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Freezing as an effective method to preserve toxin-producing *Fusarium* species over an eight-year period

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Abstract

Fusarium pathogens are among the grave small-grain cereal species with a high toxicity capacity. In global agriculture, contamination of cereal grains with toxic metabolites of *Fusarium* is one of the especially crucial problems. Effective work with *Fusarium* cultures necessities their safe long-term preservation, but limited reports have appeared on maintenance. The present research assessed the preservation of *Fusarium* spp. (6 *F. solani*, 5 *F. culmorum*, 4 *F. verticillioides* and one *F. equiseti*) by freezing. Potato dextrose agar (PDA) dishes containing grown mycelia were transferred from 22 ± 1ºC directly to a mechanical freezer at -16°C and stored in May 2015. Viability was evaluated after at 6, 12, 18, 24, 30, 36, 60, 96 months of preservation, through the revival of isolates on PDA and analysis of micro- and macro-morphological characters and contamination by other microorganisms. 100% viability was observed and efficiency did not change with individual cultures, in spite of preservation conditions or time duration. More significantly, freezing did maintain *Fusarium* cultures irrespective of their pathogenic background, showing that this method preserves successfully *Fusarium* species varying in pathogenicity. Preservation at -16°C did not modify the morphological stability during storage durations and contamination by bacteria or other fungi were not observed after 8 years of storage. This reports exhibits for the first time that the procedure for storing *Fusarium* cultures by freezing at -16°C is a simple and inexpensive technique, and can be reliably utilized over an eight-year period of toxin-producing *Fusarium* species.

Keywords: Fungal contamination, morphological integrity, toxic metabolites, viability.

1.Introduction

Bread wheat (*Triticum aestivum* L.) is the second most main cereal in terms of production across the globe, after rice (*Oryza sativa* L.). *T. aestivum* planted on about 219 million hectares provides an essential source of food for 36% of the world's population; 18% of their diurnal intake of calorie and 20% of their protein [1]. Barley (*Hordeum vulgare* L.) is a relatively drought constraint-resilient crop that can be cultivated even under inappropriate conditions. *H. vulgare* is mostly utilized as animal feed, when it comes to spring barley, for producing malt for food and brewing industries. Malting barley is a relatively high-value [1]. Both *Triticum* and *Hordeum* are considered to be main sources of numerous valuable substances of biological and nutritional importance, particularly beta-glucan polysaccharides and food fiber. On the report of the Commission Regulation (EU) No 432/2012 [2], foods fabricated from wheat and barley encompassing at least 1 g of beta-glucan per quantified part supply to the preservation of normal blood cholesterol levels. A useful influence for consumers is achieved with an intake per day of 3 g of beta-glucans.

As is right for all other kind of small-grain cereals, it is crucial to take into account the possibility for contamination of *Triticum* and *Hordeum* with *Fusarium* mycotoxins. *Fusarium* pathogens are among the grave small-grain cereal species with a high toxicity capacity. Secondary metabolites of these fungal species, such as deoxynivalenol (DON), fumonisin B1 and zearalenone are among five most principal mycotoxins on a European and global scale [3]. Mycotoxins lead to various impacts on the human and animal bodies, among others they are estrogenic, mutagenic and teratogenic [4]. They also have an important effect on the economy

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because, in the line with the articles of the legal acts, the existence of mycotoxins at a definite level leads to the exception of agricultural crops, food and feed products from commercial trade [2]. DON is the most observed *Fusarium* mycotoxin in small-grain cereals. It can result in chronic and acute unfavorable impacts on the nervous system, immune system, and the gastro-intestinal tract in humans and animals [4]. Mycotoxins are chemically stable contaminants; they survive numerous processing steps and are exist in multiple end-products, like feed, beer and flour [5]. In some sub-populations, in especially young population groups, human chronic dietary exposure to DON may exceed their respective acceptable intakes per day [3].

