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Hidden Secrets of Mangrove Swamp Rice Stored Seeds in Guinea-Bissau: Assessment of Fungal Communities and Implications for Food Security

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Abstract: Rice cultivation is one of the most important agro-economic activities in many countries, and the correct seed storage between production cycles is essential for crop success. In Guinea-Bissau, mangrove swamp rice (MSR) is a highly productive rice cropping system, thus providing surplus for sale. Depending on storage conditions, rice grains may present moisture, insects, or the development of fungi that might affect crop productivity. Considering different rice varieties and storage conditions, samples were collected from 30 farmers within 13 villages across the main MSR-producing regions. Stored rice was used to isolate and identify seed-borne fungi through DNA barcoding, to test aflatoxin content, and to evaluate seed germination rates. Polyethylene bags (the container), raised wooden platforms, and storage rooms (the structures) are the most used facilities. Hermetic containers were recorded mainly in Oio. A total fungal richness of 18 genera was found, and 16 different species were identified. The most represented fungal genera are *Aspergillus*, *Curvularia*, and *Bipolaris*. Despite the presence of aflatoxin-producing fungal samples, they did not present concerning levels for human consumption, and the germination rate was not affected regardless of storage structures. These results provide a baseline on fungi occurrence in stored MSR seeds from traditional facilities in Guinea-Bissau.

Keywords: storage structures; West Africa; seed-borne fungi; *Oryza sativa*; *Oryza glaberrima*; DNA barcoding

1. Introduction

Smallholder agriculture supports 55% of African livelihoods, serving both consumption and revenue needs [1]. Among these agriculture activities, rice production stands out by providing one of the most important staple crops. Most losses in the rice value chain happen at the farm level during storage [2]. According to Ref. [3], it is estimated that every year, 25–33% of the world's grain crops, including seeds, are lost during storage, which has a significant effect on global food security. The impact is even bigger in food-deficient developing countries, such as Guinea-Bissau, where farmers make an effort to preserve their seeds for future crop production. Causing significant losses to farmers, fungi are the major microorganisms responsible for damage or spoilage of seeds in storage, causing unpleasant odour, grain discoloration, flavour changes, loss of seed viability, loss of grain nutritional value, producing mycotoxins, and weakening the plant at its initial growth stages [1,3–8]. Under temperature- and humidity-appropriate conditions for their development, fungi can result in important economic losses [9]. Thus, the diversity of fungi in stored rice as well as the conditions in which it is stored are significant concerns in agriculture and food storage.

In Africa, where agriculture is mostly seasonal [7] and agricultural practices can vary across regions, maintaining proper seed storage conditions is crucial to ensuring the viability of seeds and preventing fungal contamination [10]. Considered a global food security concern [11], fungi pose a serious problem for human health, particularly in developing countries with hot and humid climates that favour fungal growth [12]. The major grain storage fungi in Africa are species of the genera *Aspergillus*, *Penicillium* [1,3,13], *Alternaria* [3], and *Fusarium* [14]. In the context of food security and safety, perhaps the most important pathogens of global significance are mycotoxigenic fungi, which can reduce the quality and quantity of marketable products by damaging commodities (e.g., maize, rice, and peanuts) while producing mycotoxin metabolites that can be carcinogenic in both damaged and apparently healthy products or commodities [15]. Mycotoxigenic fungi are those producing mycotoxins, which are toxic secondary metabolites [16]. Mycotoxins can contaminate food and feed crops, posing serious health risks to humans and animals if consumed in sufficient quantities. The most important mycotoxigenic fungi belong to three main genera: *Aspergillus*, *Fusarium*, and *Penicillium*, with the most dangerous aflatoxins synthesised by the *Aspergillus* species (*A. flavus*, *A. parasiticus*, and the rare *A. nomius*) [12,16]. The first two can increase the risk of liver cancer and affect growth in young children, with *A. flavus* mostly found in temperate and tropical conditions [2]. Aflatoxins are the most potent carcinogenic mycotoxins, which contaminate dietary staples, such as rice, worldwide throughout the food chain [12,16,17].

Phytopathogenic fungi are the ones that can cause plant diseases [18] and can employ diverse strategies to infect their host plants [19]. Saprophytic fungi are a large group that serve as primary decomposers of organic material. They are important for carbon and nutrient cycling, and they improve bioavailability for plants and other soil biota [20,21]. Symbiotrophic fungi create mutualistic partnerships with plants to acquire organic carbon, which includes mycorrhizal fungi important for plant uptake of nutrients and water [21]. According to Ref. [13], seed-borne fungi can be (i) seed-transmitted pathogens, (ii) seed fungi that reduce seed and grain quality, or (iii) fungi that have no detrimental effects. The pathogenic fungi that are harmless in the seeds and then affect the plant cycle, resulting in systemic and recurrent infection of the plants, are quite important both for plant reproduction and human health. Depending on whether the infection occurs mostly before or after harvest, these fungi can be considered field fungi or storage fungi [13].

In Guinea-Bissau, as well as among more than three-quarters of African smallholder farmers [7], the agricultural output is kept in the village for local consumption; therefore, it is stored using local methods and facilities. These storage facilities are made of local materials, built by the villagers, and kept in their homes [7], and agricultural products are subjected to damage due to biological, environmental, and other factors [22]. The need to maintain seeds for subsistence highlights the critical importance of the success of storage structures for the community. In an overview study of Africa, three types of storage facilities were generally classified as either endogenous or exogenous: bulk, bag, and hermetically sealed containers [1,3]. In Guinea-Bissau, the first reference to the MSR's storage was made by Espírito-Santo [23], who studied the Balanta ethnic group (the main ethnic group in Guinea-Bissau [24]) in the Tombali region (South), but few studies have been conducted since then (see [25,26]).

Fungi play a crucial role in rice storage in West Africa, particularly in the mangrove regions. A study from rice storage facilities in West Africa has shown that various fungal genera, such as *Aspergillus*, *Fusarium*, and *Rhizopus*, are prevalent, thus impacting the quality and safety of stored rice [27]. Another study focused on farmer perceptions of seed-borne fungal diseases from storage rice samples saved for planting, highlighting the prevalence of storage fungi such as *Aspergillus* [8]. However, both studies have focused on rice seeds obtained from rainfed rice production systems, bypassing the mangrove swamp rice production system. Considering the uniqueness of MSR production across several countries in West Africa, and particularly in Guinea-Bissau, research on storage fungi diversity from rice storage constitutes a significant knowledge gap. The documentation of fungal diversity is crucial for nature conservation and sustainable resource management in the region, especially considering the ongoing loss of natural habitats due to land use changes and global warming [28]. Understanding the dynamics of fungi during rice storage, including their distribution and impact, is essential for developing effective storage practices to ensure food security and safety in West Africa's mangrove regions.

The main aim of this study is to identify the fungi present in the stored MSR seeds. Specifically, we aim (i) to describe storage facilities in three mangrove swamp rice-producing regions in Guinea-Bissau; (ii) to evaluate the germination rate of seeds stored under different storage facilities (as seed-borne fungi can affect the germination); (iii) to identify the fungi present in the stored mangrove rice and characterise their distribution and trophic modes (pathotrophic, saprotrophic, symbiotrophic, and mycotrophic); and (iv) to understand the impact of storage practices on the potential health risks associated with rice consumption by analysing aflatoxin content. To accomplish these points, rice seeds belonging to 30 farmers were collected in the main mangrove rice-producing regions.

This work aims to contribute to the knowledge of fungal communities in stored MSR seeds across the three main producing regions in Guinea-Bissau. Our goal is to assess if different fungal communities are present and whether fungal diversity is associated with geographical distribution and/or specific storage containers. This will allow more specialised studies aimed at improving storage conditions, respective cultivation success, and food safety.

