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Prevalence of Seed-Borne Fungi on Soybean (*Glycine max* L. Merr.) Seeds Stored Under Medium-Term Cold Room Facilities: Implications for Genebanks

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Abstract: This study examined the prevalence of seed-borne fungi in polyethylene plastic-packaged soybean seeds stored in a genebank and identified factors influencing their incidence. Twenty-four seed lots were sampled from the collection stored at 10 °C in the World Vegetable Center genebank in Arusha, Tanzania. The seeds used were those regenerated and harvested in 2015, 2016, and 2017. A seed health test was conducted for sterilized seeds on potato dextrose agar, sterilized seeds on top of the paper, and unsterilized seeds on top of the paper. Seven-year-old sterilized seeds plated on top of the paper exhibited the highest germination percentage (74 ± 7.09%) and lowest fungal incidence (7.00 ± 4.41%). Conversely, seven-year-old unsterilized seeds plated on top of the paper had the lowest germination (22.00 ± 6.97%) and highest fungal incidence (79.00 ± 8.31%). Older seed lots showed significantly higher equilibrium seed moisture content (Eqmc), though seed age had no significant influence on germination percentage or fungal incidence. Seed germination percentage showed a significant negative relationship with Eqmc, though Eqmc had no significant effect on fungal incidence. Meanwhile, germination percentage showed a significant negative relationship with fungal incidence. Six fungi isolates were identified using their morphological features from soybean seed samples: *Absidia glauca*, *Aspergillus niger*, *Fusarium* spp., *Mucor hiemalis*, *Pestalotiopsis versicolor*, and *Sordaria macrospora*. It is concluded that high fungal incidence negatively affects seed germination but is not significantly correlated with seed moisture content. The dry and cold storage conditions in the genebank, while effective in extending seed longevity, can unintentionally allow seed-borne mycoflora to persist. Although fungi may not actively proliferate under these conditions, they may do so when favorable conditions are restored, such as during germination tests, and consequently may compromise seed viability. Therefore, this study emphasizes the importance of increased systematic seed health checks before storage in genebanks to ensure optimal seed quality, and the replacement of all polyethylene plastic bags to aluminium foil packaging.

Keywords: soybean seed; seed health; germination capacity; seed ageing; seed moisture content; seed-borne fungi

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Citation: Shango, A.J.; N'Danikou, S.; Ramadhani, S.; Sumaye, S.; Nickas, J.; Daud, M.L. Prevalence of Seed-Borne Fungi on Soybean (*Glycine max* L. Merr.) Seeds Stored Under Medium-Term Cold Room Facilities: Implications for Genebanks. *Seeds* **2024**, *3*, 589–607. https://doi.org/10.3390/seeds3040040

Academic Editors: Aria Dolatabadian and Mohammad Sayari

Received: 2 August 2024
Revised: 14 October 2024
Accepted: 17 October 2024
Published: 5 November 2024

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1. Introduction

Soybean (*Glycine max* L. Merr.) is one of the major grain legumes for nutrition and healthy diets. It is also an economically important crop cultivated in Southeast Asia and East Africa [1]. Despite this importance, the fungal incidence in seeds has been reported as one of the major limiting factors in soybean production [2]. During the preharvest stage,

fungal infections are primarily associated with environmental factors especially such as warm and humid conditions. These conditions are conducive to the invasion of soybean pods and seeds by fungi [2–5]. The fungi associated with soybean seeds are facultatively parasitic, and they can also infect and colonize seeds (seed-borne), leading to a reduction in seed quality, including viability and vigor [6,7]. This can significantly reduce crop yields and potentially lead to seedling blight and pod decay, particularly when pathogenic species are involved [8]. Preharvest infections can serve as a primary source of pathogen spread, locally and over long distances, as contaminated seeds often act as vectors for disease transmission [6,8]. Despite that, saprophytic seed-borne fungi normally have no direct damage to soybean seeds but can produce mycotoxins in stored seeds and bring health risks to humans and livestock [9,10]. Postharvest fungal infections are typically linked to inadequate drying and substandard storage facilities, which create favorable environments for the proliferation of storage fungi. These fungi can also lead to seed decay and seed quality deterioration over time, complicating the storage and marketing of soybean seeds [6,11].

Concerning pathogenic seed-borne fungi, the species in the *Aspergillus*, *Penicillium*, *Colletotrichum*, *Phomopsis*, *Fusarium*, and *Alternaria* genera are the most frequently isolated fungi from soybean seeds [11–16]. Phomopsis seed decay (PSD), storage fungi infections, and/or *diaporthe-phomopsis* complexes are among the economically important diseases of soybean that can cause the asymptomatic infection of seeds or decays and consequently impact seed viability and vigor (11–12]. The primary causal agents of PSD are *Phomopsis longicolla* (syn. Diaporthe longicolla), *D. phaseolorum* Sacc. var. sojae, and *D. phaseolorum* var. caulivora [17–19]. Apart from these, numerous *Fusarium* species have also been frequently reported to deteriorate the seed quality, cause root rot and seed decay, and pod and seedling blight in soybean, including *Fusarium graminearum* [20], *F. verticillioides* [10,21], *F. proliferatum*, *F. virguliforme*, *F. oxysporum* [22,23], *F. oxysporum* [24], *F. fujikuroi*, and *F. incarnatum*-equiseti [19]. The infection of *F. verticillioides* was found to change the flavonoid content in seeds, consequently leading to poor seed quality [5,21]. Apart from fungal infections, soybean production faces multiple challenges, including monocropping, excessive reliance on chemical inputs, and poor crop rotation, all of which contribute to the proliferation of pests [25–28]. Climate changes stimulate these issues by creating favorable conditions for both new and existing pests. Additionally, climate change increases the frequency of abiotic stresses like droughts, heat waves, and flooding, further weakening the crops and making them more vulnerable to pest attacks [29–31]. This interplay of biotic and abiotic stresses presents significant challenges for soybean farmers, necessitating the adoption of more resilient agricultural practices. In addition to the challenges posed by pests and climate change, the longevity of stored soybean seeds is crucial for maintaining crop health and productivity. Seed longevity in stored soybean seeds is significantly impacted by several factors, including seed aging, moisture content, temperature, and relative humidity [32,33]. Elevated moisture levels and high temperatures expedite seed aging, consequently diminishing germination and viability. Oxidative stress, induced by reactive oxygen species (ROS), also contributes to seed deterioration [34].

Genebanks are instrumental in conserving agricultural biodiversity, particularly in the context of soybean conservation. These repositories store plant genetic material that is crucial for breeding programs aimed at developing crops with enhanced yield, disease resistance, and environmental adaptability [35,36]. The concept of genebanks emerged in the mid-20th century in response to concerns about genetic erosion due to industrial agriculture. Initially led by agricultural research institutions, genebanks were established to store seeds for breeding purposes. Notable centers include the International Center for Agricultural Research in the Dry Areas (ICARDA), the International Rice Research Institute (IRRI), the World Vegetable Center, and the Svalbard Global Seed Vault [35,37–39]. For soybeans, genebanks provide access to a diverse range of genetic material necessary for breeding varieties with improved environmental adaptability, pest resistance, and yield [34,40]. Effective seed preservation during long-term storage is challenging due to

factors such as storage pests, seed aging, moisture content, and storage conditions [35]. Successful seed preservation requires maintaining low moisture levels and stable temperatures to reduce metabolic activities that lead to seed degradation, but also ensures high seed germination potential upon retrieval. Proper storage conditions are critical for maintaining seed viability, as seed germination percentages can decline rapidly if exposed to unfavorable environments.

