain a voucher number, the samples were taken to herbarium for identification to obtain a voucher number. Samples were cleaned with distilled water to remove dirt and debris and chopped into small segments (1-2 cm).

The leaf and stem samples of *P.guajava and V. nilotica* were surface sterilized by the method described by Hussaini et al. (2024). The samples were washed with tap water, then the washed leaf samples were treated by the immersion procedure as follows: 70% ethanol for 2 min followed by 2.5% sodium hypochlorite (NaOCl) solution for 3 min and 70% ethanol for 30 seconds. Then, samples were rinsed in double distilled, sterilized water for a couple of minutes before being dried on a blotting sheet. The surface-sterilized samples were cut, aseptically, into 1 cm × 1 cm length and were placed (4 segments on each plate) in Petri dishes containing prepared

Potato Dextrose Agar (PDA) medium supplemented with chloramphenicol (100 mg/L). The Petri dishes were sealed properly with parafilm to avoid desiccation of the medium and any contamination during this period and incubated at 28.05 ± 0.5℃ for two (2) weeks. The Petri dishes were maintained and monitored daily to check the growth of fungal colonies from the leaf and stem segments. Individual hyphal tips that emerged from the edges of each treated plant parts were transferred separately onto fresh PDA and a assigned with a code number until identification. The fungal strains in the pure culture were kept on PDA slant at 4 to 4.5℃ and were sub-cultured from time to time.

The colonization frequency (CF %) of the endophytic fungi was calculated as described by Eric et al. (2020).

$$CF = \frac{Number\ of \frac{leaf}{stem}\ segmets\ colonized\ by\ endophytes}{Total\ of \frac{leaf}{stem} analyzed} \text{ x } X\ 100$$

Identification of Endophytic Fungal Isolates

Fungal identification was based on the morphology of the cultures and characteristics of the spores. The fungal isolates were characterized macroscopically by observing the top and reverse mycelia characteristic of 7 days old fungal culture on PDA medium. Fungal hyphae were mounted on microscopic slides and stained with lactophenol cotton blue and examined in 40X light microscopy. The fungal isolates were further identified on the basis of microscopic characters, for spore shape and phenotypic characteristics, for spore type, growth colonial shape, color, texture using standard mycology manual described by Kumar et al. (2019), Hyde et al. (2019). Another fungal isolate was used for molecular identification (Bellemain et al., 2010; Schoch et al., 2012). For DNA preparation, all selected fungal cultures were centrifuged at 5,000 rpm for 5 min to collect the mycelium which was frozen in liquid nitrogen and ground into a fine powder. About 150-200 mg of this frozen and ground mycelium was used for DNA extraction using the commercial EZNA Fungal DNA kit according to the manufacturer's instructions. For PCR reaction and DNA digests, the ITS region of each fungus was amplified by Polymerase Chain Reaction (PCR) using universal fungal primers ITS1 5'TCCGTAGGTGAACCTGCGG-3 'and ITS4 5'TCCTCCGCTTATTGATATGC-3'. The PCR reactions were carried out in 50 mL of mix containing: 1.5 mM MgCl2, 0.2 mM dNTP, 0.5 mM of each primer, 0.025 U of Taq polymerase and 10 to 30 ng of fungal DNA. The reactions were carried out in a thermal cycler. PCR amplification with Initial denaturation of 95°C, 5 min, 35 cycles: 95°C, 30 sec; 55°C, 30 sec; 72°C, 1 min and Final extension of 72°C for 10 minutes using the program described by (Gao et al., 2025). The PCR products were subjected to electrophoresis using 2% agarose gels. After electrophoresis, the gels were stained with ethidium bromide and the DNA bands were visualized under UV light. Sizes were estimated by comparison with DNA size markers. DNA sequencing and sequence analysis was carried out on DNA fragments 5.8-ITS generated by PCR and was done in the two orientations. The primers ITS1 / ITS4 were used. The sequences obtained were analyzed by comparison with the sequences available in the National Center for Biotechnology Information (NCBI) GenBank using the basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These sequences were then recorded in the NCBI's online database and accession numbers assigned.

RESULTS AND DISCUSSION

Endophytic Fungal Distribution

Table 1 presents the occurrence of fungal species across different host plants and tissues. A total of 18 fungal species were identified with *Thysanophora penicilloides* and *Aspergillus fumigatus* having the most common, appearing in nearly all tissues except for *Vachellia nilotica* stems. *Guava* Leaves had *Aspergillus fumigatus* and *Fusarium verticillioides* while *P. guajava* and *V. nilotica* stem contained the following fungal isolate: *Aspergillus flavus* and *Gliocladium roseum*. *Sporangia spp* and *Aspergillus niger* were found across multiple leaf and stem samples of both plant species.