These toxins are fabricated by several *Fusarium* pathogens and lead to the plant disease Fusarium head blight (FHB). There are three principal negative outcomes of head blight invasion in wheat and barley: loss of grain harvest, deteriorate technological quality, and contamination by *Fusarium* mycotoxins [6]. Infected kernels result in several technological problems regarding safety and quality of processed *Triticum* and *Hordeum* products. It is supposed that these toxins may act as aggressiveness factors and enhance the pathogenicity of *Fusarium* fungi in cereal crops [7]. *F. graminearum*, *F. culmorum*, *F. tricinctum*, *F. cerealis*, *F. verticillioides*, *F. langsethiae*, *F. equiseti*, *F. asiaticum*, *F. sporotrichoides*, *F. solani* and *F. poae* are the most widespread fungi invading small-grain cereals, among many other species according to global regions [8]. The level of contamination of *Triticum* and *Hordeum* kernel with *Fusarium* mycotoxins relies on several factors; among others climatic conditions, cultivation system, date and method of grain harvest, as well as the level of resistance of planted cultivars to *Fusarium* spp. invasion [9]. Because of its global presence and often noxious impacts, FHB has been analyzed in a broad range of areas [10], and interest in these pathogens persists. Researches on fungicide resistance or population genetics of *Fusarium* fungi are significant to establish better management policies. These researches are built on experiments utilizing large numbers of field FHB cultures, originating from laboratory fungal collections, which should be properly stored. While the diversity of FHB fungi is well reported [7, 10], a trustworthy storage technique of isolates is requested.

Long-term storage of fungal strains is necessary for their in-depth epidemiology and quarantine, systematic, pathogen identification, and disease control researches [11]. The techniques utilized for storage should permit high-quality, long term preservation and full revival, as well as guaranteeing viability and the physiological, morphological and genetic stability of living cells during the storage duration [12]. The classical and primary technique of culture storage is continual growth: isolates are grown on suitable medium, usually are utilized for short-term preservation. Such isolates are preserved at temperatures from 5°-20°C [13]. Frequent sub-culturing, however, may have harmful impacts as losses of virulence, pathogenicity, and/or sporulation capacity but there is no procedure to predict when or whether any of these pivotal characteristics might be lost during sub-culturing [14]. To defeat these disadvantages, many other preservation techniques in silica gel, water, and oil have been evaluated; even though findings are differed accounting on the fungus group in question [11]. Nevertheless, no single storage technique can be applied generically to all fungal species [15].

Freezing methodologies, encompassing cryopreservation either at low temperature (-20 and -70°C) or in liquid nitrogen, are largely and versatile applicable to store most fungal pathogens [16]. With lyophilization, or freeze-drying, the fungal isolates are frozen and thereafter dried under vacuum. The technique is highly successful with fungal isolates that produce mitospores. Freezing below -135°C and freeze-drying are efficient techniques for constant storage, and highly recommended [17]. Nonetheless, cryopreservation in liquid nitrogen and lyophilization necessity complex and expensive equipment; liquid nitrogen must be replenished orderly, and the mechanical deep freeze utilizes electricity [11]. In good health, sporulating isolates also can be sealed tightly and preserved in a freezer at -20°C or maintained in a deep freezer at -70°C to reinforce revival and augment the interval between needed transfers [18]. However, freezing temperatures between -20 and -60°C are very challenging because they repeatedly lead to cryoinjuries. Possible freezing at below -20°C in standard and mechanical freezers has been little analyzed for fungal isolates and could bring novel options of lower cost cryopreservation. One study Sakr [19] has demonstrated that *Fusarium* species causing head blight were maintained by

freezing at -16°C successfully for up to 60 months, showing the integral maintenance of viability, purity and morphological characteristics. In addition, to the best of our knowledge and based on data accessible in the scientific literature, there have been no reports to date regarding freezing preservation impact on viability of toxin-producing *Fusarium* species. Here we extend this research to explore viability, morphological stability and purity for the same isolates (previously evaluated for five years period) after being stored for 96 months by freezing.