2. Materials and Methods

2.1. Field Collection

Rice samples (≈1.0–1.5 kg each) were collected in the pre-sowing season, from 22 June to 2 July 2022, in 13 villages belonging to three mangrove rice-producing regions in Guinea-Bissau (Cacheu, Oio, and Tombali; Figure 1 and Table S1). Samples of stored rice, harvested by farmers in the previous season, were provided by 30 farmers representing the main ethnic groups who still produce MSR (20 Balanta, 3 Felupe, 4 Baiote, 2 Nalu, and 1 Manjaco), considering different varieties and/or different storage structures. The type of structures/containers where seeds were collected was recorded. During collection, a short survey was carried out focusing on the rice variety (vernacular name), storage structure, and container. Rice species identification was performed based on previous studies in regional rice species (for African rice: *Oryza glaberrima*, see Teeken et al. [24]; for Asian rice: *O. sativa*, see Temudo [26]). The samples were collected into labelled plastic bags and processed at the Plant Protection Services Laboratory, Ministry of Agriculture of Guinea-Bissau, to study the seed germination rate, identify the fungi present, and analyse the aflatoxins content. After field samples were collected, the plastic bags were opened daily to prevent moisture accumulation until further processing. Sample collection was completed over two field trips lasting five and three days.

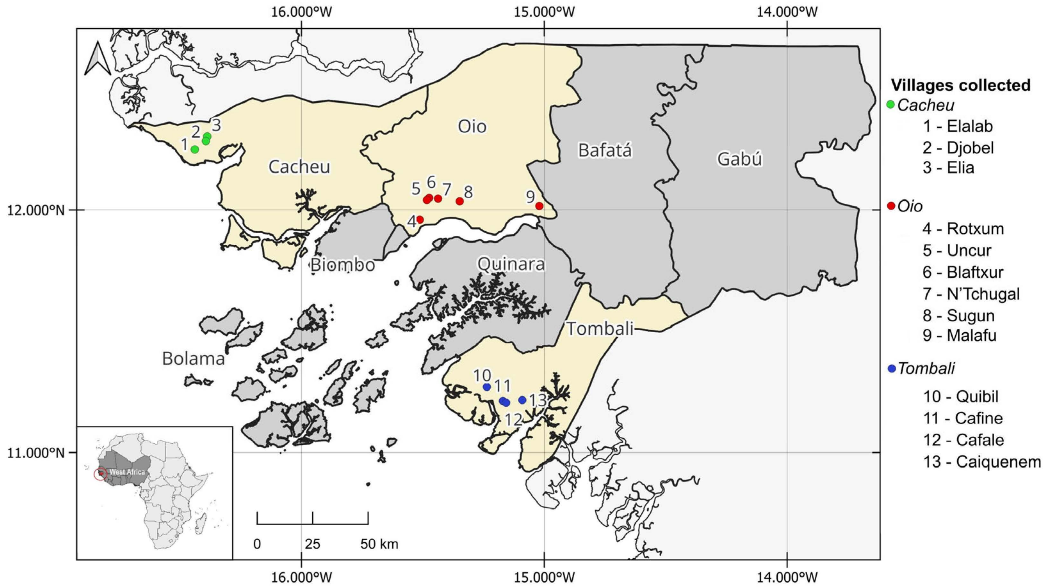

Figure 1. Map of Guinea-Bissau with the location of the 13 villages screened in major MSR-producing regions (Cacheu n = 3, in green; Oio n = 6, in red; Tombali n = 4, in blue) where the rice samples were collected.

2.2. Seed Germination Rate

Three replicas of one hundred (100) seeds were randomly processed from each rice sample, hydrated with distilled water inside a 50 mL centrifuge tube, shaken for 2 min, and placed in a 90 mm Petri dish lined with a filter paper disc. The trials were left inside the laboratory under similar conditions, with daylight, and the filter paper was daily watered with distilled water. The number of germinated seeds was counted after 3, 5, 10, and 14 days. The germination rate was determined using the following formula:

$$\%\text{germination} = \frac{\text{number of seeds germinated}}{\text{total number of seeds}} \times 100$$

The root length of 10 random seeds in each of the three replicas per sample was measured at day 5, and the seedling length of 10 random seeds was measured at day 14. Measurements were performed using a digital caliper. On day 14, it was recorded whether or not there were visible fungi present on the seeds. The seed vigour index (SVI) was calculated by determining the germination percentage and seedling length of the same seed lot using the formula: $\text{germination}(\%) \times \text{seeding length}(\text{mm})$. Seed vigour is an important attribute of seed quality assessment, ensuring high performance in diverse environmental conditions [29]. According to Abdul-Baki and Anderson [30], the seed lot showing the highest SVI is considered more vigorous.

2.3. Fungal Identification, Distribution, and Trophic Modes

2.3.1. Fungal Isolation

To identify the fungi present in the stored mangrove rice and characterise their distribution and trophic modes, samples from three representative villages, one per region, were selected based on their production dimensions and logistic factors: Elalab (8 samples) in Cacheu, Malafu (6) in Oio, and Cafine (7) in Tombali (Figure 1). First, three replicas of ten seeds were randomly taken from each sample. The surface was disinfected with 0.5% sodium hypochlorite, washed twice with sterilised water, and placed in Petri dishes with potato dextrose agar (DIFCO™, Sparks, MD, USA), supplemented with potassium thiocyanate (50 µg/mL, Sigma, Algés, Portugal), and incubated at 24 °C ± 1 °C with a 12-h photoperiod. Colony growth was observed daily for 7 days and, depending on the growth rate, was hyphae-tip-transferred to obtain pure cultures.

From single fungal colonies collected at these three representative villages, small portions sized 0.3–0.5 cm^2 were extracted into sterilised 2 µL tubes for subsequent genomic DNA extraction and species identification. All the Petri dishes were photographed using an Olympus TG6's camera (Figure S1).

2.3.2. DNA Extraction, Amplification, Sequencing, and Bioinformatics

Genomic DNA (gDNA) extraction followed the protocol of the innuPREP Plant DNA Kit (Innuscreen Gmbh, Specanalítica, Portugal), according to the manufacturer's instructions. Approximately 100 mg of mycelium was ground into a fine powder using a pestle in a mortar filled with liquid nitrogen. The nuclear ITS (Internal Transcribed Spacer) rDNA (ribosomal DNA) region was selected as the fungal DNA barcoding marker to allow genus/species identification by molecular approach. The primers ITS1F [31] and ITS4 [32] were used for the amplification of ITS regions 1 and 2, including the 5.8S rRNA gene (ITS), following previously described conditions [33]. All PCR reactions were performed in a 25 µL reaction volume using 25–50 ηg of gDNA with the addition of BSA (20 mg/mL). The thermal cycling conditions consisted of an initial denaturation step at 96 °C for 10 min; 28 cycles of 30 s at 94 °C (denaturation); 1 min at 55 °C; 3 min at 72 °C (annealing); and 8 min at 72 °C (final extension). Amplified products were purified with Sureclean Plus (Bioline, London, UK) and sent to STAB Vida company (Monte da Caparica, Portugal) for Sanger sequencing in both forward and reverse directions.

2.3.3. Sequence Processing and Putative Taxa Assignment

Raw sequences were edited using BioEdit v.7.0.9 [34] and manually aligned before final consensus sequences using forward and reverse sequences. The isolates were sequenced, and putative identification was performed by nucleotide blast (nblast) in three online databases: NCBI GenBank Nucleotide Blast (https://blast.ncbi.nlm.nih.gov, 14 January 2024), Mycobank (https://www.mycobank.org/, 14 January 2024), and BOLD Systems (https://boldsystems.org, 17 February 2024). The first three blast hits from each database and their respective crucial information (species name, total score, query cover/overlap, e-value/probability, percentage of identity/similarity, and accession number/collection number) were recorded. The taxonomy of fungi was revised to standardise according to accepted names by consulting the Global Biodiversity Information Facility database (GBIF, https://www.gbif.org/, 20 February 2024) and the NCBI Taxonomy browser (https://www.ncbi.nlm.nih.gov/taxonomy, 20 February 2024). The results were carefully analysed, and the identification of species was accepted when a similar taxonomic classification was successfully retrieved using at least two databases.