As outlined by [41] genebank standards, seeds should be dried to reach equilibrium in a controlled environment, with temperatures ranging from 5 to 20 °C and relative humidity levels of 10–25%, depending on the species. Non-oily seeds should be dried to a moisture content of 4–7%, while oily seeds may require different moisture management due to their higher oil content. Before storage, a seed health test is essential to ensure that seeds are free from pathogens and pests that could compromise their viability during storage. Once dried and tested, seeds should be stored in airtight containers for long-term preservation, unless frequent access is required, in which case non-airtight containers may be used. Long-term storage requires both primary and safety duplicate seed samples to be kept at -18 ± 3 °C with 15 ± 3% relative humidity, while medium-term storage involves refrigeration at 5–10 °C with the same humidity levels. Generally, for genebank conservation, the initial germination percentage for most cultivated crop species should exceed 85% [33,41–43]. For certain specific accessions and wild or forest species that may not normally achieve high viability, a lower percentage may be acceptable [41]. When germination percentages fall below these thresholds, seed rejuvenation or regeneration becomes necessary to preserve genetic integrity. Advanced packaging materials, such as aluminum foil or laminated bags, offer superior protection against moisture and oxygen compared with polyethylene [44,45], thereby preserving germination capacity during seed storage. Regular germination testing is also essential to monitor seed viability over time, ensuring the genetic resources remain usable for future breeding efforts.

Nevertheless, there is limited information on the major factors influencing fungal incidence in soybean seeds stored under genebank conditions, and the pathogenicity of soybean seed-borne fungi species is not fully understood. The current research is based on the hypothesis that (i) seed moisture content is higher in older seeds than newer seeds in the genebank, and (ii) moisture content and the age of stored seeds both influence the fungal incidence and germination of stored seeds. This study, therefore, aimed to generate information on the influence of seed moisture content and seed age on fungal incidence and physiological quality among selected soybean seed lots. Specifically, the study sought to (i) determine the relationship between seed moisture content and fungal incidence, (ii) determine the effect of seed age on fungal incidence, and (iii) identify the species of the seed-borne fungi isolated from the selected soybean seeds. The information generated from this work is critical for the efficient management of soybean seeds, and for enhancing seed health and quality monitoring in genebanks.

2. Materials and Methods

2.1. Study Site and Experimental Materials

The seed quality assessment experiments and pathological analyses were carried out at the World Vegetable Center (WorldVeg)—Eastern and Southern Africa (ESA), Arusha, Tanzania (3.37° south, 36.8° east, elevation 1235 m) [46]. WorldVeg-ESA is situated in Arusha in a cool, wet, and medium-altitude area, where the average temperature during the warm season (November to February) is 21.6 °C (with an average minimum temperature of 14.9 °C and an average maximum temperature of 30.1 °C), while the average temperature during the cold season (June to September) is 11.9 °C (with an average minimum temperature of 24.5 °C and average maximum temperature of 18.7 °C) [46]. Arusha has a bimodal rainfall with a mean annual rainfall of 800 mm, with the long rains starting around mid-March and ending in late May, while short rains start in early November and end in early January [47]. A total of 24 seed lots regenerated at the WorldVeg-ESA research station, harvested in 2015, 2016, and 2017, and stored under active collection at 10 °C and

45% RH (medium-term) in the genebank using polyethylene plastic packages were used in the evaluations in the year 2022 (the reference year for the seed ages) (Table 1). The 24 seed lots were selected based on availability, as they were in high demand for distribution. However, before distribution, these seed lots must undergo mandatory seed health tests to ensure they meet quality standards, especially since there were no previous records of seed health tests before storage.

Table 1. List of evaluated seed lots in the experiments.

SN	Accessions	Origin	Acquisition Year	Regeneration Year	Seed Age (Years)
1	TZA 3829	Tanzania	2014	2015	7
2	TZA 3826	Tanzania	2014	2015	7
3	TZA 448	Tanzania	2014	2015	7
4	AGS 329	Taiwan	2013	2016	6
5	AGS 382	Taiwan	2013	2016	6
6	AGS 423	Taiwan	2013	2016	6
7	AGS 430	Taiwan	2013	2016	6
8	AGS 432	Taiwan	2013	2016	6
9	AGS 437	Taiwan	2013	2016	6
10	AGS 440	Taiwan	2013	2016	6
11	AGS 456	Taiwan	2013	2016	6
12	AGS 457	Taiwan	2013	2016	6
13	AGS 458	Taiwan	2013	2016	6
14	AGS 459	Taiwan	2013	2016	6
15	AGS 460	Taiwan	2013	2016	6
16	AGS 461	Taiwan	2013	2016	6
17	TGM 1203	Nigeria	2015	2016	6
18	TGM 1257	Nigeria	2015	2016	6
19	TGM 30	Nigeria	2015	2017	5
20	AGS GS 84051-32-1	Taiwan	2013	2017	5
21	AGS 129	Taiwan	2013	2017	5
22	AGS 327	Taiwan	2013	2017	5
23	AGS 133	Taiwan	2013	2017	5
24	TGM 722	Nigeria	2015	2017	5

2.2. Measurement of Equilibrium Seed Moisture Content (Eqmc)

Eqmc was determined at room temperature (approximately 20–25 °C) using enough intact seeds to fill a 3.2 mL sample holder. Three replicates of each seed lot were placed in the non-destructive measuring chamber of an AW-D10 water activity station used in conjunction with a HygroLab 3 display unit (Model: HygroLab 3, Rotronic AG, Bassersdorf, Switzerland). Measurements [water activity (=eRH/100) and temperature] were recorded and converted into Eqmc with the help of an online tool available in the seed information database (SID) (https://ser-sid.org/viability/moisture-equilibrium) accessed on 26 January 2022. Once the instrument stabilized, the water activity obtained from the HygroLab 3 display unit was multiplied by 100 to give the equilibrium relative humidity, eRH. This was used together with the temperature reading from the HygroLab 3 display unit to estimate the seed moisture content based on an oil content of 5% using the SID. The web-based calculations are based on the in-built Cromarty's equation, which predicts the moisture content of seeds [48,49]. For this study, we used an oil content of 21.8% for the seed lots [50]. As per the manufacturer of the instrument, the method error for Eqmc determination is estimated at ±0.8% RH, and the instrument's temperature accuracy is ±0.1 °C. The mean values for temperature and relative humidity are provided for each seed lot used in this study (Supplementary Table S1).

2.3. Seed Health Test

This study was conducted to assess seed health by comparing the effects of sterilization on seed germination and microbial contamination under varying conditions. A seed health test was conducted using three treatments: sterilized seeds on potato dextrose agar (PDA) (SSP), sterilized seeds on top of the paper (SST), and unsterilized seeds on top of the paper (UST). The three methods were chosen to assess seed health by providing different levels of sterilization, allowing for the evaluation of seed viability and potential microbial contamination under controlled conditions. They were suitable as they enabled a comparison of seed viability with and without sterilization, offering insights into both seed health and the impact of microbial contamination on germination. In the treatments with sterilized seeds, a 5% sodium hypochlorite (NaOCl 6.0%; SERI PLUS LTD, Nairobi, Kenya) solution was applied for 10 min to surface sterilize the seeds. After sterilization, the seeds were rinsed thoroughly with distilled water three times to remove any residual NaOCl. Following the rinsing process, the seeds were air-dried on sterile tissue paper at room temperature for 30 min before setting the test. The test was laid out in a completely randomized design (CRD) for all selected seed lots. The test was conducted in a germination chamber (Model: Memmert IPP750 Plus, Memmert GmbH + Co. KG, Schwabach, Germany) under a controlled environment (constant temperature 25 °C and alternating 12 h of light and darkness). A hundred seeds were used per soybean seed lot. Sub-samples (4 replicates of 25 seeds each) of each seed lot were placed on two layered germination tissue papers in 9 cm Petri dishes in the germination chamber. The germination tissue paper in the Petri dishes was moistened with 5 mL of sterile distilled water and monitored daily.