2. Materials and Methods

2.1. Fungal isolates

All *Fusarium* isolates, including six *F. solani*, five *F. culmorum*, four *F. verticillioides* (synonym *F. moniliforme*) isolates and one *F. equiseti* isolate, were isolated from naturally infected wheat grain. The 16 isolates were monosporic derived cultures of the original field isolates and were selected for their contrasting pathogenicity based on previous several experimental observations [20]. On Petri dishes with potato dextrose agar (PDA) with 13 mg/l kanamycin sulphate added after autoclaving, the isolates were morphologically identified with the aid of the Leslie and Summerell [21] manual on the basis of microscopic studies of the shape and size of macro- and micro-conidia, and were molecularly distinguished by Random amplification of polymorphic DNA markers [20]. *Fusarium* monosporic-derived cultures were grown separately in Petri dishes in an incubator (JSPC, JS Research Inc., year of appliances: 2004) for 10 days at 22 ± 1ºC in the dark to allow mycelial growth and sporulation for analyzing freezing preservation methodology. The primary morphological characterization was conducted in the "day zero" and this was utilized as a standard for evaluating the modifications in viability, purity, micro-morphology, and macro-morphology over 8 years of the experiment. While the four tested *Fusarium* species, i.e., *F. solani*, *F. culmorum*, *F. verticillioides* and *F. equiseti*, are considered of FHB pathogens [19], and great pathogenic variability was observed in the tested FHB isolates [19]; it can be concluded that these tested pathogens produce toxins in the cereal grains since the toxins may act as aggressiveness factors and enhance the pathogenicity of *Fusarium* fungi in cereal crops and lead to FHB disease [7, 10]. We hypothesized that these pathogens are toxin-producing species in spite of the toxin analyses were not conducted on these fungi.

2.2. Preservation by freezing

All *Fusarium* monosporic-derived isolates were examined for purity and were consequently sub-cultured into dishes carrying PDA. The samples of PDA-Petri dishes including fungal cultures reaching appropriate growth for 16 *Fusarium* isolates were transferred from 22 ± 1ºC directly to standard and mechanical freezer at -16°C without module controls the freezing rate (Figure 1) and preserved in May 2015. Only pure isolates that were characterized by contemporary taxonomic criteria and methodologies [21] (Leslie and Summerell 2006) were involved in the present research. After storage of 96 months, stored and frozen Petri dishes were thawed at 4°C for 24 hours. Five agar plugs (5 mm diameter) of the colony were cut of for each culture, then putted onto the surface of fresh Petri dishes with PDA and incubated in conditions mentioned to allow mycelial growth for viability analysis. The success of freezing preservation system was evaluated by observation the growth of the FHB fungi on freshly prepared PDA medium. After 6, 12, 18, 24, 30, 36, 60, 90 and 96 months of preservation, fungal isolate' viability, morphological characters, and purity were assessed after 96 months of preservation.

Figure 1. Stocks and Preserved cultures of Fusarium in a standard and mechanical freezer at -16°C without module controls the freezing rate

Isolates showing revival of at least one out of five agar plugs were considered viable. In addition, the viability of each *Fusarium* isolates was successful if the rate of present growth was the same as that of the original culture and if the color of the colony and morphology matched the fungal characterization reported for each isolate. The culture was found to be nonviable if no growth occurred. For this storage technique, viable cultures were checked for morphological criteria (agreement of colony characters with the earlier known examination) and contamination by bacteria or other fungi.

3. Results and Discussion

Several *Fusarium* pathogens fabricate mycotoxins [3], secondary metabolites that can result in harmful health impacts in humans and animals upon consumption [4]. Nevertheless, limited information is accessible on the toxicity, existence, and contamination levels of these toxic metabolites in *Triticum* and *Hordeum* kernels [5]. Thus, the storage of *Fusarium* isolates is requested as reference stocks for continuous research that necessity that the preseved cultures remain viable for long time durations without any morphological or physiological modifcations. As a results of the diversity in the presence of *Fusarium* species [9] and a wide diversity in mycotoxin spectra and concentrations [6], *Fusarium* species may vary from each other in terms of tolerance to storage methodologies. The methods, in turn, vary fundamentally in relation to the vital or physiological state that preserve *Fusarium* cells, the duration of storage that they permit, the type of equipment and labor specialization needed for their realization [13]. More economical plant pathogen storage methodologies should be analyzing because of expensive procedures of cryopreservation and lyophilization techniques which are not required accessible in all mycology laboratories [16, 17] (Milosevic et al. 2007; Homolka 2013). In this report, a storage methods namely freezing preservation was used to detect the suitability of the maintenance methods for four *Fusarium* pathogens, i.e., *F. solani*, *F. culmorum*, *F. verticillioides* and *F. equiseti*, for 96 months. Indeed, Sakr [19] (2020) examined the survival of the same isolates by freezing used in the current research for a period of limited duration of five years.