2.3.4. Ecological Guild Classification

Fungal taxa were classified according to their trophic mode (pathotrophic/saprotrophic/symbiotrophic) using FUNGuild (http://www.funguild.org/, 7 April 2024), a Python-based tool that can be used to characterise fungal taxa by trophic mode [35]. The identification of fungi as mycotrophic, and specific cases of "No data available" resulting from FUNGuild, were made through bibliographic research [[36–54], Table S2]. After mapping the ecological roles obtained from FUNGuild into the resulting phylogeny, we were able to assess genera and/or species relationships according to trophic guilds. For phylogenetic analysis, DNA sequences were aligned on the command-line version of MAFFT v7.490 [55], using the default settings. Phylogenetic analysis of the 62 DNA sequences of ITS regions 1 and 2, including the 5.8S rRNA gene, was performed on the command-line version of RAxML v8.2.12 [56] for Ubuntu distributions, specifically raxmlHPC-PTHREADS-SS3. A GTRCAT nucleotidic model was applied for 100 runs (-N) with rapid Bootstrap analysis (-f a) and a search for the best-scoring maximum likelihood (ML) tree, starting with ML optimisation from a random starting tree (-d). No outgroups were assigned. The resulting tree was inspected on FigTree v1.4.4 [57], rooted at the midpoint, and imaged on Inkscape 1.1.2 [58].

2.4. Aflatoxins Content Analyses

A single subsample of 300 g per village was created by mixing 50–100 g of rice seeds from the collected samples. The resulting 13 mixed samples were prepared at the Plant Protection Lab in Guinea-Bissau and sent to the SGS-Portugal company (SGS—Sociedade Geral de Superintendência S.A., Lisbon, Portugal) for aflatoxins content analyses. The assessment was set for four major aflatoxins: B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2), following the SGSLABETS089 method [59–63].

2.5. Statistical Analysis

Statistical analyses were performed in R v. 4.2.3. [64] using taxa richness and prevalence across different regions sampled. The heatmap was produced based on the fungal prevalence detected in each region and performed with the function "heatmap.2" (package "gplots"). Relationships between the different fungi found by region were analysed using Spearman's rank correlation coefficients. Correlation values were calculated with the package "stats" using the "cor" function and the "Spearman" statistical method. To compute correlation matrix p-values, the "cor_pmat" function (package "ggcorrplot") was used, considering a significance threshold of p-value $\leq$ 0.05. The visualisation of the correlation matrices was produced with the packages "corrplot" and the function "corrplot".

3. Results

3.1. Characterisation and Distribution of Mangrove Rice Storage Facilities

Data obtained during stored rice sample collection allowed access to how the seeds are prepared from harvest to storage. Farmers separate the seeds from the rest of the harvest in roughly two ways: (a) most choose a plot where the rice grains are well developed and the variety looks fairly homogenous, and then proceed to harvest, dry, and thresh the bundles separately; and (b) when they produce only one variety and consider that it is not mixed with others, they separate the seeds after the threshing of the entire harvest. However, the choice of unfilled grains was observed among Guinea-Bissau farmers in N'Tchugal (Oio) and Cafine (Tombali), who claim that the lightest seeds germinate all at once. After the harvest, the bundles are left on the ridges and/or the dikes during a variable period that can reach more than one month, being subjected to soil moisture and night dew. Then the bundles are gathered, threshed, and the grain stored.

Representative rice storage facilities, including their structures and the containers that they hold, are shown in Figure 2 and Table 1. Four types of storage containers were observed: polyethylene bags (50 kg) (Figure 2A,B), metal barrels (200 L) (Figure 2C), plastic jerry cans (20 L) (Figure 2D), and *fuúle*/*vuúle* (Figure 2E,F) (also, rice bundles can be placed on raised wooden platforms in specific rooms, Figure 2G). These containers may be kept in distinct storage structures, either outside or within buildings: *bentém*, *biré*/*psangá*, storage room, house corridor. Storage containers, such as *fuúles* (Balanta) (Figure 2E,F), can be small and used to store the seeds of each variety or have large dimensions for the storage of rice for consumption during the rainy season. A traditional Balanta rice storage facility, named *biré* (in Southern Balanta) or *psangá* (in Northern Balanta), is a chimney-like compartment usually built in a corner inside the house with all the walls made of adobe bricks and an entrance located at the top (Figure 2H), with or without a door and also with or without a ceiling; additionally, farmers can make a raised platform to avoid the direct contact of the grains with soil moisture. Today, it is possible to find less efficient *biré*/*psangá*, where one wall is lower than the walls of the house, without a door or ceiling (Figure 2I,J). Another more recent storage structure is a room inside the house, which is becoming increasingly used; in Balanta, it can be named literally "mice's room" (*Findar ne N'òré* in Oio) or "site of rice" (*N'humbá ne Malu* in Tombali). These rooms can be used exclusively for storing food products or not, and they can have a proper door or not, allowing the circulation of people, domestic animals, and mice (hence the name "mice's room"). Rice seeds can also be stored in polyethylene bags (Figure 2A,B), plastic, or metal barrels (Figure 2C), kept in storage rooms (or proper rooms where the household head or one of his wives sleeps), and placed directly on the ground (Figure 2A) or up in wooden or bamboo log platforms (*bentém* in Creole) (Figure 2B), sometimes plastered with adobe. *Fuúle/vuúle* are hermetic structures built with mud and rice straw (Figure 2E,F). The metal barrels are only intended for storage at a stage when they are already punctured, rusty, and without a lid, not giving any advantage as a container (Figure 2C). When rice grains are collected from the field, they are always stored with the shell (paddy rice), either when destined for the next sowing season or consumption.

Figure 2. Overview of the different storage facility (*bembas*) conditions for mangrove rice. (**A**) Bags on a room's floor (Uncur, Oio); (**B**) Bags up on a wooden log platform, *bentém* (Caiquenem, Tombali); (**C**) Metal barrel sealed with straw and mud (N'Tchugal, Oio); (**D**) Plastic jerry can kept in a storage room, along with kitchen material (Uncur, Oio); (**E**) Old women building a bowl-silo (*fuúle/vuúle*), layer by layer as it dries (Cafine, Tombali); (**F**) *fuúle/vuúle* sealed with oilcloth and mud, placed in the front porch (Blaftxur, Oio); (**G**) Bundles stored on an elevated structure in a storage room; (**H**) A *biré/psangá* (Creole/Balanta) in a specific area of the house with all walls made of adobe brick and whose only entrance is a hole located next to the roof of the house (Quibil, Tombali). The roof is covered with straw, mud, and cibe beams, and the bags are piled on the floor; (**I**) A more contemporary *biré/psangá*, featuring walls that do not reach the ceiling, a more open entrance, and no independent roof—only the house's metal roof sheet (Cafine, Tomabli); (**J**) Interior view of the previous *biré/psangá*, where bags are piled on the floor (Cafine, Tombali). Photos by Sofia Conde.

Table 1. Storage facilities presented by type of container and structure for each rice variety collected in the different villages among the three study regions. (*) When rice is kept in bundles, these are placed on raised platforms.

