Analysis of Microfungi in Rice Rhizospheres from Four Rice – Producing Villages in Niger State, Nigeria Using Illumina Sequencing

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Summary

To accurately identify the fungal communities in the rhizosphere of rice plants, *Oryza sativa* L. as well as understand their relationships, an Illumina-based sequencing approach was employed in this study. Rice rhizosphere samples were collected from four large-scale rice-producing villages in Niger State: Lagun, Batavovogi, Dokogi and Jikannagi. Genomic DNA was extracted from each sample, and their fungal Internal Transcribed Spacer (ITS) regions were amplified using standard Illumina sequencing protocols. After quality-filtering the sequences, the operational taxonomic units (OTUs) in each sample were identified using QIIME software, and were assigned taxonomies through the Unite databases. The sequencing produced 340,492 ITS rDNA reads. The sample from Lagun had the highest richness according to the Chao 1 diversity index (776.4), while the Dokogi sample had the lowest richness (482). Most OTUs belonged to *Phylum Ascomycota*, followed by *Basidiomycota*, while representatives of the following classes were observed, i.e. *Sordariomycetes*, *Eurotiomycetes*, *Dothideomycetes*, *Agaricomycetes*, and *Rhizophyctidomycetes*. The Jikannagi sample showed predominance of *Sordariomycetes*, *Agaricomycetes*, and *Dothideomycetes*, while *Eurotiomycetes* and *Rhizophyctidomycetes* were dominant in the Dokogi sample. The Jikannagi sample also exhibited the highest fungal diversity (Shannon index). However, a significant portion of the fungal reads were not classifiable because the data base used in this study was not sufficient enough. Therefore, there is need for future studies to be based on an improved data base in order to reduce the number of unclassified fungi.

Key words

DNA reads, fungal communities, illumina sequencing, operational taxonomic units, QIIME

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Introduction

Rice (*Oryza sativa* L.) is an essential staple food worldwide and is widely recognized as a model plant for scientific research (Sen et al., 2020). Nigeria has emerged as the leading rice producer in Africa, driven by government initiatives focused on crop development and reducing reliance on oil. Understanding the intricate interactions within the rice rhizosphere, which encompasses the endosphere (inside the root), rhizoplane (root surface), and rhizosphere (soil surrounding the root) is critical for enhancing agricultural productivity (Ding et al., 2019). Studies have explored microbial communities in these areas, including the rhizosphere, anoxic bulk soil, and oxic surface soil (Hernández et al., 2015; Qin et al., 2019; Kumar & Dubey, 2020; Wei et al., 2023).

Hinsinger et al. (2009) describe the rhizosphere as a narrow layer of soil, approximately 1 to 2 mm wide, surrounding plant roots, where the roots influence the biological and chemical properties of the soil. This region predominantly hosts bacteria and fungi, along with oomycetes, nematodes, protists, algae, viruses, archaea, and arthropods (Afridi et al., 2022). These organisms thrive on rhizo-deposits released by the roots (Pantigoso et al., 2022). Consequently, the microbiome in the rhizosphere differs significantly from the microbial communities in bulk soil (Deng et al., 2024). These microorganisms play key roles in nutrient cycling, root health, plant growth, and disease suppression, significantly impacting crop yield (Mendes et al., 2013). The rhizosphere facilitates the exchange of materials between plants and their surrounding environment, and most fungi in this area are heterotrophic, with some being pathogenic and others beneficial, playing a central role in nutrient cycling by decomposing organic matter (Solomon et al., 2024).