A decision key for fungal pathogens that assist in selecting the best storage technique was reported

three decades ago by Ryan *et al.* [18]. An analysis of this guide connected with *Fusarium* species exhibits that while these fungal pathogens produce asexual structures and do not have motile spores in medium culture [22], then preservation by freezing is recommended. Storage of fragments of hyphae, asexual structures, and spores for *Fusarium* species, preserve fungal characters that are identical to the parent isolate. A methodology that allows for removal of material over time (such as storage by freezing permits), while storage isolates in the main collection is highly advantageous [18], and especially in culture collections of little funding and resources.

The best storage technique is considered to be the one where no reproduction and growth can occur, but where all the functional and structural features are retained [11]. It is known that frequent sub-culturing can result in alterations in some of the characters of fungi, such as a reduction on the capacity to sporulate [15], and some fungal pathogens have their sporulation capacity reduced when preserved by freezing, when compared to maintenance in medium at 4°C [23]. For the assessed storage method herein, more stable and consistent growth was achieved by the tested preservation method, i.e., freezing at -16°C. The main factor for a successful revival of fungal cultures was the capacity to survive the storage process. Over 96 months, 16 *Fusarium* isolates were examined in six months intervals for freezing methodology. There was no loss of recovery or vitality proportion (100%) for freezing storage method for 16 *Fusarium* isolates for the tested period (Table 1). 100% viability was reported and effeteness did not change with individual cultures, irrespective of time period or storage conditions (Table 1). Indeed, Sakr [19] found that the viability of the same fungal isolates was 100% after a period of limited storage duration of five years. More importantly, freezing at -16°C did stote *Fusarium* cultures regardless of their pathogenic background, showing that this technique store efficiently *Fusarium* pathogens differing in pathogenicity. Abd-Elsalam *et al.* [11] observed that storage by freezing incorporating fungal mycelium and spores had high revival rates.

Thought some reports exhibited that freezing can result in a reduction in spore survival, either by structural harm in spores, because of the formation of ice crystals on the lyophilization process prior to freezing [15], or in the thawing process [24], all tested *Fusarium* isolates seemed to resistant this kind of storage.

Table 1. Viability of 16 fungal isolates for four *Fusarium* head blight preserved in sterile distilled water by freezing at -16°C

Fungal isolates (identification)	Assessment period (months)									
	6	12	18	24	30	36	60	60	90	96
F1 (*F. culmorum*)	+	+	+	+	+	+	+	+	+	+
F2 (*F. culmorum*)	+	+	+	+	+	+	+	+	+	+
F3 (*F. culmorum*)	+	+	+	+	+	+	+	+	+	+
F28 (*F. culmorum*)	+	+	+	+	+	+	+	+	+	+
F30 (*F. culmorum*)	+	+	+	+	+	+	+	+	+	+
F7 (*F. solani*)	+	+	+	+	+	+	+	+	+	+
F31 (*F. solani*)	+	+	+	+	+	+	+	+	+	+
F35 (*F. solani*)	+	+	+	+	+	+	+	+	+	+
F20 (*F. solani*)	+	+	+	+	+	+	+	+	+	+
F26 (*F. solani*)	+	+	+	+	+	+	+	+	+	+
F29 (*F. solani*)	+	+	+	+	+	+	+	+	+	+
F15 (*F. verticillioides*)	+	+	+	+	+	+	+	+	+	+
F16 (*F. verticillioides*)	+	+	+	+	+	+	+	+	+	+
F21 (*F. verticillioides*)	+	+	+	+	+	+	+	+	+	+
F27 (*F. verticillioides*)	+	+	+	+	+	+	+	+	+	+
F43 (*F. equiesti*)	+	+	+	+	+	+	+	+	+	+

Viability response of 16 fungal isolates over 6, 12, 18, 24, 30, 36, and 60 months was analyzed previously and cited by Sakr (2020), however, the viability response in the current study, was reanalyzed of 16 fungal isolates at the following different time points of storage (6, 12, 18, 24, 30, 36, and 60 months). +: Presence of colony growth.