Region	Village	Rice Variety [Vernacular Name]	Storage Container	Storage Structure
Cacheu	Djobel	*Iacai branco*	bag	*bentém*
		Edjur	bag	storage room
		Iacai preto	bundles	raised platform *
		Edjur	bag	storage room
		Edjur	plastic jerry can	storage room
		Iacai preto	plastic jerry can	storage room
		Etele	bag	*bentém*
	Elalab	*Batumpaiabo*	bag	*bentém*
		Caublak/Caublac	bag	storage room
		Tomor/Etomoray	bag	storage room
		Bakongabu	bag	storage room
		Batumpaiabo	bag	storage room
		Iacai iadi	bag	storage room
		Balenabu	bag	storage room
		Tomor/Etomoray	bag	*bentém*
	Elia	*Iacai vermelho*	bag	*bentém*
		Edjur	bag	*bentém*
		Etele	bag	*bentém*
		Edjur	plastic jerry can	house hallway
		Edjur	bag	*bentém*
		Etele	plastic jerry can	house hallway
		Etele	bag	*bentém*
Oio	Blaftxur	*Caublak/Caublac*	*fuúle/vuúle*	front porche
		Caublak/Caublac	*fuúle/vuúle*	front porche
		Caublak/Caublac	*fuúle/vuúle*	front porche
		Seli/Sili	*fuúle/vuúle*	front porche
		Tanham/Atanham	*fuúle/vuúle*	front porche
	Malafu	*Sampena/Quisampena*	metal barrel	storage room
		DEPA	bag	storage room
		Yaca sau/xau	bag	storage room
		DEPA	bag	*bentém*
		Caublak/Caublac	bag	*bentém*
		Caublak/Caublac	bag	storage room
	N'Tchugal	*Malu rassa*	bag	storage room
		Caublak/Caublac	metal barrel	house hallway
		Caublak/Caublac	*fuúle/vuúle*	front porche
		Yaca sau/xau	bag	storage room
		Caublak/Caublac	metal barrel	storage room
	Rotxum	*Yaca ieie*	bag	storage room
		Barakonde	bag	front porche
	Sugun	*Caublak/Caublac*	metal barrel	storage room
		Caublak/Caublac	metal barrel	front porche
		Yaca sau/xau	metal barrel	front porche
		Yaca ieie	bag	storage room
		Yaca sau/xau	bag	front porche
	Uncur	*Caublak/Caublac*	bag	storage room
		Caublak/Caublac	plastic jerry can	storage room

Table 1. *Cont.*

Region	Village	Rice Variety [Vernacular Name]	Storage Container	Storage Structure
Tombali	Cafale	*Caublak/Caublac*	bag	*bentém*
		Caublak/Caublac	bag	*bentém*
		Yaca sau/xau	bag	*bentém*
	Cafine	*Caublak/Caublac*	bag	*bentém*
		Yaca sau/xau	bag	*bentém*
		Var 29 (Ianda Arruz project)	bag	*biré/psangá*
		Mamusso	*fuúle/vuúle*	front porche
		Yaca branco	bag	*biré/psangá*
		Yaca sau/xau	bag	storage room
		Yaca branco	bag	*bentém*
	Caiquenem	*Yaca sau/xau*	bag	storage room
		Yaca sau/xau	bag	*bentém*
		Caublak/Caublac	bag	storage room
	Quibil	*Yaca sau/xau*	bag	*biré/psangá*
		Caublak/Caublac	bag	storage room
		Caublak/Caublac	bag	*biré/psangá*
		Yaca sau/xau	bag	storage room

From data obtained in fieldwork, Balanta is the only ethnicity that uses *fuúle/vuúle* (18% among sampled Balanta), but polyethylene bags (63%) are the storage container mostly used at present, along with records of metal barrels (16%) and one record of a plastic jerry can. Baiotes mostly use polyethylene bags (64% among sampled Baiotes), 29% use plastic jerry cans, and only one farmer presented rice bundles on top of a platform in Djobel, Cacheu (Figure 2G). For the Felupe, Manjaco, and Nalu ethnic groups, only the use of polyethylene bags was recorded. A regional overview shows that polyethylene bags are currently the most used container, being highly represented in the three study regions. With the exception of bundles (placed on raised wooden platforms in specific rooms), all types of containers were recorded in Oio, and more than polyethylene bags, plastic jerry cans and bundles were recorded in Cacheu, and *fuúle/vuúle* was recorded mainly in Oio but also in Tombali regions. Concerning storage structures, *bentém* and storage rooms are used in the three regions, while *biré/psangá* was only recorded in Tombali (Cafine and Quibil), the use of the front porsch was recorded in Oio and Tombali for the placing of *fuúle/vuúle* (Blaftxur and N'Tchugal, Oio; Cafine, Tombali) and metal barrels (Sugun, Oio), and the house hallway was recorded as a place of storage for metal barrels (N'Tchugal, Oio, Figure 2C) and plastic jerry cans (Elia, Cacheu). The distribution of storage facilities by region is presented in Figure 3. Regarding the distribution of storage facilities, polyethylene bags are the most prevalent across the three regions (94% in Tombali, 77% in Cacheu, and 48% in Oio; Table S3). Oio is the region with more diverse storage facilities used, being the only one with records of 200 L barrels (24%), and the region with higher use of hermetic mud containers, *fuúle* (24%). Plastic jerry cans were mainly recorded in the Cacheu region, and rice bundles were only found in one Cacheu village (Djobel).

Out of the 30 farmers from 13 villages in 3 regions, a total of 64 samples were collected (Table 1). The vernacular names of the varieties provided by farmers were categorised into two rice species: African rice (*Oryza glaberrima*) and Asian rice (*O. sativa*). Overall, for the African rice (named *Emanamanai* in Felupe), a total of six different varieties were collected, with vernacular names in different ethnic groups' languages (Table 2).

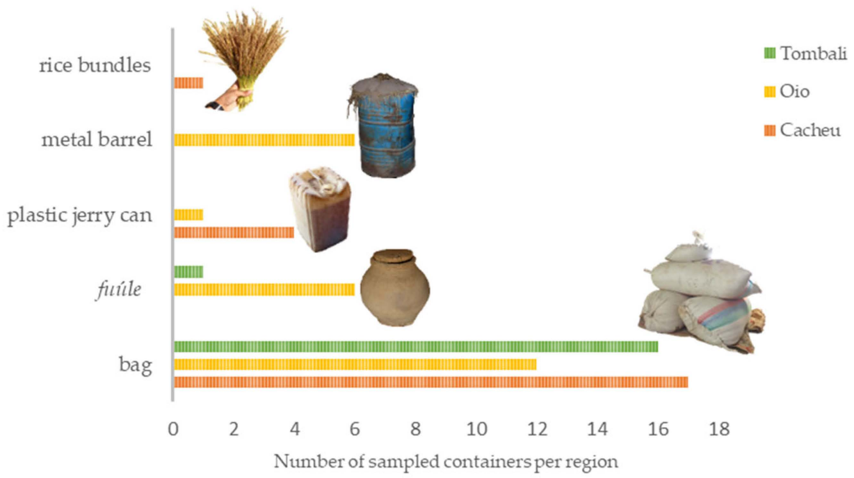

Figure 3. Number of storage containers sampled in each region.

Table 2. List of rice varieties and respective ethnic origin of the name, divided between African rice (*O. glaberrima*) and Asian rice (*O. sativa*). The presence of each rice variety in the regions studied is represented by the codified letters, where C = Cacheu, O = Oio, and T = Tombali.