Metagenomic approaches, especially next-generation sequencing (NGS) techniques like Illumina MiSeq, have revolutionized microbial community studies, revealing the vast diversity of unculturable microbes (Zhang et al., 2021). It involves analyzing soil nucleic acids using methods like DGGE and T-RFLP after PCR (Billington et al., 2022), and profiling microbial communities based on fatty acid analysis (PFLA) (Fan et al., 2017). Unlike older methods that use dideoxynucleotides to terminate DNA chains, next-generation sequencing (NGS) identifies pyrophosphate release when nucleotides are added, allowing for the sequencing of individual DNA molecules. This results in more comprehensive community profiling and more accurate species identification compared to DGGE and T-RFLP (Satam et al., 2023). Pyrosequencing and Illumina Miseq, both NGS methods, have been employed to study the rhizosphere microbiome and soil microbial communities (Sun et al., 2014; Chen et al., 2021). The Illumina Miseq platform is especially valued for its higher read depth, comparable sequencing quality, and lower cost (Razali et al., 2017). For instance, Dong et al. (2021) used Illumina sequencing to explore the rhizosphere microbiomes of rice (*O. sativa* L.) under increased nitrogen fertilization, revealing a diverse range of bacteria and fungi.

Despite the global advancements in rhizosphere microbiome studies, limited research has focused on the fungal communities associated with rice rhizospheres in Nigeria. This knowledge gap is particularly pronounced in Niger State, where rice cultivation is integral to the local economy and food security.

Therefore, this study employed Illumina MiSeq sequencing to analyze the fungal diversity in rice rhizospheres from four major rice-growing areas in Niger State. By elucidating the functional roles of these fungi, this research aimed to provide a foundation for leveraging fungal biodiversity to enhance rice production, contributing to sustainable agricultural practices and improved food security in the region.

Materials And Methods

Sample Collection, Physicochemical Analysis and DNA Extraction

Rice rhizosphere samples were obtained during the harvest stage from Batavovogi, Dokogi, Lagun, and Jikannagi, key rice-growing villages in Niger State, on December 28, 2019. Rice plants were uprooted from five randomly selected points in each village, following the method described by Oberholster et al. (2018). The samples from each village were pooled into a single composite sample. Each sample was placed in sterile polythene bags labeled 1 (Batavovogi), 2 (Dokogi), 3 (Lagun), and 4 (Jikannagi) and transported to the Biology Laboratory at the University of Ilorin on the same day. Then, 20 grams of each sample were weighed into separate sterile ziplock bags and stored at -80 °C for further analysis. The physicochemical parameters of the rhizosphere soils were analyzed, including available phosphorus (P) using the sodium bicarbonate solution and molybdenum blue (MB) method, available nitrogen (N) estimated through potassium persulfate oxidation, pH determined following Makut and Owolewa (2011), and organic carbon, organic matter content and soil texture assessed using the methods of Oyeyiola and Agbaje (2013). Genomic DNA (gDNA) from each 10-gram rhizosphere sample was extracted using the ZymoBIOMICS™ DNA Miniprep kit, with a modification in which the disruption step was performed using a vortex mixer instead of a bead beater, following the guidelines of Zymo Research (2020). The purity of the gDNA was evaluated on a 0.8% agarose gel according to the protocol of Animasaun et al. (2015), and its concentration was measured using a Nanodrop 8000 spectrophotometer (Thermo Scientific, US).

Genomic DNA Amplification Using PCR and Illumina Miseq Sequencing

The DNA amplification, sequencing and library preparation followed the detailed procedure outlined in the 16S metagenomic sequencing library assembly (Part # 15044223 Rev. B) at Macrogen in Seoul, South Korea. Amplification was performed using Herculase II fusion polymerase and the Nextera XT Index Kit V2, generating paired-end reads with specific primers ITS_3F (5′-GCATCGATGAAGAACGCAGC-3′) and ITS_4R (5′-TCCTCCGCTTATTGATATGC-3′) (Yang et al., 2022). The DNA sequences obtained were analyzed to classify fungi from the phylum level down to species.

Metagenomic DNA Sequence Processing and Analysis

Paired-end reads were merged using the Fast Length Adjustment of Short (FLASH) tool, available online (http://ccb.jhu.edu/software/FLASH/), following the method by Magoč &

Salzberg (2011). After merging, the quality of the combined reads was carefully checked. Sequences shorter than 300 base pairs, those with barcode issues, ambiguous nucleotides, or primer mismatches were excluded. Sequences that passed the quality checks were grouped and annotated into Operational Taxonomic Units (OTUs) using tools like CD-HIT-OTU-MiSeq and UCLUST as described by Wei & Zhang (2019). Taxonomic classification was carried out using QIIME, with a 97% similarity threshold based on the UNITE+INSD All Eukaryotes database and 99% similarity based on the UNITE Fungi2 database (Li et al., 2019). This taxonomic analysis provided a comprehensive view of the microbial communities in each sample, from broad classifications to specific species.