Preservation at -16°C did not modify the morphological stability during maintenance durations and contamination by bacteria or other fungi was not observed after 8 years of storage (Figure 2). In harmony with our data, Legard and Chandler [25] reported no obvious morphological alterations in strawberry pathogenic fungi, i.e., *Colletotrichum gloeosporioides*, *C. obscurans*, *C. acutatum*, and *Phomopsis fragariae* of strawberry (*Fragaria×ananassa* Duchesne), preserved by freezing at −95°C. Indeed, Sakr [19] reported that morphological modification and microbial contamination were not determined for the same fungal isolates. Choosing of vigorous and good sporulating cultures stored by freezing at -16°C was the most significant factor influencing survival of *Fusarium* pathogens by freezing during our investigation. Our data seem to reinforce Bunse and Steigleder's hypothesis [17], while we found the highest re-growth assessments for frozen *F. culmorum*, *F. verticillioides*, *F. solani*, and *F. equiseti* culture which produce abundant spores. This assumption can clarify the better findings when freezing agar was used as growth medium, because freezing agar is known to reinforce the production of fungal spores [17]. In this study, the samples of PDA Petri dishes containing fungal cultures achieving suitable growth can easily be preserved and transported (Figure 1). Neither employed preservation methods, duration in preservation nor taxonomic identification was linked with a loss of viability, microbial contamination or morphology modification in the present investigation.

The non-biotic constraint that cells are exposed to through freezing and thawing are well-documented [11]. When freezing is started in a dilute aqueous solution only an amount of the water undergoes transition to ice and the gases and solutes in the remaining aqueous solution become more concentrated. Cells in suspension are exposed to hypertonic solutions through freezing and the cellular viability and morphology are detected by the rate of cooling. Based on no effect of our analyzed conditions of freezing on culture morphological and revival features of tested *Fusarium* isolates, we can debate that uncontrolled and rapid condition freezing may be conducted for the preservation of tested 16 fungal cultures of *Fusarium* genus. Pathogens' storage is established on the reversible transition between biosis (an active vital situation) and anabiosis (an inactive state) or hypobiosis (low activity situation). The low-temperature freezing methodology stores the cell in an anabiosis status, while techniques such as refrigerated sub-culturing, refrigeration and stock in sterile or saline water (4–10 °C) tend to save the cell

in a situation of hypobiosis [11]. Our results agree with storage data observed by Legard and Chandler [25] for strawberry pathogenic fungi and Sakr [19] for several *Fusarium* species.