The first seed germination count was recorded three days after plating, while the last seed germination count was eight days after plating, and the seed germination percentage was established per seed lot and treatment [51]. The percentage of seed germination was computed using periodic counts (i.e., number of germinated seeds) as shown in Equation (1).

$$\text{Seed Germination percentage}(\%) = \frac{n}{N} \times 100 \quad (1)$$

where

n is the number of germinated seeds and N is the total number of seeds tested.

The incidence of fungi was recorded as a percentage of the total population for each soybean seed lot (Equation (2)). Fungal incidence reflects the overall prevalence of fungi within a seed lot as the percentage of seeds within a seed lot that show the presence of any fungi.

$$\text{Fungi Incidence}(\%) = \frac{n}{N} \times 100 \quad (2)$$

where

n is the number of soybean seeds on which the fungus appears, and N is the total number of seeds tested in each lot.

2.4. Identification of Fungi Isolates from Infested Soybean Seeds

The agar plate method, as described by the International Seed Testing Association [52], was utilized to isolate both pathogenic and saprophytic fungi from the seeds. Fungi grown from seeds on tissue paper and potato dextrose agar (PDA) media plates during the germination test were subsequently purified and cultured on PDA for further identification. To inhibit bacterial contamination, the PDA media was supplemented with streptomycin. Hyphal tips or spores were aseptically transferred to fresh sterile PDA plates using an ethanol-flamed inoculating needle to ensure the isolation of pure fungal cultures. These plates were then incubated under alternating 12 h light and dark cycles at 25 ± 2 °C for seven days to allow fungal growth and colony formation. Once the pure cultures were established, the fungal isolates were identified based on their morphological characteristics. The isolation frequency (%) of each fungal species was calculated by determining the proportion of samples in which each fungus was observed relative to the total number of

samples analyzed (Equation (3)). Isolation frequency measured how common a specific fungi species was among all samples processed.

$$\text{Isolation frequency}(\%) = \frac{\text{n}}{\text{N}} \times 100 \quad (3)$$

where

n is the number of samples with a specific fungi species and N is the total number of samples processed.

The morphological features of the fungi isolates on plates were examined. Temporary slides were prepared for each of the isolates, and the slides were then observed under a stereomicroscope to examine the morphological features of the grown fungi, and the macroscopic and microscopic features were recorded. These features were matched with those described in the standard reference for identifying the isolates [53].

Despite successful identification, the two fungi isolates *Absidia glauca* and *Pestalotiopsis versicolor* displayed morphological changes upon reculturing, making it challenging to obtain a consistent spore suspension for testing. This variability underscores the importance of ensuring reproducibility in experimental conditions and highlights the limitations of relying solely on morphological traits for fungi identification. Koch's postulates were applied to assess the effects of the remaining four fungi isolates—*Aspergillus niger*, *Fusarium* sp., *Mucor hiemalis*, and *Sordaria macrospora*—on seed germination (thus confirming pathogenicity), following the method described by [2]. In this study, Koch's postulates were used to confirm that the observed effects on seed germination were caused by these specific fungi species. According to Koch's postulates, (i), a pathogen should consistently affect the host (in this case, seeds), (ii) it must be isolated from affected seeds and cultured in pure form, (iii) the same effects on germination should be reproduced when healthy seeds are inoculated with the cultured pathogen, and (iv) the same pathogen species must be re-isolated from the inoculated seeds. This approach allowed us to verify the role of each fungus isolate in affecting seed germination, rather than inducing disease symptoms. To meet Koch's postulates, the following steps were taken: Postulate 1: each of the four fungi species (*Aspergillus niger*, *Fusarium* sp., *Mucor hiemalis*, *Sordaria macrospora*) was first isolated from the affected seeds. Postulate 2: pure cultures of the fungi were obtained by transferring 8 mycelium plugs to 30 mL of PDA media, incubating them in an orbital rotator at 150 r·min^{-1} and 25 °C for 4 days to produce a spore suspension at a concentration of approximately 1×10^5 spores per mL. For spore enumeration, we refer to the dilution protocol described by [2], which provides a consistent and reliable spore density under similar conditions. Postulate 3: ten healthy seeds of soybean accession "AGS 292" were surface-sterilized (as described in Section 2.3), then soaked in the spore suspension of three PDA plates per fungi species isolate, and incubated under alternating 12 h light and dark cycles at 25 ± 2 °C. Postulate 4: to complete the postulates, the same fungi species were re-isolated from the inoculated seeds that exhibited symptoms, confirming pathogenicity that the fungi responsible for affecting seed germination were the same as the original isolates.

The pathogenicity of each fungus isolate was tested, and symptoms were observed on the seeds over a seven-day incubation period. The disease severity grade was recorded according to [2], using a scale from 0 to 4, where 0 = healthy seed germination without discoloration inside the seeds; 1 = delayed germination with negligible or no discoloration inside the seeds; 2 = low germination with slightly water-soaked and yellow symptoms inside the seeds; 3 = no germination with partially water-soaked, yellow or brown, softened decay inside the seeds; and 4 = no germination, brown, and severe seed decay. The disease severity index (DSI) was calculated according to [2] as presented in Equation (4). In addition, the seed germination percentage was computed (Equation (1)) and documented.

$$\text{DSI} = \frac{\sum_i^{\text{o}}(\text{Severity grade} \times \text{Seeds per grade})}{\text{Total seeds} \times \text{Highest severity grade}} \times 100 \quad (4)$$

2.5. Statistical Analysis

All data analyses were carried out with R software, version 4.1.1 [54] to uncover similarities and differences between treatments and between seed lots for the studied seed quality parameters. Data cleaning was performed using the R package "tidyverse" [55]. The generalized linear model (GLM) following the binomial error distribution using the logit link function was used to analyze the germination counts of the selected soybean seed lots. Seed moisture content and fungal incidence were subjected to analysis of variance, followed by Tukey's post hoc honestly significant difference test for multiple comparisons to determine significant differences at a $p = 0.05$ significance level using the R package "multcomp" [56]. The rcorr() function in the "Hmisc" package [57] was used to obtain both Pearson's simple correlation coefficients (r) and the significance levels (p-values) among variables, i.e., the incidence of different microorganisms detected during seed health tests, seed moisture content, and seed age.

3. Results

3.1. Soybean Seed Moisture Content at Varying Seed Ages

Overall, the Eqmc of tested seed samples ranged from 8.10 ± 0.02% to 10.90 ± 0.02%. The soybean seed lots regenerated and stored for different years in polyethylene plastic packages exhibited a significantly different mean Eqmc ($p = 0.004$), whereby older seed lots regenerated in 2015 (7-year-old seeds) exhibited higher mean Eqmc (9.30 ± 0.11%) than those that were regenerated in 2016 (6-year-old seeds) (9.10 ± 0.06%) and 2017 (5-year-old seeds) (8.90 ± 0.04%) (Figure 1). However, the initial seed moisture data before seed storage were not available for comparison.