To assess the diversity within the soil samples, alpha diversity metrics such as the Shannon index, which measures community diversity in terms of species richness and evenness, were used. The Chao-1 index was also applied to estimate community richness as described by Chiu (2023). These analyses, performed using QIIME, offered valuable insights into the species distribution and diversity in the studied environments.

Results

Soil Physicochemical Analysis

In this study, the recorded pH values of the rhizosphere soils from Batavovogi, Dokogi, Lagun, and Jikannagi were 9.50, 9.16, 9.81, and 9.71, respectively. The organic carbon content (%) measured 0.65, 0.44, 0.70, and 1.18 for Batavovogi, Dokogi, Lagun, and Jikannagi, respectively. Available nitrogen (Cmol kg^{-1}) levels were 0.028, 0.028, 0.0238, and 0.046 for Batavovogi, Dokogi, Lagun, and Jikannagi, respectively. The organic matter content (%) was 1.31, 1.17, 1.21, and 2.03 for Batavovogi, Dokogi, Lagun, and Jikannagi, respectively. Available phosphorus (ppm) values were measured at 4.42, 5.13, 4.83, and 3.91 for Batavovogi, Dokogi, Lagun, and Jikannagi, respectively. Clay content (%) varied with values of 16.48, 16.48, 8.48, and 18.48 for Batavovogi, Dokogi, Lagun, and Jikannagi, respectively. Sand content (%) ranged from 65.52, 75.52, 67.52, to 52.48 for Batavovogi, Dokogi, Lagun, and Jikannagi, respectively. Silt content (%) was 18, 8, 16, and 39 for Batavovogi, Dokogi, Lagun, and Jikannagi, respectively (Table 1).

Metagenomic Results

The analysis yielded a total of 414,666 DNA sequence read counts (Table 2). After applying stringent filtering and rarefaction, including clustering at a 97% similarity threshold and eliminating low-quality, short, ambiguous, chimeric and denoised reads, 340,492 high-quality reads remained. Of these, the Lagun sample had the highest read count at 95,660, followed by Jikannagi with 87,830, Dokogi with 81,166, and Batavovogi with the lowest at 75,836 (Table 2). These 340,492 quality reads were then used for taxonomic identification through the UNITE_IND_All_Eukaryotes and UNITE_Fungi databases, resulting in the detection of 2,551 Operational Taxonomic Units (OTUs). Lagun had the highest OTU count (767), while Dokogi had the fewest (482) (Table 2). The Chao1 values corresponded with the OTU distribution, and Jikannagi exhibited the highest Shannon index, while Lagun showed the lowest (Table 2).

Table 1. Physicochemical Properties of the Rhizospheres

Parameters	Batavovogi	Dokogi	Lagun	Jikannagi
Soil pH	9.50	9.16	9.81	9.71
Organic carbon (%)	0.65	0.44	0.70	1.18
Total nitrogen (Cmol/kg)	0.028	0.028	0.0238	0.046
Organic matter (%)	1.31	1.17	1.21	2.03
Clay (%)	16.48	16.48	8.48	18.48
Sand (%)	65.52	75.52	67.52	52.48
Silt (%)	18	8	16	39
Available phosphorous (ppm)	4.42	5.13	4.83	3.91

Table 2. Sequencing Statistics, Diversity and Richness Index of Fungal Communities in Rice Rhizospheres from Four Localities Sampled in This Study

Sample Name	Sample ID	Read Count before clustering	Read count after clustering	OTUs	Chao1	Shannon Index
Batavovogi	1	96,032	75,836	610	610.8	6.9480
Dokogi	2	99,107	81,166	482	482.3	6.8592
Lagun	3	114,406	95,660	767	776.4	6.6557
Jikannagi	4	105,121	87,830	692	694.0	7.1679