Table 1: Endophytic Fungal Species Isolated from Stem and Leaves of *Psidium Guajava and Vachellia nilotica*

Plant name	Name of fungi isolated
PGS	*Thysanophora penicilloides*
	Aspergillus fumigatus
	Sporangia spp.
	Aspergillus niger
PGL	*Thysanophora penicilloides*
	Aspergillus fumigatus
	Sporangia spp.
	Aspergillus fumigatus
	Fusarium verticillioides
VNL	*Thysanophora penicilloides*
	A.niger
	A.fumigatus
	Sporangia sp.
	Aureobasidium pullulans
VNS	A. *flavus*
	A.fumigatus
	F.verticillioides
	Gliocladium roseum

Molecular Characterization

Molecular identification was conducted to provide definitive taxonomic labels for the endophytic isolates (Table 2). Representatives endophytic isolates were selected for molecular analysis: PGL and PGS from *P. guajava*, and VNL and VNS from *V. nilotica*. The isolates showed high similarity to established sequences in the GenBank database, such as PQ 072653.1 for *A. fumigatus*, OR473115.1 for *Fusarium*

verticillioides, PQ753628.1 for A. flavus and JN676125.1 for *A. niger*. With regards to sequence regions, identification was based on a conserved ribosomal DNA region, including ITS1, 5.8S rRNA, and ITS2. PGL and VNL had a larger ribosomal subunit (SSU and LSU) than PGS and VNS. DNA fragment Length varied significantly, with PGL yielding the longest sequence at 1056 bp, while VNL (*F. verticillioides*) was the shortest at 558 bp.

Table 2: Sequence Identification and Genebank Accession Details of *Psidium Guajava* and *Vachellia Nilotica*

Sample Code	Closest GenBank match	Accession number	Organism identified	Sequence region	DNA fragments length (bp)
PGL	A. *fumigatus isolate S2*	PQ 072653.1	A. *fumigatus*	SSU rRNA, ITS1, 5.8S rRNA, ITS2, LSU rRNA	1056
VNL	*Fusarium verticillioides* isolate HSRF41	OR473115.1	*F.verticilliodes*	SSU rRNA, ITS1, 5.8S rRNA, ITS2, LSU rRNA	558
PGS	*Aspergillus flavus* isolate SMS6	PQ753628.1	*flavus*	ITS1, 5.8S rRNA, ITS2, LSU rRNA	585
VNS	*Aspergillus niger* isolate B6	JN676125.1	*niger*	ITS1, 5.8S rRNA, ITS2, 28S rRNA	584

Note: Host plants, PGL, and PGS = *Psidium guajava* leaves and stem while VNL and VNS = *Vachellia nilotica* leaves and stem

Phylogenetic Observations

Phylogenetic analysis was used to confirm the evolutionary placement of the fungal isolates relative to known reference strains (Table 3). The *A. fumigatus* isolate (PGL) had a distinct, well-supported monophyletic group, confirming it belongs to a single evolutionary lineage with its reference sequences. Clade Consistency (VNL and VNS) VNL revealed a clustered clade within the *F. verticillioides* clade, showing clear separation from other *Fusarium* species. VNS showed a close relationship to the reference isolate JN676125.1, positioning it firmly within the *A. niger* clade. Species Confirmation revealed that PSI which harbors *A. flavus* isolate grouped consistently with reference strains, providing high-confidence species-level identification.

Table 3: Summary of Phylogenetic Analysis of Endophytic Fungi Obtained From *Psidium Guajava and vachellia Nilotica*

Sample Code	Closest relative in phylogeny	Phylogenetic observation	Percentage identity (%)
PGL	A. *fumigatus*	Forms a distinct, well-supported monophyletic group with other A. fumigatus sequences	99.99
VNL	*F. verticilliodes*	Clusters definitively within the F. *verticillioides* clade, separate from other Fusarium species	100.00
PNS	*A. flavus*	Groups consistently with reference strains of A. *flavus*, confirming its species-level identification	100.00
VNS	*A. niger*	Clusters within the A. *niger* clade, showing a close relationship to the reference isolate JN676125.1.	100.00

Note: Host plants, PGL, and PGS = *Psidium guajava* leaves and stem while VNL and VNS = *Vachellia nilotica* leaves and stem

Discussion

The present study revealed a rich diversity of endophytic fungi associated with the stem and leaves of *Psidium guajava* and *Vachellia nilotica*, reinforcing the concept that higher plants serve as reservoirs of diverse microbial and insecticidal communities. Endophytic fungi colonize internal plant tissues without causing harm and often establish mutualistic relationships that enhance host survival and adaptability (Hussaini et al., 2022). The results demonstrated that several fungal taxa, including *Thysanophora penicilloides*, *Aureobasidium pullulans*, and *Sporangia sp*, were identified from both plant species and tissues. This suggests that these fungal isolates were endophytes with broad ecological tolerance. *Aureobasidium pullulans*, in particular, is widely reported as a ubiquitous endophyte with significant ecological plasticity and the ability to produce extracellular enzymes and antimicrobial compounds that contribute to host plant defense (Eric et al. 2020). Its repeated occurrence across samples in the present study supports previous findings that it is among the most dominant fungal endophytes in many plant species. This observation corroborates with the findings of Gupta *et al.* (2023). Identification of *Aspergillus spp* (*A. niger, A. fumigatus, and A. flavus*) further highlights their adaptability and ecological significance as endophytes in the examined plants. Although these fungi are often regarded as saprophytes or opportunistic pathogens, their presence as endophytes suggests a dual ecological role that may shift depending on environmental conditions as reported by Pandy *et al*. (2023). For instance, *Aspergillus niger* has been reported to produce secondary metabolites with antimicrobial properties, while *A. fumigatus* is known for its enzymatic capabilities that may facilitate antiprotozoal nutrient acquisition within host tissues. This is in-line with the work of Meshra *et al.* (2023). The high total colonization frequency of 80.95% observed across the examined plants suggests that the internal tissues of *Psidium guajava and Vachellia nilotica* provide a hospitable environment for fungal endophytes. Specifically, *Psidium guajava* leaves (PGL) and stems (PGS) were reported to support a more robust fungal population compared to *Vachellia nilotica*. This variation is attributed to the different phytochemical compositions or tissue densities of the host plants. This observation is in agreement with the work of Hussaini *et al.* (2024). Similarly, the detection of *Fusarium verticillioides and Gliocladium roseum* indicates the presence of fungi with diverse ecological functions. While *Fusarium* spp is commonly associated with plant diseases,

