Fungi in Seed Germplasm Collections

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Seeds can harbor a wide range of microorganisms, especially fungi, which can cause different sanitary problems. Seed quality and seed longevity may be drastically reduced by fungi that invade seeds before or after harvest. Seed movement can be a pathway for the spread of diseases into new areas. Some seed-associated fungi can also produce mycotoxins that may cause serious negative effects on humans, animals and the seeds themselves. Seed storage is the most efficient and widely used method for conserving plant genetic resources. The seed storage conditions used in gene banks, low temperature and low seed moisture content, increase seed longevity and are usually favorable for the survival of seed-borne mycoflora. Early detection and identification of seed fungi are essential activities to conserve high-quality seeds and to prevent pathogen dissemination.

seed-borne fungi seed storage gene bank

1. Introduction

Seeds have been the basis for agriculture and foodstuffs since Neolithic times. Most important crops are grown from seeds, and cereal and legume grains constitute the main raw material of human food and animal feed. Seeds can harbor a wide range of microorganisms, especially fungi, which can cause significant sanitary problems in the seeds themselves, in growing crops, as well as to human and animal health. The main harmful effects of seed-associated fungi are mainly related to the transmission and dissemination of plant diseases, the reduction of seed quality and seed longevity, and the production of mycotoxins that can be highly toxic to humans and animals through the consumption of contaminated materials.

Seeds can be passive carriers of pathogens which can be transmitted to growing plants when environmental conditions are suitable. The term seed-borne describes the state of any microorganism that is carried with, on, or in the seed. The term seed-transmitted refers to the act of infecting seedlings from seed-borne inoculums. The first records of seed-borne diseases date back to the middle of the 18th century and reported bunt and smut diseases of cereals. One century later, the fungus causing bean anthracnose was demonstrated to be seed-borne [1]. At the beginning of the 20th century, the growth of the seed industry and the global seed market worldwide were the most likely causes of many new outbreaks of diseases [2]. In the following years numerous examples of destructive diseases disseminated by seeds were reported [3]. Although to a lesser extent, the increase in the international exchange of plant germplasm for breeding programs and research purposes in the last few decades has also increased the risk of spreading seed-borne pathogens into new areas [4][5].

Many seed-borne fungi may drastically reduce the germination of seeds stored for genetic preservation or plant reproduction [6]. It has long been known that some of the fungi that infect seeds before harvest, in the field, can reduce seed quality. However, the effects of other fungi that mainly invade seeds after harvest, the so-called “storage fungi”, were not reported until the 1940s [7].

Mycotoxins are secondary metabolites produced by filamentous fungi capable of causing serious diseases in humans and animals [8]. The major mycotoxin-producing fungi are species of *Aspergillus*, *Fusarium*, *Alternaria* and *Penicillium*. The mycotoxins that pose the greatest potential risk as food and feed contaminants are aflatoxins, altersolanol, trichothecenes, fumonisins, zearalenone, ochratoxin A and ergot alkaloids, among others [9]. Mycotoxigenic fungi are generally not aggressive pathogens, but they are often well-adapted to growing on substrates with low moisture, and they can colonize stored seeds. A major concern related to mycotoxins arose from the discovery of aflatoxins in poultry feed in England in the early 1960s [10], and since then extensive research has been carried out A area [11].

An outlook on some basic aspects of seed-borne fungi, such as the fungal types and pathways of infection and transmission, has been included to help the understanding of the interactions and processes that occur between fungi and seeds. An overview of seed health methods is also provided, from conventional techniques to the latest molecular analyses, as early detection is a key aspect for controlling problems caused by seed-borne pathogens.

2. Fungi in Seed Germplasm Collections

2.1. Effect of Cold and Dry Storage on Fungi Longevity

The longevity of seed-borne fungi depends on diverse factors, including fungus type, its location in the seed, the severity of infection and the presence of antagonistic microbiota. However, storage conditions are the major factor affecting the survival of seed-borne fungi.

Several months or a few years of storage under normal room or warehouse conditions may substantially reduce the inoculum of many pathogens, and most of them were found to disappear after 10–15 years [3][12]. However, prolonging the period of commercial storage is generally not considered a practical method to free seeds from seed-borne fungi, due to the parallel decrease in seed viability. In a work by Russell [13], the longevity of *Helminthosporium sativus* (=*Bipolaris sorokiniana*, sexual morph *Cochliobolus sativus*) and *Alternaria tenuis* (=*A. alternata*) on wheat seed, stored under room conditions, was studied for 16 years. The viability of both fungi progressively decreased with time. However, *Alternaria* died out more rapidly and disappeared after 7 years, while *Helminthosporium* was found in a small percentage of seeds after 15 years, when the germination rate was below 30%. In an experiment conducted in Poland, fungi from *Fusarium* genera were not detected on barley grains after 3–5 years of uncontrolled storage, while *Bipolaris sorokiniana* and *Alternaria* were still present after this period [14].