In this study, fungi belonging to the phyla *Ascomycota*, *Basidiomycota*, *Aphelidiomycota*, *Mucoromycota*, *Glomeromycota*, *Mortierellomycota*, *Monoblepharomycota* and *Entorrhizomycota* occurred in that order of dominance (Fig. 1). Fungal species diversity in the sample from Jikannagi was the highest, with fungi belonging to *Phylum Ascomycota* having 74%, while those belonging to the *Phylum Basidiomycota* have 5% Percentage Dominance respectively (Fig. 1). Fungal species diversity in the sample from Lagun was the lowest, with fungi belonging to *Phylum Ascomycota* having 58%, and fungi belonging to *Phylum Basidiomycota* having 4% Percentage Dominance respectively (Fig. 1). This same sample had the highest dominance of unassigned microbes (23%) in this study (Fig. 1). However, other organisms belonging to the following phyla were detected in each of the samples analysed in this study, i.e. *Anthophyta*, *Chlorophyta*, *Ciliophora*, *Nematoda* and *Protista* (Fig. 1).

At the class level, fungi belonging to *Sordariomycetes*, *Eurotiomycetes*, *Dothideomycetes*, *Agaricomycetes*, and *Rhizophlyctidomycetes* were dominant in all the samples analysed in this study (Fig. 2). However, fungi belonging to classes *Eurotiomycetes* and *Rhizophlyctidomycetes* were most dominant only in the sample from Dokogi, while fungi belonging to classes *Sordariomycetes*, *Dothideomycetes* and *Agaricomycetes* were dominant only in the sample from Jikannagi (Fig. 2). At the generic level, fungi belonging to genera *Thielavia*, *Aspergillus* and *Pleurotus* were dominant in that order in the sample from Dokogi, while fungi belonging to the genera *Acroiostachys* and *Curvularia* were dominant in the sample from Jikannagi (Fig. 3). Other fungi belonging to other genera such as *Periconia*, *Cladosporium*, *Penicillium*, *Talomyces*, *Fusarium*, *Stachybotrys*, and *Sarocladium* were detected in all the samples analysed.