Rice species	Rice Varieties [Vernacular Name]	Language	Region
Oryza glaberrima	*Edjur*	Baiote	C
	Etele		C
	Malu rassa	Balanta	O
	Bakongabu	Felupe	C
	Balenabu		C
	Batumpaiabo		C
Oryza sativa	*Tanham/Atanham*	Balanta	O
	Caublak/Caublac	Creole	C/O/T
	DEPA		O
	Seli/Sili		O
	Var 29 (Ianda Arruz project)		T
	Yaca branco		T
	Yaca ieie		O
	Yaca sau/xau		O/T
	Tomor/Etomoray	Felupe	C
	Iacai branco	Felupe/Baiote/Creole	C
	Iacai adi		C
	Iacai preto		C
	Iacai vermelho		C
	Barakonde	Mandinga	O
	Mamusso	Susu	T
	Sampena/Quisampena		O

3.2. Germination Rate Success of Stored Mangrove Rice

Germination trials (n = 64 samples with 25 rice varieties) demonstrate that, on average, all varieties had high germination rates (Table 3). Some *Oryza glaberrima* varieties, such as *Etele* and *Edjur*, and *Oryza sativa* varieties, such as *Iacai preto*, *Yaca sau/xau*, *Sampena/Quisampena*, and *Yaca branco*, showed a lower percentage of germination (<70%) in the first 3 days, but after 5 days, all varieties had a germination rate of more than 80%. At the end of the 14-day germination trial, all the samples had a germination rate of >95%. The variety with the highest seedling length was *Tanham/Atanham* (112.2 mm), followed by *Seli/Sili* (91.9 mm), both *O. sativa* species. Considering *O. glaberrima* species, the higher seedling length resulted from the *Balenabu* and *Batumpaiabo* varieties, with 73.4 and 79.3 mm, respectively. The seed vigour index (SVI) is higher in *Tanham/Atanham* (11,219), *Seli/Sili*

(9128), and *Caublak/Caublac* (8103) varieties, thus being the most vigorous according to the highest SVI values (Table 3). At the end of the trials, out of the 64 samples, only two rice varieties (*Edjur* and *Iacai preto*) from one single village (Djobel, in Cacheu) did not present visible fungi during germination trials.

Table 3. Average values resulting from seed germination rate trials. The values presented correspond to the average of the three replicates of each sample in each rice variety. The seed vigour index (SVI) is calculated by multiplying the percentage of final germination by the seedling length.

	Rice Varieties [Vernacular Name]	% Germ. Day 3	% Germ. Day 5	Root Length [mm]	% Germ. Day 10	% Germ. Day 14	Seedling Length [mm]	SVI
Oryza glaberrima	*Bakongabu*	93%	95%	43.3 ± 13.1	99%	100%	64.3 ± 14.6	6425
	Balenabu	85%	88%	49.8 ± 12.4	99%	100%	73.4 ± 9.1	7341
	Batumpaiabo	88%	93%	36.4 ± 15.5	99%	100%	79.3 ± 15.3	7891
	Edjur	51%	92%	55.4 ± 15.9	95%	97%	70 ± 11.8	6806
	Etele	8%	96%	48.2 ± 18.8	99%	100%	69 ± 12.8	6869
	Malu rassa	92%	95%	70.4 ± 16.6	98%	99%	67 ± 12	6636
Oryza sativa	*Barakonde*	97%	99%	60.9 ± 17.2	100%	100%	79.9 ± 11.5	7965
	Caublak/Caublac	94%	95%	54.4 ± 13.9	98%	99%	80.8 ± 13.9	8013
	DEPA	73%	95%	39.3 ± 18.9	98%	100%	69.1 ± 15.9	6873
	Iacai branco	93%	98%	50.8 ± 19.4	98%	99%	69.7 ± 10.5	6905
	Iacai iadi	73%	83%	51.3 ± 13.6	96%	99%	70.3 ± 13.3	6959
	Iacai preto	58%	97%	53.3 ± 18.8	98%	100%	72.9 ± 13.9	7254
	Iacai vermelho	98%	98%	56.8 ± 12.4	98%	100%	68.1 ± 14.8	6785
	Mamusso	95%	96%	42.5 ± 7.2	98%	99%	62.9 ± 8.9	6208
	Sampena/Quisampena	68%	93%	16.8 ± 11.8	98%	99%	57.9 ± 16.8	5737
	Seli/Sili	97%	97%	51.8 ± 10.1	99%	99%	91.9 ± 14.8	9128
	Tanham/Atanham	97%	97%	44.3 ± 9.3	99%	100%	112.2 ± 12.8	11,219
	Tomor/Etomoray	95%	97%	54.4 ± 13.9	99%	100%	77.2 ± 13	7694
	Var 29 (Ianda Arruz project)	88%	97%	20.6 ± 10.1	95%	99%	75.6 ± 11.6	7454
	Yaca branco	68%	82%	32.3 ± 13.5	98%	99%	71.2 ± 11	7052
	Yaca ieie	97%	98%	55.8 ± 12.9	99%	100%	78.7 ± 12.7	7829
	Yaca sau/xau	67%	86%	45.6 ± 12.9	95%	99%	72.9 ± 11.4	7188

3.3. Identification and Distribution of Stored Fungi

Seventy isolates from 21 samples were processed for DNA barcoding. Of these, 62 sequences were obtained and deposited in GenBank under the accession numbers PP786320–PP786381 (Table S4). DNA barcoding allowed for the identification of 26 taxa with a total fungal richness of 18 genera and 16 different species (Table S4), with 9 genera present in Cacheu, 9 in Tombali, and 11 in Oio (Figure 4A). The most represented genera were *Aspergillus* (40% in Cacheu, 28% in Tombali, and 18% in Oio), *Curvularia* (24% in Oio), and *Bipolaris* (20% in Tombali) (Figure 4B), and only 4 genera were shown to be shared in the three regions: *Aspergillus*, *Curvularia*, *Bipolaris*, and *Chaetomium*. From the fungi detected, 3 genera are considered to pose health security problems: *Aspergillus*, *Fusarium*, and *Talaromyces* [65], due to aflatoxins production. Seed-borne fungi can be of human health concern, like *Aspergillus* spp. (mycotoxins producer), or of plant health concern, such as *Bipolaris oryzae* (responsible for Brown Spot disease of rice). In the analysed samples, *Aspergillus* spp. was only found in bag containers, and *Bipolaris oryzae* was found both in bag and *fuúle* containers.

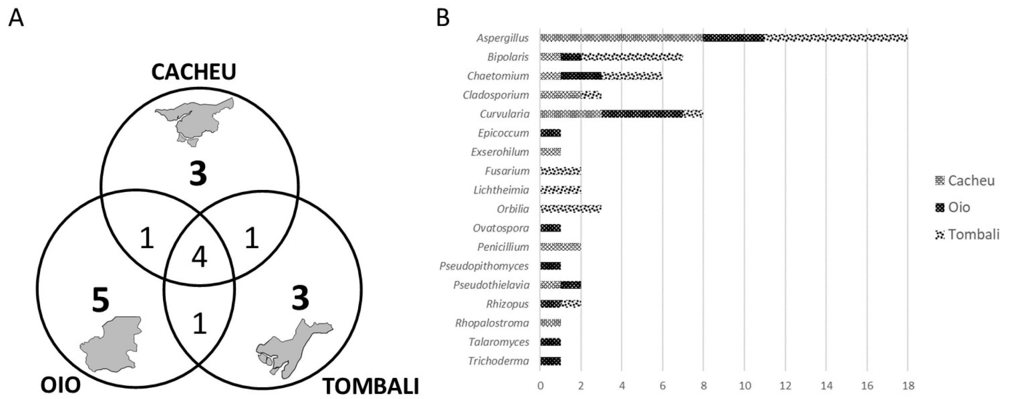

Figure 4. (**A**) Venn diagram of the number of fungal genera between regions; (**B**) Fungal genera abundance by region.

Regarding fungal taxa classification according to ecological guild, all genera (with the exception of *Pseudothielavia* genus) are saprotrophic, 78% of the genera are pathotropic, 44% are symbiotrophic, and 44% are mycotrophic (Figure S2, Table S2).

