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Suitable methods for storage of *Fusarium* head blight, spot blotch and common root rot pathogens for extended periods

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Abstract

Fusarium head blight (FHB), spot blotch (SB) and common root rot (CRR) are the economically ones of the most important fungal disease of wheat, barley, and many other plants of Poaceae family. Efficient and suitable methods for storage of the pathogens can help in achieving the target of sustainable management of these diseases in a relatively short period of time. In this study, we aimed to test suitable methods for storage of *Fusarium* spp. and *Cochliobolus sativus* isolates differing in their phenotypical characters collected from the FHB, SB and CRR-infected wheat and barley fields. Our preservation procedure included prolongation of storage duration of two existing protocols, sterile distilled water and freezing, that were available for 16 FHB isolates of four species, 32 SB isolates and 22 CRR isolates. Two storage treatments started in May 2015 and included fungal suspension (spores and hyphae) in sterile distilled water at 4°C and fungal cultures on Petri-dishes after reaching suitable growth by freezing at -16°C were tested in periods ranging up to 9 years. The survival, morphological changes and contamination by other microorganisms were then assessed: phenotypic features were analyzed qualitatively at 6, 12, 18, 24, 30, 36, 60, 96, and 108 months. All these experimental procedures were conducted under aseptic conditions. Fungal isolates were recovered from the two storage treatments having a full viability and maintaining their morphological stability and purity at each time point. Neither employed preservation technique, storage period nor taxonomic identification was linked with a decrease of survival, microbial contamination or morphology changes. Our observations propose that Castellani's technique and freezing are easy and inexpensive methods for long-term preservation of *F. culmorum*, *F. verticillioides*, *F. solani*, *F. equiseti* and *Cochliobolus sativus*, and can be principally used in the creation of stock collections of restricted resources. The present research is the first work showing the possibility of storage for FHB, SB and CRR causal agents utilizing fungal suspension at 4°C in cold water and mycelial cultures by freezing for 9 years.

Keywords: freezing, microbial contamination, morphological stability, viability, water storage

1. Introduction

The prolonged outstanding issue of "living reproduction parts" patterns for fungi will be fixed when it is found that the life, features and properties of authentic cultures can be maintained [1, 2]. Fungal pathogens are highly changeable eukaryotic organisms with a slow development velocity in culture media relative to most of the bacteria. In addition, colonial storage has been probably damaged with the contamination by other saprophytic fungi or bacteria [3, 4].

The storage of disease causal agents in viable, stable, and pure cultures is of main importance in plant pathology issues for genetic analysis, taxonomy for identification/comparison, identification of plant resistance against pathogens and fungicides, and breeding programs [5]. To preserve culture collections, trustworthy long-term maintaining and storage techniques are needed. No single preservation technique has been widely employed to all fungi due to the particular character of each species [6].

The selection of a particular storage treatment for fungi not only relates on the success of the application but also the anticipated employment of the fungal pathogen, time, facilities and preservation costs [7].

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In principal, preserved culture should remain viable for prolonged duration by decreasing the metabolism until it reaches a phase of artificial dormancy [4]. This process inhibits the accumulation of mutations resulting in morphological and biochemical modifications [2]. The repeated sub-culturing technique, in which cultures are grown on nutrition medium at constant intervals, is efficient for short-term storage [1]. Nevertheless, this application is not optimal for storing large number of fungal cultures over time due to accumulation of spontaneous mutations during the continual subculture, risk of contamination, culture deterioration and loss. In addition, it necessities continuous attention and special equipment [5]. To defeat these problems, several long term techniques have been analyzed, even though data are varied [5, 7, 8].

The preferable preservation media for the maintaining for several years of numerous fungal isolates are accepted as cryopreservation in liquid nitrogen or in a mechanical deep freezer or lyophilization [9, 10]. However, these treatments necessity expensive and complex equipment; the mechanical deep freeze utilizes electricity, and liquid nitrogen must be replenished regularly. Thus, rapid, simple, inexpensive storage treatment has been applied successfully for diverse fungal pathogens as preservation in sterile distilled water, mineral oil, sand, silica gel, and by freezing [11, 12, 13, 14].

Storage of fungi under sterile distilled water, a technique reported by Castellani seventy years ago, has been employed to successfully preserve several diverse fungal species for many years [11, 15, 16, 17]. This rapid, easy, and economically treatment retained both survival and morphological, physiological and genetic integrity of the particular species included. Castellani's procedure should be supplemented by a second preservation application as a back-up to maintain survived fungal isolates for extended period of time [5, 11]. Maintaining fungal isolates by freezing in standard freezers (working at -20°C and easily obtainable in almost mycology laboratories) seems to be a back-up storage treatment. Morever, this economical tool has been yielded satisfactory findings in maintaining viability of frozen cultures for many years [18].