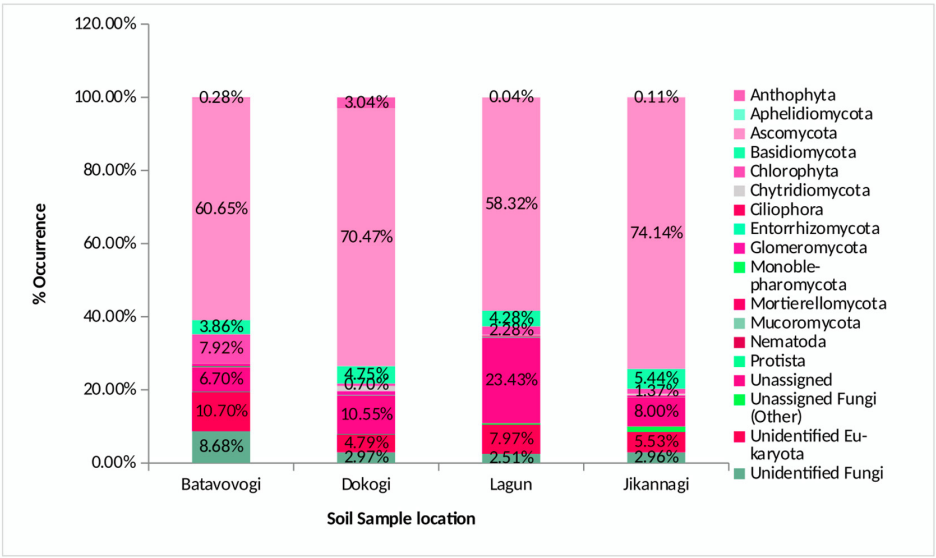

Figure 1. Percentage Dominance of Fungal Phyla in the Samples

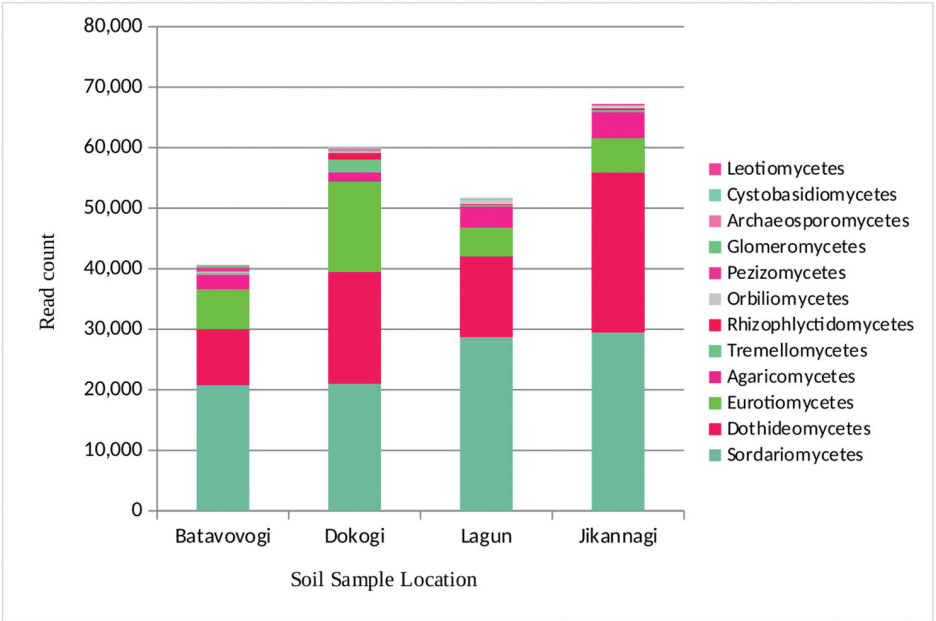

Figure 2. Percentage Dominance of Fungal Classes in the Samples

Table 3. Fungal Read Counts of the First 30 Fungal Species Detected in This Study

S/N	Species	Sample Collection Centres				
		Batavovogi	Dokogi	Lagun	Jikannagi	Total read counts
1	*Thielavia terricola*	1,810	7,338	1,701	7,022	17,871
2	*Aspergillus alabamensis*	2,044	5,892	581	1,571	10,088
3	*Achroiostachys saccharicola*	264	1,019	1,948	5,620	8,851
4	*Curvularia lunata*	796	2,278	1,224	2,217	6,515
5	*Stachybotrys elegans*	2,910	0	2,236	0	5,146
6	*Cladosporium cladosporioides*	1,496	2,733	561	276	5,066
7	*Dokmaia* sp.	51	467	2,920	1,458	4,896
8	*Nigrospora oryzae*	746	1,482	1,547	982	4,757
9	*Sarocladium strictum*	79	203	4,159	79	4,520
10	*Edenia* sp.	50	418	1,560	1,741	3,769
11	*Periconia macrospinosa*	0	1,586	236	1,925	3,747
12	*Aspergillus* sp.	0	2,518	690	29	3,237
13	*Echria* sp.	126	752	64	1,952	2,894
14	*Gaeumannomyces* sp.	1,560	11	1,166	131	2,868
15	*Zopfiella* sp.	143	0	37	2,264	2,444
16	*Phaeosphaeriopsis musae*	147	547	819	559	2,072
17	*Sarocladium oryzae*	374	332	1,063	262	2,031
18	*Cordana terrestris*	232	0	210	1,303	1,745
19	*Penicillium oxalicum*	929	134	156	518	1,737
20	*Rhizophlyctis rosea*	0	1,123	155	342	1,620
21	*Epicoccum sorghinum*	62	990	61	473	1,586
22	*Serendipita* sp.	383	147	324	708	1,562
23	*Phaeosphaeria* sp.	39	30	0	1,447	1,516
24	*Penicillium lineolatum*	187	709	13	479	1,388
25	*Trichoderma lixii*	43	0	307	1,030	1,380
26	*Penicillium parvum*	289	587	185	319	1,380
27	*Thanatephorus cucumeris*	0	0	917	449	1,366
28	*Sarocladium hominis*	32	554	703	59	1,348
29	*Alternaria longissimi*	332	371	267	369	1,339
30	*Chaetomium* sp.	918	232	166	14	1,330