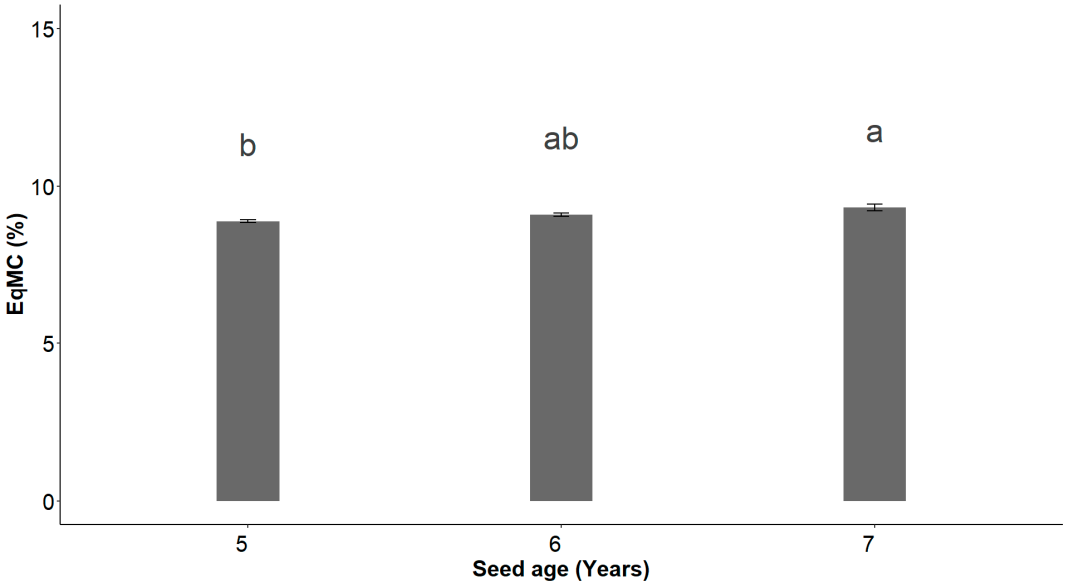


Figure 1. Variation of mean equilibrium moisture content among seed lots regenerated and stored in different years. Number of replicates (r) = 3, number of samples (n) = 72. Bars bearing the same letter(s) are not significantly different ($p > 0.05$) according to Tukey's post hoc honestly significant difference (HSD) test.

3.2. Germination Percentage of Soybean Seeds at Varying Seed Ages and Seed Treatments

Seed germination percentage did not significantly differ among seed lots with different ages ($p = 0.216$). However, the interaction between seed age and seed treatment significantly ($p = 0.017$) affected the germination percentage of soybean seeds (Figure 2). Seven-year-old sterilized seeds plated on top of the paper exhibited the highest germination percentage (74.00 ± 7.09%). Conversely, the lowest germination (22.00 ± 6.97%) was observed on seven-year-old unsterilized seeds plated on top of the paper. Older seeds were poorly

germinated, as shown by the trend in the control seed lots, which received no seed health treatment (UST). However, the initial seed germination data at the time of regeneration and the start of storage were not available for comparison.

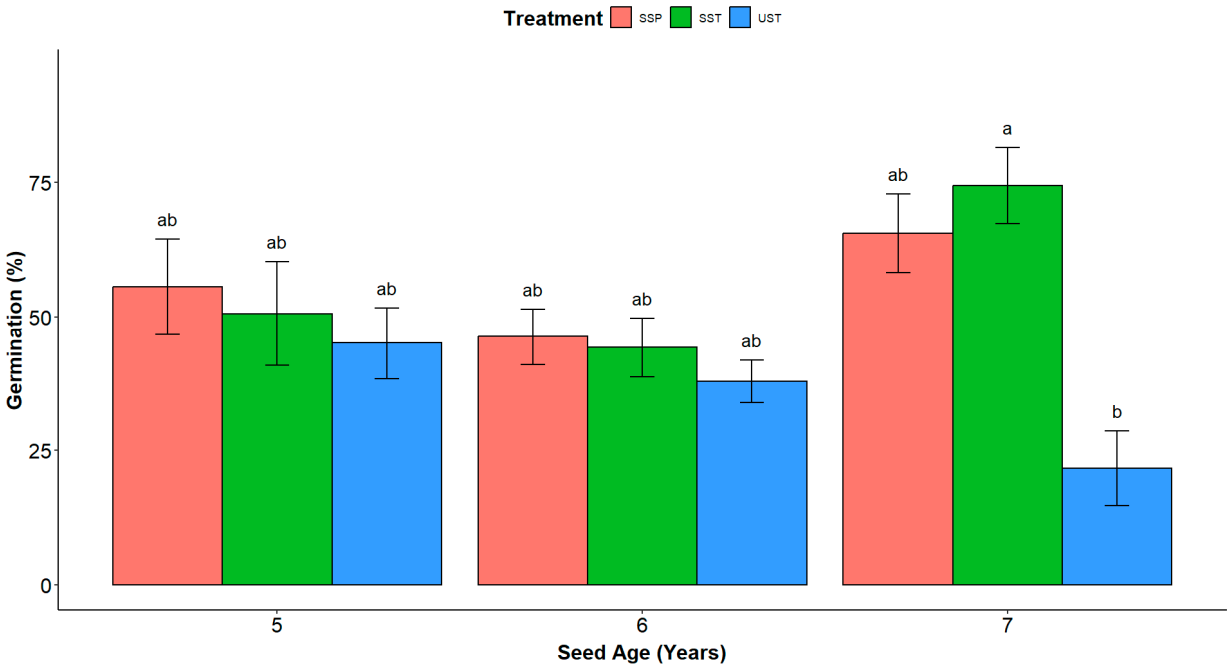


Figure 2. Variation of mean germination percentage with seed age and seed treatments among soybean seed lots. UST—unsterilized seeds plated on top of the paper, SSP—sterilized seeds plated on PDA media, SST—sterilized seeds plated on top of the paper, number of samples (n) = 240. Bars bearing the same letter(s) are not significantly different ($p > 0.05$) according to Tukey's post hoc honestly significant difference (HSD) test.

3.3. Fungal Incidence Among Soybean Seeds of Varying Seed Ages and Seed Treatments

Fungal incidence did not differ significantly among the seed lots with different ages ($p = 0.889$) but differed significantly among the treatments ($p < 0.001$). The interaction between seed age and seed treatment significantly affected fungal incidence in soybean seeds ($p = 0.001$) (Figure 3). Older (seven-year) unsterilized seeds plated on top of the paper (UST) exhibited higher fungal incidence (79.00 ± 8.31%). In contrast, the seven-year-old sterilized seeds plated on top of the paper (SST) had low fungal incidence (7.00 ± 4.41%).