Upon ecological guild classification, a phylogenetic tree based on the DNA sequences obtained (Figure 5) was built as part of the FunGuild v1.0 software pipeline [66], which allowed for the validation of taxa identification made through the three nucleotide databases (Table S4). Four clades can be easily recognised with strong bootstrap support (BS > 80%; see Figure S3). These clades belong to the Ascomycota phylum, and the outgroup clade belongs to the Mucorales order. These three ingroup clades can also be further separated as (a) Aspergillaceae, with Trichocomaceae as an outgroup; (b) a clade composed mostly of Sordariomycetes, but with a heterogeneous mix of families; and (c) Pleosporaceae, with two outgroups belonging to the Didymosphaeriaceae and Didymellaceae families, all belonging to the Pleosporales order.

Correlation analyses between fungal occurrence (by genera) and screened regions allowed us to determine that the Oio and Cacheu regions (north of Guinea-Bissau) exhibited similar fungal compositions when compared with Tombali (Figure 6A). *Bipolaris*, *Chaetomium*, *Fusarium*, *Lichtheimia*, and *Orbilia* genera are positively associated with Tombali, while *Cladosporium*, *Exserohilum*, *Penicillium,* and *Rhopalostroma* are the genera most positively related to Cacheu. Concerning Oio, *Curvularia*, *Epicoccum*, *Ovatospora*, *Pseudopithomyces*, *Talaromyces*, and *Trichoderma* are the most positively associated genera. In addition to Tombali, *Chaetomium* is also positively associated with Oio. *Aspergillus* is positively related to Tombali and Cacheu, *Pseudothielavia* is related to Oio and Cacheu, and *Rhizopus* is related to Oio and Tombali. The correlation analyses conducted revealed several significant relationships (p-value ≤ 0.05) among the genera identified (Figure 6B). *Bipolaris*, *Fusarium*, *Lichtheimia*, and *Orbilia* showed significant positive relationships with each other. *Epicoccum*, *Ovatospora*, *Pseudopithomyces*, *Talaromyces*, and *Trichoderma* are also significantly positively correlated but present a negative relationship with the *Aspergillus* genus. *Cladosporium* only exhibited a significant positive correlation with *Penicillium*, while *Rhopalostroma* exhibited a positive correlation with *Exserohilum*. The genera *Chaetomium*, *Curvularia*, *Pseudothielavia*, and *Rhizopus* showed no significant relationships with other genera. Given the small number of replicas of each storage container, it was not possible to correlate fungal diversity with storage facilities.

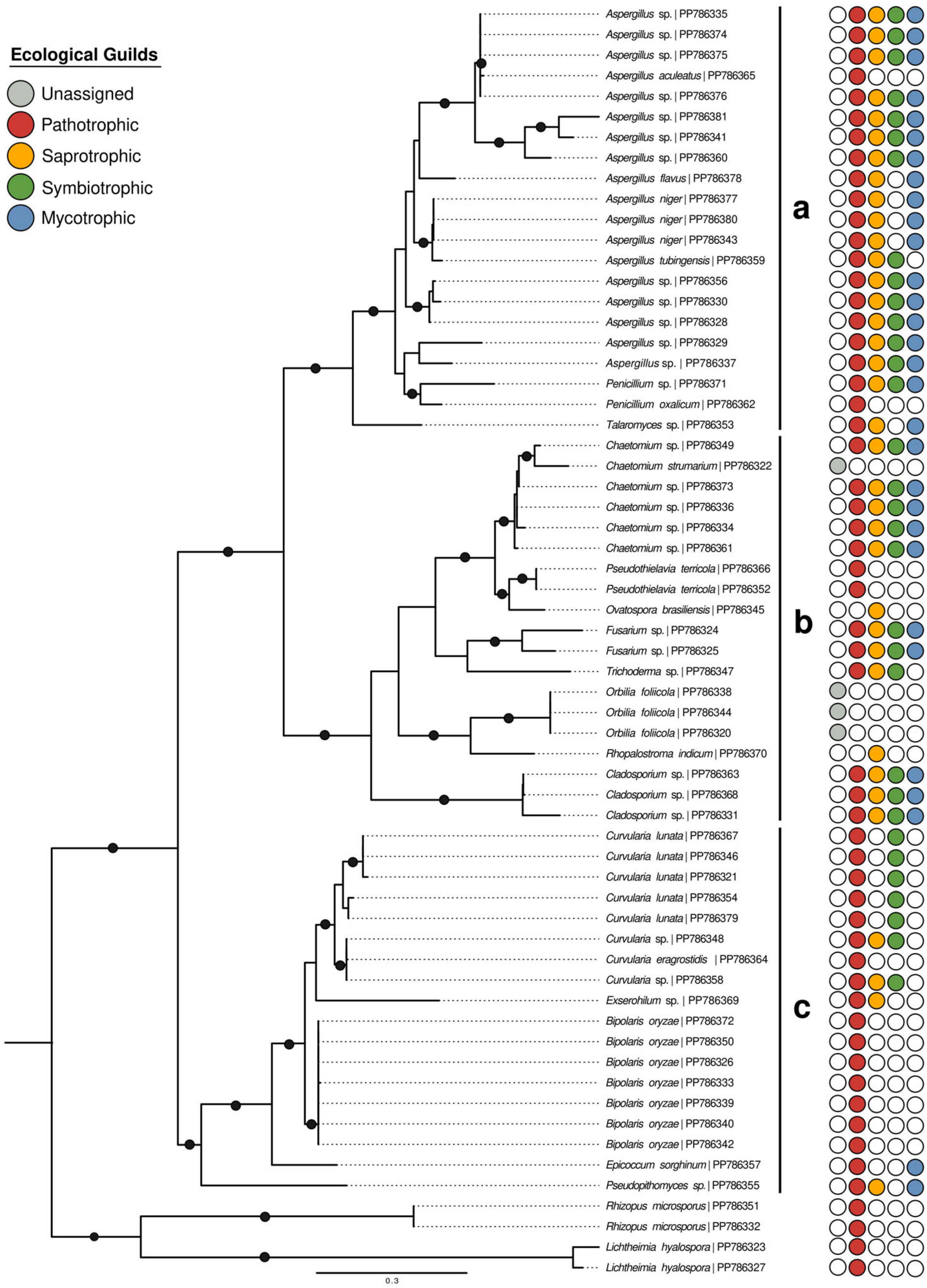

Figure 5. Maximum likelihood phylogenetic tree retrieved from 62 DNA sequences of ITS regions 1 and 2, including the 5.8S rRNA gene. The scale bar represents the number of estimated changes per branch length. The root length was truncated for imaging purposes. Clades are indicated with the letters (**a**–**c**), corresponding to (**a**) Aspergillaceae, (**b**) mostly Sordariomycetes, and (**c**) Pleosporales. Full circles indicate bootstraps equal to or up to 80%. Ecological guild classifications are presented in full circles and with different colours obtained via FUNGuild or bibliographic search (for the mycotrophic guild). For an unedited version of this tree, please refer to Figure S3.