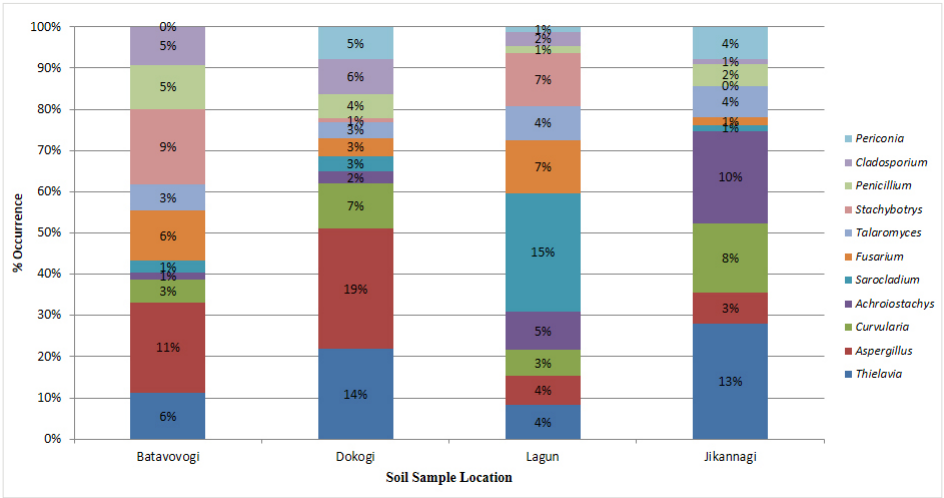

Figure 3. Percentage Dominance of Fungal Genera in the Samples

At the species level, *Thielavia terricola* and *Acroiostachys saccharicola* were dominant in the sample from Jikannagi, while *Aspergillus alabamensis* and *Curvularia lunata* were dominant in the sample from Dokogi (Table 3). Several species which include *Talaromyces ucrainicus*, *Pestalotiopsis* sp., *Acremonium flavum*, *Nigrospora oryzae*, *Penicillium* sp., *Talaromyces varians*, *Stachybotrys microspora*, *Neoscytalidium dimidiatum*, *Sarocladium oryzae*, *Exserohilum rostratum*, *Eremothecium gossypii*, *Curvularia hawaiiensis*, *Penicillium restrictum*, *Rhizopus microsporus*, *Choanephora cucurbitarum*, *Aspergillus spinosus* and *Parasola* sp. were detected as singletons, appearing only in specific samples.

Discussion

The study revealed that the sample from Lagun, which had the highest pH value, contained the greatest number of operational taxonomic units (OTUs), while the sample from Dokogi, with the lowest pH, exhibited the fewest OTUs. Additionally, the fungal community in the Lagun sample demonstrated the highest Chao1 values, indicative of greater community richness, compared to samples from other rice fields. These findings align with the observations of Tedersoo et al. (2020), who emphasized the significant influence of soil pH on OTU counts and Chao1 values. Conversely, the sample from Jikannagi had the highest Shannon diversity index, reflecting greater evenness and diversity, while the Lagun sample recorded the lowest Shannon index. Although soil pH, organic carbon and total nitrogen are widely recognized as major determinants, factors such as soil texture, moisture content, cation exchange capacity (CEC), salinity and micronutrient availability also likely influence fungal diversity. The sequencing results, with 340,492 reads and 2,551 OTUs, highlight the complex interactions among these factors in shaping fungal community structure, supporting the view of Zhong et al. (2019) that multiple soil properties govern microbial diversity.