In the case of gene banks, seeds are usually dried to a low moisture content and stored at low temperatures [15]. It has been well demonstrated that cold storage enhances the survival of seed-associated organisms, and, thus,

gene banks may serve as reservoirs of seed-borne pathogens. Temperatures of −18 °C are recommended for the long-term conservation of germplasm [16]. Under similar sub-freezing conditions, Hewett [17] found that the viability of several seed-borne fungal pathogens was almost unaffected after 8–14 years of storage. *Ascochyta lentis* was also isolated from seed accessions stored at 4–6 °C for 33 years at the National Seed Storage Laboratory (NSSL) in the USA [18]. In a later study, Menzies et al. [19] found that mycelium of *Ustilago tritici* survived in infected wheat seeds after 32 years of storage at −15 °C. These authors point out that the conservation of infected seeds can be an advisable method to maintain collections of this fungus. Recently, data from the first 30 years of a 100-year seed storage experiment located in the Svalbard permafrost (−3.5 °C) have shown that all of the seed-borne pathogens have survived, and only a few of them have shown a reduction in the infection percentages [20].

In some cases, the increase in fungi survival at low storage temperatures has negatively affected the seed quality. Gilbert et al. [21] reported that *Fusarium*-infected wheat seeds had better germination and emergence rates after 24 weeks of storage at 10 °C and 20 °C than at −10 °C and 2.5 °C. Kaiser et al. [22] and Singh et al. [23] showed that the number of seeds infected by *Ascochyta fabae* and *Rhizoctonia bataticola* (=*Macrophomina phaseolina*) in lentils and chickpeas increased after several years of storage at sub-freezing temperatures. The reasons for these increases are not clear, because fungal growth cannot occur under these conditions. These results could be due, among other possibilities, to a shift in survival in other components of the mycoflora that formerly inhibited the growth of the fungus [22][24].

At the other extreme, the inverse relationship between temperature and fungal longevity has been widely used to control seed-borne pathogens through seed heat treatments [25][26][27][28].

To survive in seeds, many fungi are able to endure dehydration by producing xerotolerant propagules, such as drought-resistant conidia, chlamydospores, sclerotia or dormant mycelium [3], and desiccation tolerance has been established in the spores of a wide variety of fungi [29]. An inverse relationship between relative humidity and viability has been determined for the fungal spores of several entomopathogenic and phytopathogenic fungi [30]. In the study, a model of the effects of moisture content and temperature on conidia survival was developed. This model was analogous to that established for orthodox seeds [31], although the relative humidity range in which this model could be applied varied considerably among fungi. The relationship between relative humidity and fungal spore viability appears to be highly species-specific and more complex than in seeds. Thus, there are even examples of higher losses of conidia viability at intermediate relative humidity than at lower or higher values [32][33].

At the CRF-INIA gene bank, an experiment was conducted to test the effect of seed drying on seed-borne fungi on bean seeds. Seeds were dried at 13% and 5% relative humidity and 20 °C until a seed moisture equilibrium was reached. Desiccation treatments decreased the proportion of infected seeds for most of the fungi found (*Penicillium*, *Cladosporium*, *Alternaria*, *Ulocladium*, *Fusarium*, *Botrytis*, *Rhizoctonia*, among others). In contrast, the percentage of seeds in which *Aspergillus* and *Mucor* were detected tended to increase with seed drying [34].

Cold and low humidity storage environments also contribute to maintaining the viability of endophytic fungi of grass seeds [35][36]. Endophyte viability, however, generally decreases at a faster rate than seed viability [37].

In summary, the dry and cold storage conditions used in seed gene banks to increase seed longevity usually favor the survival of seed-borne mycoflora. Differences in fungi longevity under low temperatures and in sensitivity to desiccation might alter the initial composition of seed inoculums. This may have consequences on germinability that are difficult to predict after long storage periods, when seed susceptibility to diseases can also be higher due to the ageing process.

2.2. Risk of Pathogen Dissemination

According to the information compiled in the Second Report on the State of the World's Plant Genetic Resources for Food and Agriculture [38], about 7.4 million germplasm accessions are conserved worldwide, the majority of which are maintained in seed collections. Gene banks play a central role in the movement of germplasm within and among countries, and hundreds of thousands of seed samples are supplied to plant breeders, researchers, or farmers each year. Data provided by the CGIAR [39] and the U.S. National Plant Germplasm System (NPGS) [40] indicate that these two gene bank networks alone distribute about 400,000 samples and more than 200,000 samples per year, respectively.