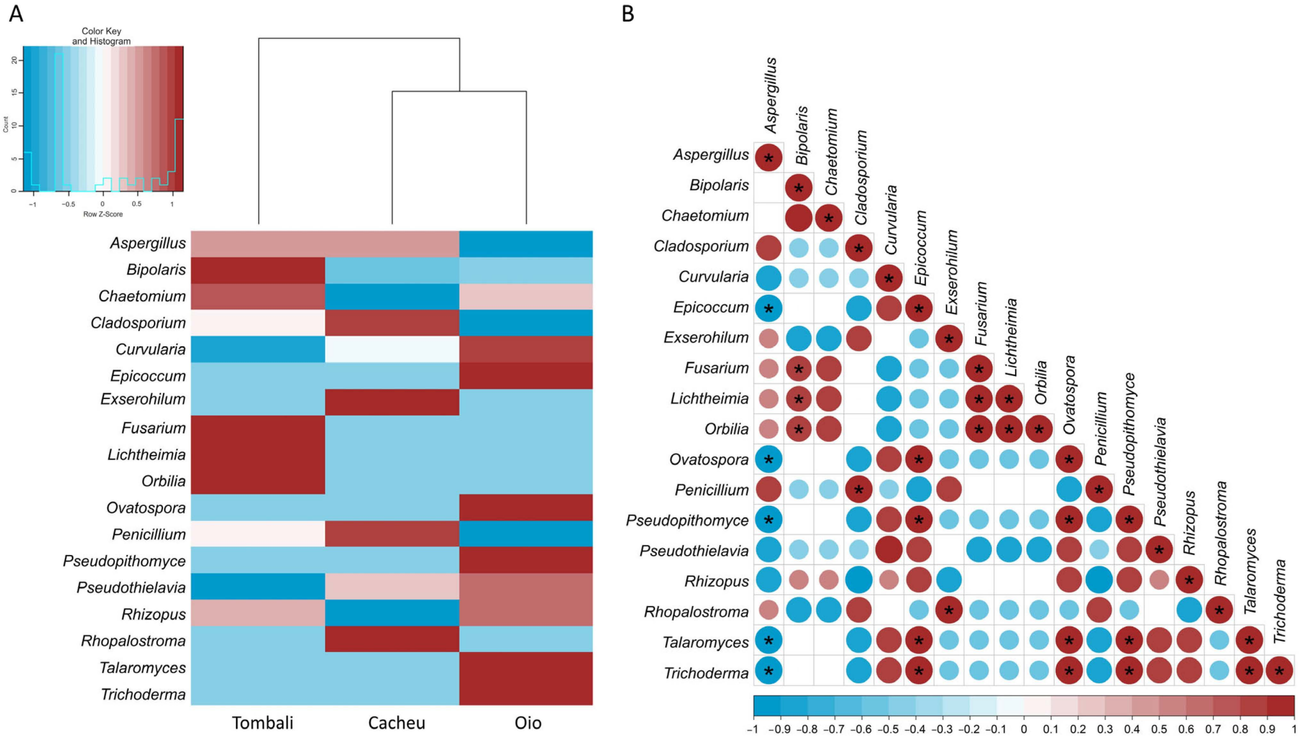

Figure 6. (**A**) Heatmap of the distribution of the fungal genera across the three studied regions of Guinea-Bissau. Darker red and blue boxes indicate high positive and negative values, respectively; (**B**) Spearman correlation coefficients between fungal genera. A colour-coded correlation scale is provided below the plot (red represents positive correlations and blue represents negative correlations); darker colour tones and larger circles represent larger correlation coefficients. Only values marked with an * are statistically significant (p-value ≤ 0.05).

3.4. Aflatoxin Content of Stored Mangrove Rice

Results obtained from all 13 villages screened revealed an aflatoxin content (total and B1, B2, G1, and G2) of < 1.0 µg/kg for all samples (Table S5).

4. Discussion

4.1. Traditional Storage Facilities for Mangrove Swamp Rice in Guinea-Bissau

In each village, storage facilities are used according to customs based on ethnicity, the volume of rice production, personal preferences, and the existence of people with knowledge to build traditional storage structures; however, integration into the market economy brought the growing abandonment of former traditional storage practices and structures/containers, as well as the adoption of storage rooms (where other items can be kept), polyethylene bags, 200-litre metal or plastic barrels, plastic jerry cans, and other random containers [67]. *Bemba* is the general Creole name given to granaries in the country. Espírito-Santo [23] made the first reference to the MSR's storage in Guinea-Bissau in 1949, studying farmers from the Tombali region (South), and reported two types of storage structures: (a) small circular huts of variable size, with an opening at the top of the wall, where the rice lays on trunks covered with mud to avoid soil moisture; and (b) a hermetically sealed amphora-like mud silo (*fuúle*). Later, both Oliveira et al. [25] and Temudo [26] portrayed a more diversified picture by including other ethnic groups. The amphora-like mud silos (*fuúles*) are still used by the Balanta ethnic group, being generally built with soil from mounds of termites due to a secretion that confers greater plasticity to the mud [68]. *Fuúles* are hermetically sealed and thus the best storage facility, while the small circular hut (*fram* in Balanta) fell into disuse. Our data confirm that the Balanta still uses *fuúle* containers with polyethylene bags, now widely used by all ethnic groups. The *fuúle* is the type of traditional container less susceptible to attack by diseases and pests because of its hermetic sealing; the same might apply to sealed barrels with rice straw and mud if they have no holes (see Figure 2C). Unfortunately, the knowledge of how to craft *fuúles* is not being transmitted to younger generations, considering the present availability of plastic or metal containers. Plastic jerry cans, although not hermetic, can reduce the attack by insects and lessen the likelihood of fungal development; metal or plastic barrels are also present, and their effectiveness might depend on their condition, such as the presence of holes, having a cover (or the use of straw and mud to isolate the top), and whether they are placed on top of a raised platform to avoid soil moisture in the case of metal containers.

Oliveira et al. [25] and Temudo [26] have concluded that farmers are cautious with the conservation of rice seeds and that, while still in the field, they separate the seeds for next year's production. However, some aspects of farmers' post-harvest practices can affect the quality of the seeds more than the absence of selection for seed purity. Leaving the harvested rice bundles in the field for many days exposed to dew on the moist soil most likely induces the development of fungi, even when farmers do not detect them or separate the bundles where they observe black seeds (*funguli* in Creole); open bags and *birés* can also favour humid conditions, leading to the development of fungi; placing rice directly on the ground, either in bags or in *birés* (without the construction of platforms, such as *bentém*), can also favour the development of fungi due to soil humidity. Both the *birés* and the storage rooms are prone to attacks by mice and insects, and mice's urine can promote humidity conditions and, consequently, the development of fungi.

Considering that the number of villages studied per region was not even, mainly due to logistics and accessibility constraints, and the existence of relevant differences in terms of temperature and air moisture among villages, the comparison of the impact of storage facilities on seeds' health between regions requires a further detailed study, including a higher number of samples of stored rice for each specific container.

Regarding the regional distribution of rice varieties, Cacheu showed the highest number of samples belonging to *Oryza glaberrima* (African rice) species (64% of the samples and 5 varieties), while Oio was only represented by 4% of the samples corresponding to one African rice variety, and the Tombali region presented 100% of the samples belonging to *Oryza sativa* (Asian rice) varieties.

4.2. Traditional Storage Practices and the Impact of Seed Storage Fungi on Food Safety and Plant Cycle

Informal conversations with the farmers during field surveys have revealed several endogenous practices for preventing the occurrence of pests and diseases on rice stored after harvest: the seeds are spread on the ground or on straw mats near the house or in the sun to dry completely (as reported in other African countries by Mobolade et al. [69]). Children are instructed to supervise to avoid consumption by animals, such as chickens or small birds, and the mixing of seeds with impurities. Only when properly dried are the seeds stored. This traditional process reduces humidity levels and helps prevent fungal development. Some farmers place local plants (as per Mobolade et al. [69]), either working as a charm for witchcraft or as traditional pesticides (like chilli pepper or *neem*), to prevent pest attacks. The lack of comprehensive cleaning of the facilities between storage cycles mentioned by farmers is likely to promote the persistence and proliferation of both fungi and pests. Despite the widespread cultivation of this crop in diverse production systems, including rainfed uplands, lowlands (rainfed and irrigated), and mangrove swamps [70], local rice still does not meet food needs, which leads to the purchase of imported rice at high prices during periods of food insecurity. Imported rice, mainly from China, Vietnam, and Thailand [71], is generally of low quality, and transport in poor storage conditions increases the likelihood of attacks by pests and diseases. Although most of the imported rice is used for consumption, it can be stored alongside rice for sowing, which can become contaminated. This scenario might cause damage to the stored seeds and conditions for the next year's rice cycle.