In all samples analyzed, the most commonly identified phyla were *Ascomycota* and *Basidiomycota*, reflecting global soil trends (Egidi et al., 2019). Continuous rice cultivation enriches *Ascomycota*, a phylum known for its role in humus decomposition and enhancing soil fertility (Zhang et al., 2019). Globally, *Ascomycota* is the dominant fungal phylum, comprising 36.7 to 93 % of OTUs in various habitats (Orgiazzi et al., 2013). In a similar way, Kim (2013) identified *Ascomycota*, *Basidiomycota*, and *Glomeromycota* in soil areas associated with *Tricholoma matsutake* infections. However, O'Brien et al. (2005) reported a higher percentage of *Basidiomycota* compared to *Ascomycota* in environmental samples analyzed through Sanger sequencing. Buée et al. (2009) found that *Basidiomycota* constituted 65 percent, while *Glomeromycota* made up 2.24 percent of the fungal community in their study of forest soils using pyrosequencing.

In this study, the most prevalent fungal classes included *Sordariomycetes*, *Eurotiomycetes*, and *Dothideomycetes* (all part of *Ascomycota*), as well as *Agaricomycetes* (from *Basidiomycota*) and *Rhizophyctidomycetes* (from *Chytridomycota*). These findings align with reports from Xu et al. (2015). Hedeler et al. (2007) also identified representatives of *Eurotiomycetes* and *Sordariomycetes* within *Ascomycota*. Additionally, Buée et al. (2009) noted the dominance of *Agaricomycetes* in their study. The findings of Lee et al. (2014), which examined methane emissions and the dynamics of methanotrophic and methanogenic communities in flooded rice fields, support the observation of significant proportions of unclassified fungi in the rice rhizosphere from Lagun in this study. Liu et al. (2015) suggested that the presence of a large number of unclassified fungi in soil samples may stem from an inadequate database for fungal identification, advocating for the establishment of a curated sequence library for this purpose.

In this study, *Thielavia* and *Aspergillus* emerged as the fungal genera with the highest relative abundance, consistent with the findings of Moussa et al. (2017), where *Thielavia* was the most dominant, followed by *Aspergillus*. These genera exemplify the ecological diversity within soil fungal communities, encompassing both beneficial and antagonistic taxa. *Thielavia*, primarily composed of saprophytic species, plays a beneficial role in decomposing complex organic matter such as lignin and cellulose, contributing to nutrient cycling and soil fertility. Conversely, *Aspergillus* includes a wide spectrum of species ranging from saprophytes, which assist in organic matter decomposition, to opportunistic pathogens known to cause diseases in plants and humans. At the species level, *Curvularia lunata*, *Aspergillus* sp., *Penicillium* sp., and *Nigrospora oryzae* were identified as prevalent

plant pathogenic fungi across all analyzed rice rhizospheres. This is consistent with the findings of Ahmed et al. (2013), who documented these species as dominant in rice seed contamination. *Curvularia lunata* is known to cause leaf blight disease, while *Aspergillus* sp. negatively impacts rice seed germination (Singh & Sinha, 2016). *Penicillium* sp. produces Ochratoxin A and Patulin, which degrades rice seed quality (Ben Miri et al., 2024). *Nigrospora oryzae* is the causative agent of panicle branch rot disease, which not only reduces yield and milling but can also be mistaken for rice blast, leading to unnecessary fungicide applications (Liu et al., 2021). *Sarocladium oryzae* is identified as the primary fungus responsible for sheath rot disease in rice (Bigirimana et al., 2015).

Conclusions

This study highlights significant variability in fungal diversity across rice fields, with Jikannagi showing the highest diversity and Lagun the lowest. *Ascomycota* emerged as the dominant phylum, with *Thielavia* as the most abundant genus. The results underscore the fungal community structure and emphasize the coexistence of beneficial, antagonistic, and pathogenic taxa, offering insights into the ecological roles of fungi in rice field ecosystems.

CRediT Authorship Contribution Statement

Olahan Ganiyu Shittu: Conceived the project and supervised the work. **Olahan Ganiyu Shittu and Ajadi Ibrahim:** Conceptualization, Investigation, performed experiments, analyzed the data and drafted the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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