This extensive exchange of germplasm poses a risk of unintentionally introducing and disseminating plant pathogens by means of infected seeds [5]. Furthermore, the diversity of stored germplasm is often directly related to the diversity of seed-associated microorganisms. Thus, crop diversity centers are also expected to be areas of high variability of crop-specific pathogens, where exotic strains or races may occur [41]. In some seed-borne diseases, the introduction of compatible mating types from one region to another may lead to the development of the sexual state in new areas, and if this occurs the pool of virulence genes in the pathogen could be greatly increased [42].

Surveys carried out on seed lots received, conserved, or distributed by different national and international gene banks have revealed the presence of economically important pathogenic fungi. In Brazilian germplasm collections, 13 important pathogenic fungi were isolated from approximately 10,000 accessions of 24 crop species before long-term storage [43]. From 1982–1997 about 0.5% of the samples exported by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) were detained due to severe fungal or bacterial infections [44]. The Seed Health Laboratory at the International Center for Agricultural Research in the Dry Areas (ICARDA) reported that 22.03% of incoming cereal seeds from 1995–2004 were infected with seed-borne pathogens, and *Tilletia caries* and *T. foetida* (=*T. laevis*), the common bunts of cereals, were the most frequently detected [45]. In India, during 2012–2014, thirty pathogenic fungi and one bacteria of quarantine significance were intercepted on the introduced germplasm and trial material of 63 crop species from 35 countries [46]. In 2018 and 2019, the Germplasm Health Unit (GHU) or the CGIAR centers tested and removed 7% of the 335,928 gene bank samples, including those for import, export, and regeneration, due to pest interception during phytosanitary processing [47].

Although seeds generally present a lower sanitary threat than vegetative material, germplasm recipients and providers should be aware of the phytosanitary hazards associated with seed exchange and take precautionary measures to minimize the risk of spreading pests and diseases. Bioversity International (formerly IPGRI and

IBPGR) has published technical guidelines that contain useful information for safe germplasm transfer, two of which refer to the seeds of legumes and cereals [48][49].

To prevent the introduction of quarantine or regulated pests, most countries have legislation, regulations, or procedures which should be contemplated in the movement of germplasm. The IPPC (International Plant Protection Convention) is an international plant health agreement that coordinates regional and national phytosanitary measures and provides information related to import and export requirements, pest status and regulated pest lists provided by each member country (https://www.ippc.int, accessed on 1 September 2022).

The CGIAR gene banks, which conserve large amounts of germplasm from many geographical regions and send shipments to a broad range of countries, have a special concern for the safety of the transfer of materials. All CGIAR gene banks have GHUs that operate in coordination with the National Plant Protection Organization (NPPO) or the quarantine agency of the host country [5]. All newly introduced materials are subject to seed health checks before being included in the gene bank, and the health of outgoing seeds must also be controlled. The GHU of the International Rice Research Institute (IRRI) maintains a 12-hectare post-entry quarantine area for the initial planting of imported rice seeds, a genetic resource center nursery area for wild races, and a phytotron for transgenic materials. It makes crop health monitoring, treatment, and isolation of the imported germplasm more efficient and safer [50]. The guidelines followed by CGIAR gene banks for the safe transfer of germplasm are available in the CGIAR portal “Crop Genebank Knowledge Base” (http://cropgenebank.sgrp.cgiar.org, accessed on 1 September 2022).

According to Koo et al. [51] who provided information on gene bank costs in the CIMMYT (International Maize and Wheat Improvement Center), ICARDA, ICRISAT, IRRI and CIAT (International Center for Tropical Agriculture), the cost of seed health tests ranged from 4.2 to 18.8% of the annual operational costs of seed processing, conservation and distribution, with the lowest and highest values corresponding to IRRI and CIAT, respectively. In comparison, the operational costs of seed health testing were 1.4 (IRRI) to 3.6 (ICARDA) times higher than those corresponding to seed viability testing.

Fulfilling seed health requirements for international transfer can be a significant problem for many gene banks with limited resources and for many countries where there is a lack of technical and human resources for plant health inspection. Furthermore, national regulations often focus on commercial trade and large volumes of consignments and they are not adequate for the purposes of the international transfer of germplasm [5]. This situation has even led some national scientists to stop making requests as they know the plant material or seeds will be refused or will not reach them in a timely manner [52]. Ideally, quarantine services and germplasm scientists should cooperate to find a balance between protecting crops and promoting global agricultural research [4].