Considering the high per capita rice consumption (219.75 kg/capita/year in 2021 [72]), the country's dependence on MSR surplus, the constant political instability that often disrupts food imports, and the lack of diversification of food production [73], studies on rice (and other food agricultural products) post-harvest losses are of extreme importance for ensuring food security and nutritional safety.

4.3. Fungal Communities in Seed Storage Mangrove Rice

By assessing fungal taxa on stored mangrove rice, three of the four major grain storage genera reported in Africa (*Aspergillus*, *Penicillium*, and *Fusarium*) were found [1,3,13,14]. The *Aspergillus* genus was identified in the three regions, *Penicillium* in both Cacheu and Tombali, and *Fusarium* only in Tombali. Several species of both genera have been associated with economically important rice diseases, such as Brown Spot disease and Bakanae disease, respectively. In both seed-borne fungi, their fungal hyphae become established in the seed and become dormant, remaining in the seed without harming germination. After germination in the field, infection develops in the plants; thus, seeds are likely to be contaminated for the next planting cycle [8,74]. Recent studies have shown that various fungal genera, such as *Aspergillus*, *Fusarium*, and *Rhizopus*, are prevalent in West Africa, impacting the quality and safety of stored rice [8,27]. Despite the existing studies that have been conducted in rainfed and upland rice production systems [8,27,75,76], our results showed similar fungal genera in MSR. Both bag and *fuúle* containers presented *Bipolaris oryzae*, a seed-borne fungus that affects plant health and is responsible for Brown Spot disease, an economically important disease affecting rice productivity. *Bipolaris oryzae* detection may indicate a systemic and systematic persistent infection within the crop cycle [77].

Storage fungi are a type of fungi that invade seeds during storage, with little expression before harvest, being saprophytes or weak pathogens, able to decrease the germinability of the seeds and produce mycotoxins [13]. Considering the identified fungi, *Aspergillus* and *Penicillium* are common storage genera, and less relevant species belong to the genera *Rhizopus* and *Chaetomium*. The production of mycotoxins leads to human health consequences. This type of fungus is associated with the type of storage container and its respective temperature and humidity conditions [78]. *Aspergillus flavus* produces the most worrying aflatoxins (B1, B2, G1, and G2) and can be found contaminating storage facilities, being a potent human carcinogen. It also has adverse effects on animals, especially chickens [6]. Our study detected the three most important genera of mycotoxigenic fungi, *Aspergillus*, *Fusarium,* and *Penicillium*, including the species *A. flavus*. Surprisingly, aflatoxin test results showed very low detection levels, concluding that these grains are safe for human and livestock consumption, sowing, and trading. Although two samples from the germination rate trials did not present visible evidence of fungal presence, both showed the presence of pathogenic fungi (at least *Aspergillus* spp.) in the isolation trials.

According to the maximum level accepted by Commission Regulation (EU) for aflatoxin in foodstuffs [79], the results obtained from all of the 13 villages screened revealed that the aflatoxin content (total and B1, B2, G1, and G2) is much lower (<1.0 μg/kg) than the regulated maximum amount allowed (<5 μg/kg for B1 and <10 μg/kg for total content) (Table S5). These results indicate that, despite the seemingly simple storage facilities used for mangrove swamp rice, no risks for human consumption were identified in the farmers' diverse traditional storage facilities in the three regions studied in Guinea-Bissau.

The results of this work showed that, despite the presence of mycotoxigenic fungi, the quality of the seeds was not compromised from a food safety perspective. Additionally, some of the fungi found are considered not harmful, with the genus *Trichoderma* considered beneficial as a seed treatment to control diseases and enhance plant growth [13]. *Trichoderma* species are extensively used in rice biocontrol, with several studies highlighting the effectiveness of different *Trichoderma* strains in combating diseases in rice plants, like bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* [80], sheath blight, caused by *Rhizoctonia solani* [81], and Brown Spot disease, caused by *Bipolaris oryzae* [82]. It is interesting that

Trichoderma sp. not only reduces the lesion length caused by pathogens but also induces resistance in rice plants [80,83], in addition to being used as a biofertilizer to improve rice growth [84]. This genus was only found in the Oio region in *DEPA* variety rice seeds stored inside a bag and placed in a separate room. The rice bundles are left in the fields during the growing period, and seeds are not selected for symptoms before sowing, which can create optimal conditions for fungal growth, leading to increasingly aggressive fungi in the next harvest.

Notwithstanding mycotoxins being a general concern of rice producing systems [8,75], the results obtained show that, despite the presence of a highly mycotoxin-producing fungus (*Aspergillus*), the MSR sampled does not present risks to human consumption.

5. Conclusions

The study performed on stored rice seeds in the three main mangrove swamp rice-producing regions of Guinea-Bissau allowed us to obtain an overall characterisation of the main storage structures and rice varieties used in MSR production, to evaluate rice germination levels of stored seeds, and to determine fungal communities on stored rice seeds used for human consumption. Fieldwork data revealed that polyethylene bags are the most used storage containers across the three regions. Oio is the region with major storage container diversity, and Tombali is the only region with records of *fuúle*/*vuúle*. The most common storage structures recorded were the *bentém*, while *biré*/*psangá* was only recorded in Tombali. Despite the differences in storage facilities, germination trials demonstrate that, on average, all varieties had a high germination rate (>95%), indicating that storage structures did not impact rice seed germination.

The fungal diversity assessment disclosed that *Aspergillus*, *Curvularia*, and *Bipolaris* were the predominantly represented genera in all MSR regions studied. Most of the identified fungi belong to the saprotrophic and pathotrophic ecological guilds, with some of the identified fungi associated with rice diseases, with potential effects on systemic and systematic infections, impacting crop productivity.

Despite the exclusive use of local storage structures/containers, the results showed that the samples are safe for human consumption. However, there is potential for the co-development of innovations to improve post-harvest management. In summary, this study establishes the first baseline of fungal communities present in stored rice seeds from mangrove rice swamp production in Guinea-Bissau, providing tools for future research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14081870/s1, Figure S1: Photographic records of the Petri dish fungi isolations of the three representative villages (corresponding data available in Table S4); Figure S2: Distribution of fungi typology in the three study regions; Figure S3: Raw TREE file output from RAxML of the 62 ITS DNA sequences generated in this work. Table S1: Geographical coordinates of the 13 villages in the study; Table S2: Characterisation of the fungi taxa according to the FUNGuild platform. Mycotrophic classification was performed through bibliographic research. Taxa assignment by FUNGuild and "No data available" were additionally classified by bibliographic research (*). When no data resulted either from FUNGuild or bibliographic research, both "No data available" and (*) are presented; Table S3: Number of samples distributed by regions and storage container typologies; Table S4: Details of the fungi species and isolates generated by our study and included in the phylogenetic analyses. The table presents the identified species, number of isolates, respective accession number, host, and origin. The isolates were sequenced, and the identification was performed by nucleotide blast (nblast) in three online databases: NCBI GenBank Nucleotide Blast (https://blast.ncbi.nlm.nih.gov, 14 January 2024), Mycobank (https://www.mycobank.org/, 14 January 2024), and BOLD Systems (https://boldsystems.org, 17 February 2024); Table S5: Aflatoxins result from villages in the three regions.

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data curation, S.C. (Sofia Conde), A.B., S.C. (Sílvia Catarino), M.P.T., G.J.C. and F.M.; writing—original draft preparation, S.C. (Sofia Conde), S.C. (Sílvia Catarino), and F.M.; writing—review and editing, S.C. (Sofia Conde), A.B., S.C. (Sílvia Catarino), G.J.C., S.F., M.P.T. and F.M.; visualization, S.C. (Sofia Conde), S.C. (Sílvia Catarino), G.J.C. and F.M.; supervision, F.M. and M.P.T.; funding acquisition, M.P.T. and F.M. All authors have read and agreed to the published version of the manuscript.

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