3.4. Relationship Between Seed Age in Storage, Seed Moisture Content, and Fungal Incidence on Seed Germination Percentage

The correlation and regression analysis (Figure 4) show that seed Eqmc had a significant negative relationship with the seed GP ($r = -0.17$, $R^2 = 0.025$, $p = 0.008$) but had no significant influence on FI ($r = -0.006$, $R^2 = 0.0011$, $p = 0.390$). On the other hand, GP was negatively affected by FI ($r = -0.41$, $R^2 = 0.17$, $p < 0.001$). While SA had a significant positive relationship with the seed Eqmc ($r = 0.21$, $R^2 = 0.041$, $p < 0.001$), it had no significant influence on GP ($r = -0.02$, $R^2 = 0.0037$, $p = 0.720$) or FI ($r = 0.00$, $R^2 = 0.0042$, $p = 1.000$).

3.5. Fungi Isolated from the Soybean Seed Samples

A total of six fungi isolates were identified from the soybean seed samples based on morphological features (Table 2). These fungi isolates were identified as *Absidia glauca* (Figure 5a), *Aspergillus niger* (Figure 5b), *Fusarium* sp. (Figure 5c), *Mucor hiemalis* (Figure 5d), *Pestalotiopsis versicolor* (Figure 5e), and *Sordaria macrospora* (Figure 5f). Other unidentified isolates represented 26.7% of the fungi population.

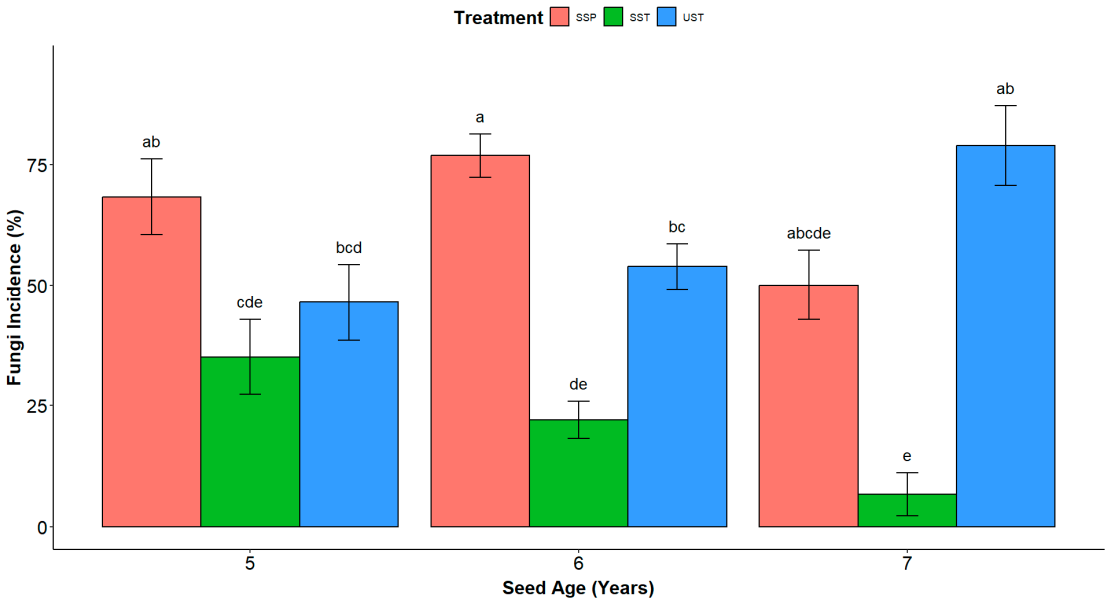


Figure 3. Variation of mean fungal incidence with seed age and seed treatments among soybean seed lots. UST—unsterilized seeds plated on top of the paper, SSP—sterilized seeds plated on PDA media, SST—sterilized seeds plated on top of the paper, number of samples (n) = 240. Bars bearing the same letter(s) are not significantly different ($p > 0.05$) according to Tukey's post hoc honestly significant difference (HSD) test.

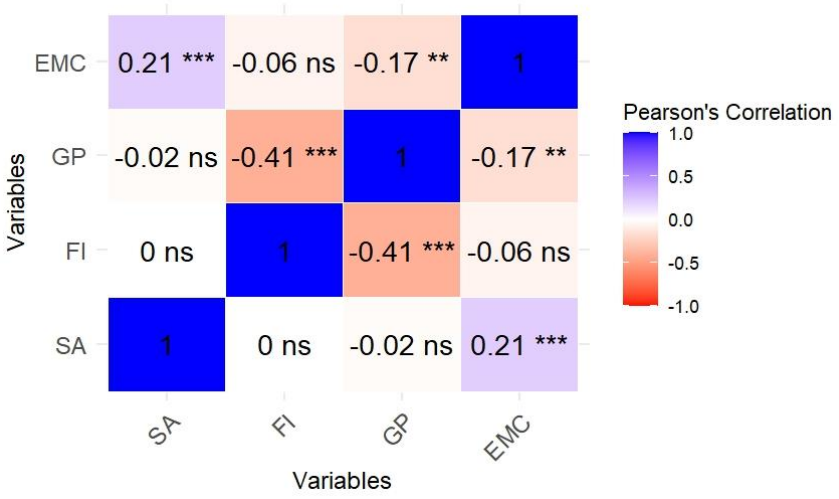


Figure 4. Relationship between seed age, seed moisture content, germination percentage, and fungal incidence. GP—germination percentage, FI—fungal incidence, EMC—equilibrium seed moisture content, SA—seed age, number of samples (n) = 240, ns—$p \geq 0.05$; **—$p < 0.01$; and ***—$p < 0.001$.

Table 2. Characterization and identification of fungi isolates from soybean seed samples.

SN	Fungi Isolate	Number of Observations of Fungi Species from Samples	Isolation Frequency (%)	Macroscopic Features	Microscopic Features
1	*Absidia glauca*	2	1.3	Whitish colonies at first, then slate-olive after a week	Sporangiophores solitary, non-septate hyphae
2	*Aspergillus niger*	75	50.0	Black and powdery-like colonies	Conidiophores smooth-walled and non-septate

Table 2. *Cont.*

SN	Fungi Isolate	Number of Observations of Fungi Species from Samples	Isolation Frequency (%)	Macroscopic Features	Microscopic Features
3	*Fusarium* sp.	3	2.0	Yellow pink creamy colonies	Cylindrical to ovoid conidia, curved septate conidiophores
4	*Mucor hiemalis*	8	5.3	Creamy-yellow colonies	Round, conidia non-septate
5	*Pestalotiopsis versicolor*	1	0.7	Brown colonies	Narrow fusiform conidia, hyaline apical appendages
6	*Sordaria macrospora*	21	14.0	White with abundant aerial mycelium	Hyphae hyaline, thin-walled, septate, irregularly branched
7	Unidentified fungi spp.	40	26.7	-	-
	Total	150	100.0		