2.3. Effects of Fungi on Seed Longevity

The initial quality of samples, together with seed moisture level and storage temperature, are the main factors that influence the longevity of orthodox seed in gene banks [15]. Samples with high germination and vigor survive longer

than low viability samples. Therefore, storing seeds of the highest initial quality is a priority of gene bank curators.

Seed infection by fungi in the field may be an important factor affecting seed quality. In legumes, species of the complex *Phomopsis*/*Diaporthe* are among the most thoroughly studied seed-borne fungi due to their severe effects on yield and seed quality in soybean growing areas [53]. In grain cereals, *Bipolaris sorokiniana* and *Fusarium* spp. are major seed-borne pathogens which are widespread around the world and can reduce seed quality and cause seed rot and seedling blight [54][55]. In grasses, some fungal endophytes decrease seed longevity [56], and some species of *Alternaria* can negatively affect the seed quality of crucifers and other horticultural crops [57][58].

Some specific studies have been conducted under gene bank storage conditions to determine the effect of fungal pathogens on legume seed longevity. Groundnut seeds infected with *Macrophomina phaseolina* experienced a drastic decrease in germinability after five years of storage at 20, 4 and −18 °C, whereas healthy lots maintained their viability during the same period at all storage temperatures. Initial germination rates were also lower in infected lots than in healthy seeds (about 83% and 99%, respectively) [23]. Similar results have been obtained in lentil and chickpea seeds infected with *Ascochyta fabae* [22] and *A. rabiei* [59].

Other studies on seed germplasm point out the negative effects of some fungi on seed conservation, although they do not present correlations between fungal incidence and germination. Duan et al. [60] reported declines in germinability in Chinese gene bank wheat accessions infected with *Fusarium verticilliodes*, *Bipolaris nodulosa* and *Cladosporium herbarum*. In the germination tests of several species, Faiad et al. [43] noted that the main genera of pathogenic fungi isolated from abnormal seedlings were *Phoma*, *Pyricularia*, *Fusarium*, *Gerlachia*, *Diaporthe*, *Colletotrichum*, *Macrophomina,* and *Rhizoctonia*. A survey of seed-borne fungi on cruciferous seeds stored in a Japanese gene bank reported that seed lots were frequently infected by pathogens, especially by *Alternaria* spp., and *A. brassicicola* and *A. japonica* apparently inhibited seed germination and caused seed rot [61].

After harvest, storage fungi, mainly *Aspergillus* and *Penicillium*, may invade and deteriorate seed samples if drying is delayed and seeds are held under humid ambient conditions during the pre-storage period. Once the seeds have been desiccated to appropriate moisture contents for germplasm conservation, seed fungal growth is completely halted.

Many of the fungi that reduce seed quality are important producers of mycotoxins which are highly dangerous for human and animal health. *Fusarium* genus generates trichothecenes, zearalenone (*F. graminearum*, *F. culmorum*), and fumonisins (*F. verticilloides*, *F. proliferatum*, *F. subglutinans*). *Aspergillus flavus* is the main source of aflatoxin contamination, *Penicillium* spp. produce ochratoxin, and *Neotypodium* may originate a diverse array of alkaloids with antimammalian activities [11]. *Alternaria* species are also capable of producing several mycotoxins, including alternariol or tenuazonic acid [62]. Although comparatively fewer studies have been carried out on the effect of mycotoxins on plants than on human and animal health, these compounds can have negative effects on seed germination and seedling development [63][64][65][66].

In spite of the detrimental effect of some seed-borne fungi on seed quality, many other mycoflora commonly found in seeds do not have a negative influence on seed viability. For example, *Alternaria* is almost always present as mycelium beneath the pericarp of wheat but normally cause no seed quality problems [7].

Another aspect that should be considered is the effect of fungal contamination on germination tests carried out in gene banks to periodically monitor seed viability. Germination tests are performed in temperate and humid conditions that favor the rapid growth of saprophytic fungi such as *Rhizopus*, *Penicillium,* or *Aspergillus*. The initial inoculum may be in the seeds themselves, in the substrates, or in the air. Saprophytic fungi can readily colonize dead or weakened seeds and then spread to healthy seeds. Thus, seeds that are initially viable may die or produce abnormal seedlings due to secondary infections, leading to an underestimation of the viability of the seed lot, which may result in unnecessary field regenerations of the samples. Especially in large-seeded legumes, the use of sand or similar substrates is a very effective method for reducing fungal spreading in germination tests (**Figure 1**).

Figure 1. Germination test in vermiculite substrate. Fungi growth remains localized around the dead tissues: (**a**) bean seeds with growth of *Rhizopus*; (**b**) chickpea seeds with growth of *Rhizopus*, *Aspergillus,* and *Penicillium*.

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