several studies have demonstrated their occurrence as asymptomatic endophytes, where they contribute to plant growth promotion or stress tolerance (Utami *et al*., 2020). *Gliocladium roseum* (now often referred to as *Clonostachys rosea*) is particularly notable for its biocontrol potential, as it produces antifungal compounds that suppress plant pathogens (Elawady *et al*., 2023). The coexistence of these fungi within the same host tissues suggests a complex microbial and anti-protozoan network that may influence plant health and productivity.

The molecular identification results in Table 2 provided robust confirmation of the fungal species through sequence analysis of ribosomal DNA regions, including SSU, ITS, and LSU. The internal transcribed spacer (ITS) region is widely regarded as the universal DNA barcode for fungi due to its high interspecific variability and reliable species-level resolution (Bora and Devi, 2023). In the present study, high similarity between the obtained sequences and GenBank reference strains (e.g., *Aspergillus fumigatus, Fusarium* verticillioides, *A. flavus, and A. niger*) confirms the accuracy of the identifications and underscores the reliability of molecular tools in fungal taxonomy investigation. This is in consistent with the findings of Eric *et al.* (2020).

Phylogenetic analysis (Table 3) further validated fungal isolate identifications by demonstrating that all isolates clustered within well-supported monophyletic clades corresponding to their respective species. Isolate PGL grouped with reference sequences of *A. fumigatus*, while VNL clustered distinctly within the *Fusarium verticillioides* clade. Such clustering patterns indicate a strong evolutionary relationships and minimal genetic divergence from known strains. This observation is in agreement with the work of Sun et al. (2023) and Xuezhen and Longfei (2023) reported that phylogenetic approaches were critical in resolving taxonomic ambiguities, confirming species identity, particularly in groups with overlapping morphological characteristics.

The high percentage identity observed in the present study among the isolates suggests that the endophytic fungi identified are not novel species but rather well-characterized taxa with established ecological roles as observed by Nguyen et al. (2024). This finding aligns with previous studies that report a high prevalence of cosmopolitan fungal genera such as *Aspergillus* sp. and *Fusarium* sp. in tropical and subtropical plants (Pandy *et al*., 2023). Their widespread distribution may be attributed to efficient dispersal mechanisms, adaptability to diverse environmental conditions, and the ability to establish stable associations with multiple host plants as observed by Chancellor *et al*. (2014).

Furthermore, the presence of similar fungal species in both *P. guajava* and *V. nilotica* suggests a degree of host overlap, indicating that some endophytes are not strictly host-specific. Similar observation was reported by Digra and Nonzom, (2023). However, the occurrence of certain species in specific tissues or plants also points to possible host preference or tissue specificity, which may be influenced by factors such as plant chemistry, microenvironmental conditions, and interspecific microbial interactions (Moglad et al., 2023).

CONCLUSION

The diversity and distribution of endophytic fungi observed in the present study reflects a dynamic and complex symbiotic system. The examined fungal isolates contribute to host plant fitness by enhancing resistance to pathogens, improving nutrient acquisition, and producing bioactive secondary metabolites. Given the increasing interest in endophytes as sources of novel drugs and agricultural biocontrol agents, the fungi identified in this study represent promising candidates for further biotechnological exploration, the study provides comprehensive insights into the diversity, molecular identity, and phylogenetic relationships of endophytic fungi associated with *Psidium guajava and Vachellia nilotica*. The results demonstrated that both plant species harbors a wide range of fungal endophytes, including members of the genera *Aspergillus, Fusarium, Aureobasidium, Thysanophora, and Gliocladium.* The integration of morphological characterization with molecular and phylogenetic analyses ensured accurate species identification and revealed strong genetic similarities between the isolates and known reference strains. The dominance of well-known fungal taxa suggests that these endophytes are ecologically stable and widely distributed species with significant functional roles. The study highlights the potential of these endophytic fungal isolates as sources of bioactive compounds and agents for agricultural and pharmaceutical applications. It is therefore recommended that future research should focus on the functional characterization of these isolates, including their metabolic profiles and interactions with host plants, to fully explore their biotechnological potential.

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