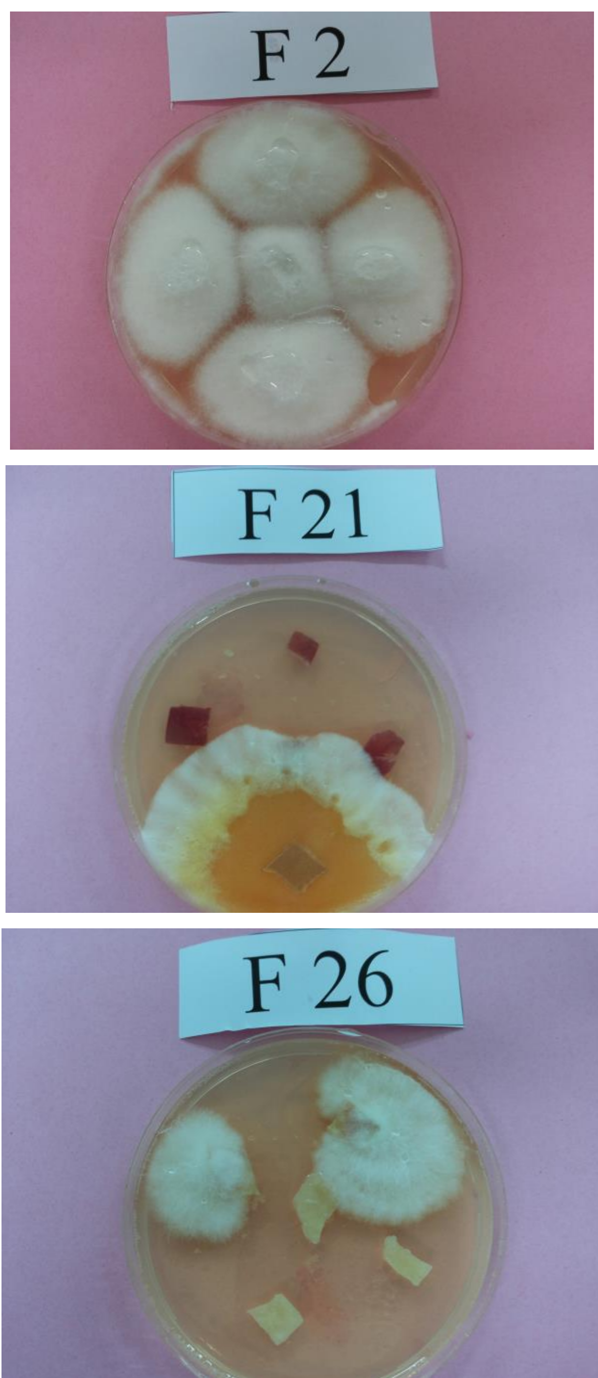

Figure 2. *Fusarium* cultures of three isolates, F2 (F. culmorum), F21 (F. verticillioides) and F26 (F. solani) causing Fusarium head blight, on Petri dish with potato-dextrose agar obtained from cultures preserved by freezing at -16°C for 96 months

In spite of the majority of metabolic activity is suspended below –70 °C, ice re-crystallization and other biophysical processes that can influence cell viability may still be active at temperatures above –130 °C [26]. The alterations in morphological characters of hyphae over freezing and thawing processes and varying revival following thawing necessity the use of several freezing and thawing modules [11, 26]. This requests species-specific data to be highlighted. The revival of the isolates preserved and thawed without freezing and thawing modules was excellent in the present study. Our data are in line with findings found by Legard and Chandler [25] for *Colletotrichum gloeosporioides*, *C. obscurans*, *C. acutatum*, and *Phomopsis fragariae* of strawberry. Regarding storage by freezing, genetic harm in fungal cultures could be resulted from the multiple freeze-thaw cycles of frozen isolates [25]. In our study, the frozen vigorously growing mycelial cultures on Petri-dishes PDA were used once due to the multiple freeze-thaw cycles of frozen fungal cultures could have caused genetic damage [25]. Survival of *Cochliobolus sativus* isolates causing spot blotch on cereals was not best stored for 2 yeas by utilizing frozen conidia at -20°C [27]. Larger fungal spores (such those for *Fusarium* employed in this report) tend to collapse during freezing, and the injury is not reversible by hydrate process. In addition, the formation of ice crystals physically harmed and killed important number spores of identical size [28]. Ishikawa *et al.* [29] found that deforming of membranes, resulting in harm, develops not only by osmotic shrinkage of cell tissues due to freezing-induced hydrate process, but also by physical malformation of cell tissues due to growth of extracellular ice. Due to these potential difficulties, freezing processes were regulated at -16 °C through our study.

4. Conclusion

The presented findings can be accepted as encouraging for the storage of 16 *Fusarium* isolates in an acceptable condition without any morphological modification by freezing methods for long durations. This research exhibits for the first time that the procedure for preservation *Fusarium* cultures by freezing at -16°C is an inexpensive and simple technique, and can be reliably employed over an 8-year period of toxin-producing *Fusarium* species.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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