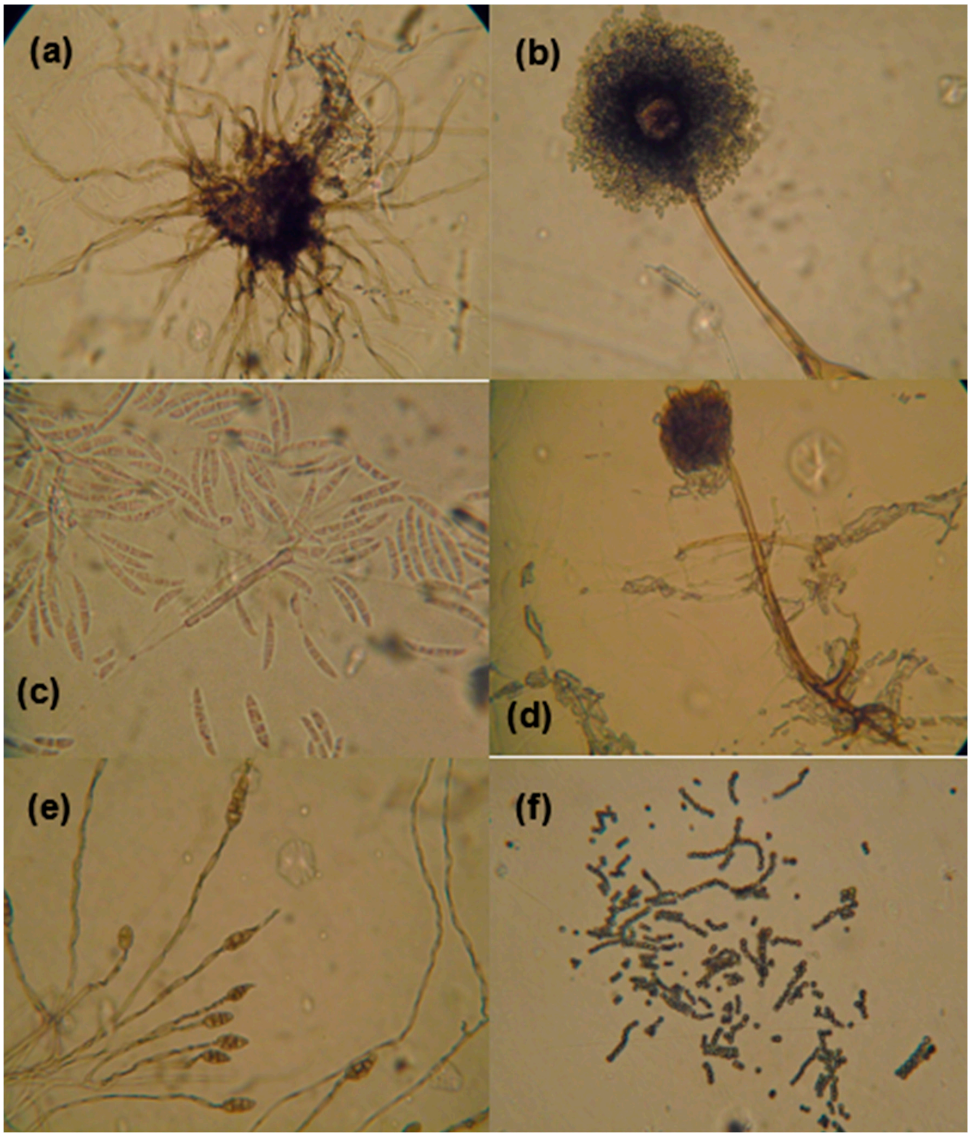


Figure 5. Microscopic features of the identified fungi species. (**a**) *Absidia glauca*, (**b**) *Aspergillus niger*, (**c**) *Fusarium* sp., (**d**) *Mucor hiemalis*, (**e**) *Pestalotiopsis versicolor*, (**f**) *Sordaria macrospora*.

3.6. Pathogenicity of the Isolated Fungi Species Associated with Soybean Seeds

After a seven-day culture on PDA plates, the mycelium growth of the fungi species on the seed surface and the infection symptoms inside the seeds were observed. The infection led to water-soaked and decay symptoms on and inside the seeds (Figure 6). The germination in inoculated seeds was notably impeded by the fungi species and exhibited a high disease severity index. The lowest germination percentage and the highest disease severity index (DSI) were exhibited by fungi species *Sordaria macrospora* (0.0%; 75.0%, respectively), followed by *Mucor hiemalis* (13.3%; 71.7%%, respectively), *Aspergillus niger* (16.7%; 70.8%, respectively), and *Fusarium* sp. (26.7%; 68.3%%, respectively) (Table 3).

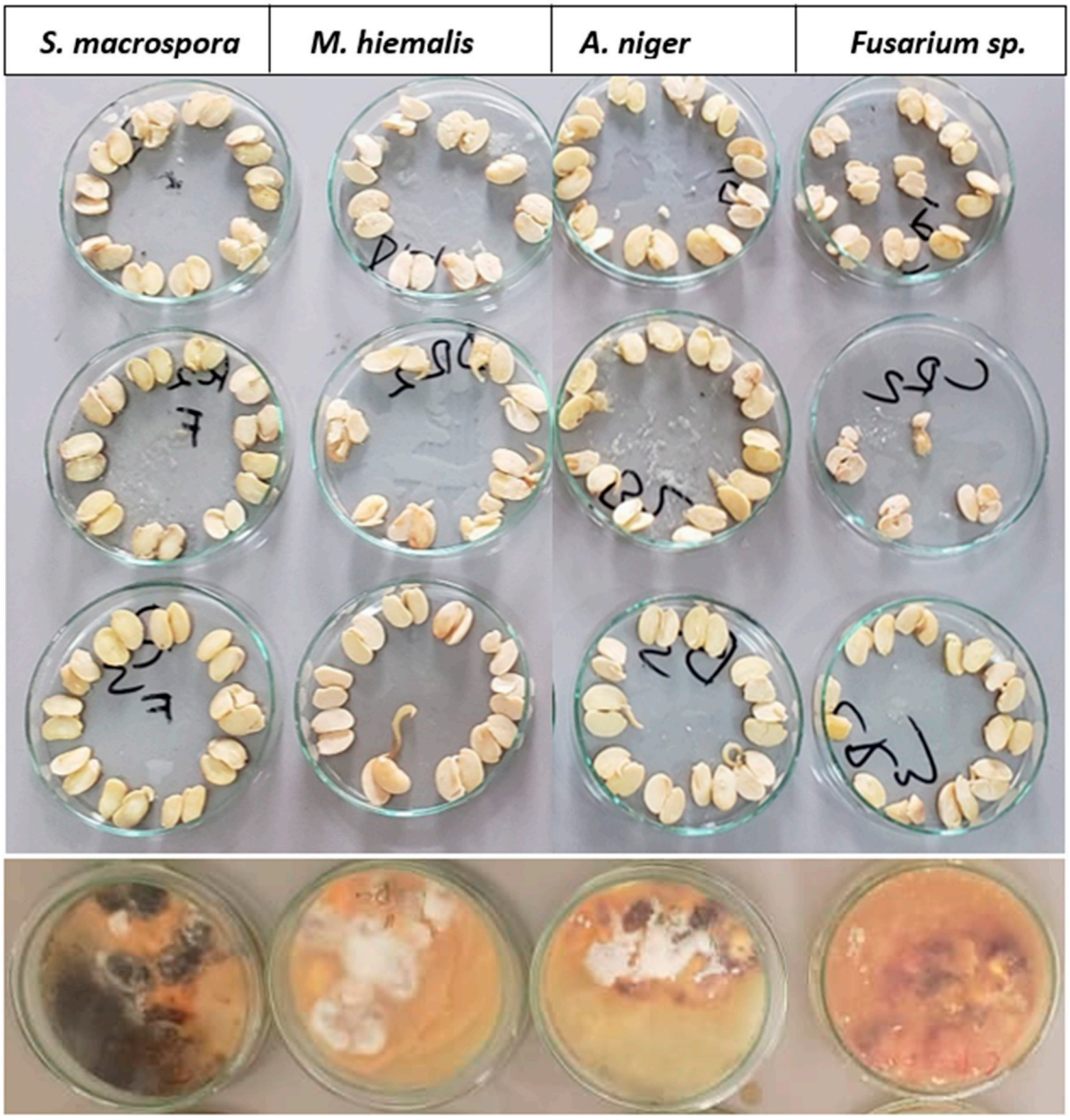


Figure 6. Symptoms exhibited by the four isolated fungi species associated with soybean seed decay and impeded germination. The figure shows the effects of four fungal species—*Sordaria macrospora*, *Mucor hiemalis*, *Aspergillus niger*, and *Fusarium* sp.—on seed germination and decay due to mycelial growth. Columns represent the fungi species, while the first three rows show seed germination responses across three replications. The final row displays the mycelial growth for each fungus species on the seed after incubation.

Table 3. Variation of mean disease severity index and seed germination percentage among fungi isolates.

Isolate	Disease Severity Index (DSI) (n = 12)	Seed Germination Percentage (GP) (n = 12)
Sordaria macrospora	75.0 ± 0.0 a	0.0 ± 0.0 b
Mucor hiemalis	71.7 ± 2.2 ab	13.3 ± 8.8 ab
Aspergillus niger	70.8 ± 1.7 ab	16.7 ± 6.7 ab
Fusarium spp.	68.3 ± 0.8 b	26.7 ± 3.3 a
p-value	0.0683	0.012

n = number of samples. Means (± SE) followed by the same letter(s) within a column are not significantly different ($p > 0.05$) according to Tukey's post hoc honestly significant difference (HSD) test.

4. Discussion

4.1. Seed Moisture Content Increased with Soybean Seed Age in Cold Room Storage

The seed moisture content varied significantly between seed lots regenerated in different years. The older seed lots showed significantly higher seed moisture content compared with fresh seeds. Such difference in the moisture content between seed lots stored at the same cold condition is usually attributed to various factors such as difference in harvesting time, drying methods, seed packaging material, genetics, and seed structure and composition [58]. The study of [44] described the significant increase in seed moisture content in soybean seeds stored under cold room conditions. Since the soybean seed lots used in the current study were packaged in polyethylene plastic bags and stored under cold room conditions, the packaging material could create heat. Therefore, this could significantly increase seed moisture content as storage duration increased, since the seeds stored under such conditions could absorb humidity, unlike if they were packaged in air-tight containers like aluminum foil bags. Polyethylene plastic bags are not entirely airtight, they can slow down but not completely prevent moisture and air exchange. A previous study by [59] emphasized adopting appropriate packaging material (i.e., aluminum foil bags) and optimal seed moisture content to maintain better seed quality in soybean. Seed moisture content plays a critical role in determining the rate and extent of physiological activities in seeds [60]. Storing soybean seeds in aluminum foil bags kept moisture content constant. This delayed seed deterioration, as phospholipids and protein content of mitochondria inner membrane were highly maintained. Hence, the seeds exhibited higher germination and coefficient velocity of germination, unlike the seeds stored in polyethylene bags [59]. Therefore, maintaining seed moisture content to an optimal level throughout the storage period can minimize the detrimental physiological changes to the seed, along with fungi contamination [61]. Depending on the crop species, lower seed moisture content (for instance, below 5%) is considered optimal for seed storage under cold conditions in genebanks [62]. The cold dry conditions preserve seed viability for a longer period as they reduce the rate of seed deterioration [63]. Therefore, it is required to adequately dry the seeds before storage to lower seed moisture content for long-term storage and seed storage moisture content has to be determined at the time of storage beginning.

Seed age greatly affects germination, with older seeds often losing viability due to physiological and biochemical damage. Reactive oxygen species (ROS) cause deoxyribonucleic acid (DNA), protein, and lipid degradation over time, disrupting essential germination processes [43,64]. Aging also leads to membrane integrity loss, weakening vital functions. High temperature and humidity speed up aging, although optimal storage conditions like low temperature and moisture can slow it down but not completely stop it [65]. Viability loss rates differ among species and seed lots, based on genetics and initial quality.

4.2. Fungi Survived in Fresh and Older Seeds in Cold Room Storage

The stored seeds of soybean showed a significant level of fungal contamination on the surface and potentially within the seed lots. However, the incidence of surface fungi was reduced upon seed sterilization using 5% sodium hypochlorite (NaOCl) before ger-

mination, as shown in treated seeds. This clearly suggests that the fungi observed were already present in and on the surface of the soybean seeds before sterilization and became active due to the change in environmental conditions during the germination test. The 5% NaOCl solution is technically used for surface sterilization while cleaning contaminated seeds [66,67]. Surface sterilization disinfects the seeds, allowing only any internal pathogens to grow when exposed to the media for germination. In this context, cold storage conditions (low temperature, 10 °C) likely suppress the sporulation of resting spores on seeds, which can become active when the environmental conditions become favorable again, e.g., during seed germination. The genebank conditions (i.e., low temperature and low seed moisture content), other than enhancing seed longevity [51], also favor the survival of seed-borne mycoflora contaminating the seeds. The cool and dry conditions typically used for seed storage can also be favorable for the survival of some types of microorganisms, including fungi [68]. Other than fungi, other microorganisms like bacteria and viruses can survive in the cool dry conditions of genebanks. Spore-forming bacteria can lie dormant on seed surfaces or within tissues, becoming active during germination [69]. Similarly, some viruses can remain viable in stored seeds for long periods [70], surviving within the seed coat or embryo and infecting new plants when they germinate. While a 5% sodium hypochlorite (NaOCl) solution can remove many external contaminants, it may not be enough to eliminate internal pathogens such as bacteria and viruses, which need specific treatments to ensure seed health and prevent disease transmission [71,72]. Pathogens can be situated beneath the seed coat or even inside other seed organs (near the embryo). These cannot be affected by NaOCl sterilization and pose another threat. Previous studies have shown that some seed-associated organisms like *Ascochyta lentis* were also isolated from seed accessions stored at 4–6 °C for 33 years [73]. Also, various seed-borne fungi pathogens can remain viable after long-term storage (after 8–14 years) at low or sub-freezing temperatures (−20 °C) [74]. The mycelium of *Ustilago tritici* survived in infected wheat seeds after 32 years of storage at −15 °C, similar to temperatures of −18 °C, which are recommended for the long-term conservation of germplasm [41]. Recently reported data from the first 30 years of a 100-year seed storage experiment in permafrost conditions (−3.5 °C) at the Svalbard Seed Vault showed that all the seed-borne pathogens survived, with some showing reduced infection percentages [75]. Therefore, timely detection through routine checks by genebank staff and identifying these microorganisms are essential to conserve high-quality seeds and prevent pathogen dissemination through seed distribution [76,77]. ISTA [52] details seed health protocols for different seed types, ensuring their quality during storage and use. Methods to detect fungi in seeds include visual inspections, blotter tests, and the agar plate method for observing fungal growth. Molecular techniques like polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) offer precise fungal DNA detection, while next-generation sequencing (NGS) provides extensive fungal profiling but is costly [78–81]. Immunoassays such as enzyme-linked immunosorbent assay (ELISA) rapidly identify fungal antigens or toxins and are suitable for large-scale screenings [82]. Microscopic examination and viability staining also help detect and assess fungal cell viability [83].

4.3. Eqmc Impaired Seed Germination but with No Direct Link to Fungal Incidence

In the present study, Eqmc had a significant negative relationship with seed germination but did not significantly influence fungal incidence. One would typically assume that, if Eqmc negatively affects seed germination, it might also have some influence on fungal incidence; however, the data suggest otherwise in this study. It is important to note that there may be other factors at play that could explain this outcome, such as contamination before storage and the use of inappropriate seed packaging materials. Under field conditions, the fungi may invade the seeds at any point throughout the seed formation, from ovule initiation to seed maturation [84]. Cleaner seed lots before storage in a cold room may maintain lower fungal incidence, even if they could later absorb humidity during storage due to inappropriate packaging materials. Fungi infestation can significantly reduce seed viability

and negatively impact seed germination. In our study, the sterilized seed lots stored for seven years in the genebank showed a better germination percentage (74.00 ± 7.09%) than unsterilized seed lots stored for seven years (22 ± 6.97%). The 7-year-old seeds seemed to tolerate fungi infection because they could germinate well despite having an important level of fungi contamination (79.00 ± 8.31%). Fungi can cause detrimental effects, including seed rot, reducing water availability and nutrients necessary for germination [85]. Furthermore, some fungi can produce toxic compounds that can damage the seedling, leading to reduced growth or seedling death.

4.4. Fungi Isolates and Their Impact on Soybean Seed Quality

The pathogenicity test revealed aggressive fungi species causing severe decay and hampering germination in inoculated seeds. This underscores their strong pathogenic nature and also the seed interactions with other microbes, including fungi species. The presence of pathogens in seeds causes a decrease in viability, a musty odor, and a change in seed color [86]. Seed damage can be exacerbated if stored in a location that promotes the development of the pathogen. Physical conditions such as temperature, moisture content of the seeds, and the humidity of storage are among the factors that favor the growth of pathogens in soybean seeds [86]. It is commonly known that the seed-borne fungi genera *Aspergillus* and *Penicillium* can grow in seeds whose moisture content is in equilibrium with the relative humidity ranging from 65 to 90% [68]. Species of Absidia thrive in mesophilic conditions, exhibiting rapid growth at temperatures between 25 °C and 34 °C [87]. *Aspergillus niger* thrives in environments with high temperatures and high relative humidity [88]. Its optimal growth occurs between 25 °C and 40 °C, with rapid decay and spread observed at 30 °C to 35 °C. *Aspergillus niger* does not grow at temperatures below 15 °C, and no growth is seen under refrigeration. *Fusarium* species generally thrive in humid environments with water activity levels above 0.86 and can grow across a broad temperature range, from approximately 0 °C to 37 °C. However, unlike thermophilic organisms that prefer very high temperatures (45 °C to 80 °C), *Fusarium* species do not exhibit optimal growth in such conditions. Their optimal growth temperature typically falls between 20 °C and 30 °C [89]. Different fungi species have different levels of pathogenicity, meaning that some fungi are more likely to cause disease and reduce seed germination than others. Fungi such as *Fusarium* are commonly seed-borne and can infest seeds during their development or storage. *Fusarium* sp. is a well-known seed-borne fungus and has been reported to infest a wide range of crops, including soybeans [2]. Others, such as *Aspergillus niger*, may colonize and infest seeds after they have been harvested, although it may also be soil-borne [68]. Various studies have reported the negative effects of the mycotoxin compounds (i.e., trichothecenes, zearalenones, fumonisins, aflatoxin contamination, ochratoxin, Neotypodium, alternariol, or tenuazonic acid) generated by the *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp. and *Alternaria* spp. on seed germination and seedling development [90–93]. In addition, the dissemination method depends on each fungus's biology and the conditions under which the seeds are produced. It is also possible that these fungi can already be present in the soil and infect the soybean seeds as they germinate after sowing. The production of xerotolerant propagules, such as drought-resistant conidia, chlamydospores, sclerotia, or dormant mycelium, to endure dehydration are among the strategies of many fungi to survive in seeds [94], and the spores of a wide variety of fungi have been proven to be desiccation tolerant [95]. A recent study on beans has shown that seed drying or desiccation treatment (i.e., seed drying at 13% and 5% relative humidity and 20 °C until a seed moisture equilibrium was reached) reduced the proportion of infected seeds for most seed-borne fungi, including *Penicillium*, *Cladosporium*, *Alternaria*, *Ulocladium*, *Fusarium*, *Botrytis*, and *Rhizoctonia*. However, the percentage of *Aspergillus* and *Mucor* fungi species tended to increase with similar treatments [96]. This study calls for future avenues for research, including investigating fungi diversity in genebank collections to inform targeted monitoring; assessing the impact of fungi infections on seed viability and genetic integrity; developing robust protocols for routine seed health tests, emphasizing

early detection; implementing integrated pest management strategies tailored to genebank settings; capacity building through training programs for genebank staff in hygiene and fungi management. Overall, addressing these aspects will enhance seed health monitoring practices and sustain genebank collections.

5. Conclusions

This study identified *Absidia glauca*, *Aspergillus niger*, *Fusarium* sp., *Mucor hiemalis*, *Pestalotiopsis versicolor*, and *Sordaria macrospora* as prevalent fungal pathogens in soybean seeds under medium storage conditions. Our findings suggest that soybean seeds were either contaminated with fungi before storage or during storage due to inappropriate packaging materials and seed handling. This calls for a need to improve seed handling before storage, including possibly treating seeds before storage. However, best practices in handling and storing seeds in storehouses and genebanks are commendable to prevent contamination. The findings also signal the importance of maintaining proper seed storage conditions to ensure optimal seed germination, since initial seed quality and the type of fungi present in the seeds can significantly impact the soybeans' fungal incidence and germination percentage. While polyethylene plastic bags offer some degree of protection against moisture exchange, they are not entirely air-tight. For long-term seed storage with minimal moisture absorption, air-tight containers like vacuum-sealed aluminum foil bags offering better moisture and gas barrier properties should be used. To gain a more comprehensive understanding of the potential impact of fungi infection on seed viability in genebanks, it is crucial to conduct in-depth investigations that focus on examining the longevity and pathogenicity of seed-borne fungi following extended storage periods (long-term). While certain seed-borne fungi can have detrimental impacts on seed quality, it is important to note that numerous endophytic fungi species do not adversely affect seeds and may even contribute to pathogen control and other beneficial functions. Therefore, a thorough examination of these fungi is warranted, including an investigation of the mode of dissemination of each fungus species isolated in the soybean seed samples.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/seeds3040040/s1, Table S1: Parameters recorded during the determination of equilibrium moisture content of seed samples.

Author Contributions: Conceptualization, A.J.S. and S.N.; methodology, A.J.S.; validation, S.N.; resources, S.N.; data curation, S.R., S.S., J.N., M.L.D. and A.J.S.; formal analysis, A.J.S. and M.L.D.; writing—original draft preparation, A.J.S., J.N. and M.L.D.; writing—review and editing, S.N., J.N. and M.L.D.; supervision, S.N.; project administration, S.N.; funding acquisition, S.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the "Taiwan-Africa Vegetable Initiative (TAVI)" project, and the Genebank Long-Term Grant from the Global Crop Diversity Trust. Funding for the genebank of Africa's Vegetable Genebank is provided by strategic long-term donors to the World Vegetable Center: Republic of China (Taiwan), UK aid from the UK government, United States Agency for International Development (USAID), Australian Center for International Agricultural Research (ACIAR), Germany, Thailand, Philippines, Korea, and Japan.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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