Fusarium head blight (FHB) associated with more 17 several pathogens of the *Fusarium* genus is a noxious disease of wheat, barley and other plants of Poaceae family in many fields globally. Spot blotch (SB) and common root rot (CRR) caused by the fungus *Cochliobolus sativus* are common barley and wheat diseases. FHB, SB and CRR are generally of the most significant diseases of cereals worldwide [19]. Reliable and accurate morphological and molecular identification of the causal agents is a pre-required for monitoring and management of fungal plant diseases. An efficient preservation method for fungal pathogens can speed up their pathological and molecular distinguishing and additional research on the biology, epidemiology and ecology of pathogens [5]. There is a rarity of observations about the preservation of causal agents of FHB and SB diseases. For instant, some *Fusarium* pathogens were stored in constant conditions up to 10 years when maintained in lyophilization, soil, and silica gel [9, 20, 21]. In silica gel and sand preservation techniques, survival and virulence were achieved for three SB isolates for 2 years, with an observed mutation risk [12]. Recently, Sakr [22, 23] and Sakr *et al.* [24] reported that fungal cultures of FHB, SB and CRR diseases can retain survival by two storage techniques (sterile distilled water and freezing) for 96 months. The present work investigates the revival, purity and morphological integrity for 70 fungal cultures of five main cereal phytopathogenic species (*F. culmorum*, *F. verticillioides*, *F. solani*, *F. equiseti* and *C. sativus*) after being preserved for nine years in two simple, safe, and easy techniques: sterile distilled cold water at 4°C and freezing at -16°C.

2. Materials and Methods

2. 1. Fungal isolates

Seventy fungal cultures sampled from naturally infected wheat and barley plants in several regions of Syria in 2015 growing season of five major cereal phytopathogenic species were invoved. Of these cultures, 16 were of four Fusarium head blight pathogens (*F. culmorum*, *F. verticillioides*, *F. solani*, and *F. equiseti*), 32 were of *Cochliobolus sativus* causing spot blotch and 22 were of *C. sativus* showing common root rot symptoms. Tables 1 and 2 present identification for 70 isolates employed in this current report. At 22 ± 1ºC for 10 days under continuous darkness, fungal isolates were incubated separately in 9 cm Petri-dishes including potato dextrose agar (PDA, HiMedia, HiMedia Laboratories) in an incubator (JSPC, JS Research Inc., year of appliances: 2004) to allow sporulation and mycelial growth. The initial

morphological identification was carried out in the "day zero" and this was employed as a standard for assessing the alternations in survival, purity, micro-morphology, and macro-morphology over 9 years of the experiment. All these experimental procedures were conducted under aseptic conditions.

2. 2. Storage in sterile distilled water

For 70 *Fusarium* spp. and *C. sativus* cultures, the fungal suspension containing spores, hyphae and fragments of mycelium was prepared by gently agitating 10 ml of sterile distilled water (autoclaved at 121 °C for 15 minutes) on the surface of Petri-dishes carrying fungal cultures reaching appropriate growth. Under aseptic conditions, the inoculation of sterile glass ampoules, hermetically closed and sealed with 2 cm Parafilm strips (Pechiny, Thomas Scientific) to inhbit water evaporation, including fungal suspension was carried out. Then, sterile glass ampoules were stored in a standard refrigerator at 4°C in May 2015 (Figure 1, A). The success of water storage tretement was evaluated by observation of the growth of the FHB and *C. sativus* pathogens on freshly prepared PDA medium. After 6, 12, 18, 24, 30, 36, 60, 90, 96 and 108 months of storage, fungal isolate' revival, morphological features, and purity were assessed.

Following storage for 108 months, 100 µL of fungal suspension were seeded in Petri-dishes with PDA and incubated in the above-mentioned conditions to allow sporulation and mycelial growth. Viable cultures were assessed for (1) morphological monitoring (approval of existence/none-existence of chlamydospores, the size and shape of the macroconidia, and supporting structures of the microconidia and macroconidia with the earlier known distinguishing), and (2) contamination by other fungi or bacteria that harm *Fusarium* and *Cochliobolus* cultures. Fungal cultures were recorded as viable if they grew.

2. 3. Storage by freezing

All *Fusarium* and *Cochliobolus* cultures were tested for purity and were then sub-cultured into PDA-dishes. The samples of PDA-Petri dishes involving fungal cultures reaching suitable growth for 16 FHB isolates of four species, 32 SB isolates and 22 CRR isolates were transferred from 22 ± 1ºC directly to standard and mechanical freezer at -16°C without module that monitor the freezing rate (Figure 1, B) and stored in May 2015. Only pure cultures that were identified by contemporary taxonomic methodologies and criteria were included in the present work.

After maintaining for 108 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, and then placed onto the surface of PDA Petri-dishes and incubated in conditions mentioned to allow sporulation and mycelial growth for revival test. The success of freezing storage system was assessed by observation the growth of the FHB and *Cochliobolus sativus* fungi on freshly prepared PDA medium. After 6, 12, 18, 24, 30, 36, 60, 90, 96 and 108 months of storage, fungal isolate' revival, morphological features, and purity were evalauted after 108 months of storage. Isolates showing viability of at least one out of five agar plugs were shown to be viable.

2. 4. Viability analysis

Survival was measured by inoculating each stored fungal culture onto PDA followed by incubation under darkness at 22±1ºC for 10 days at the several time points of preservation for 9 years. A mycelium colony established from each FHB and *Cochliobolus sativus* culture referred that the culture was viable. In non-viability, no fungal growth was found.

Figure 1. Stocks and Preserved cultures of *Fusarium* spp. and *Cochliobolus sativus* in (A) a standard refrigerator at 4°C, and in (B) a standard mechanical freezer at -16°C

3. Results and Discussion

Survived fungal isolates deposited in culture collection for extended durations can be employed for agricultural, taxonomic, and genetic goals. The obtainable techniques for the mycology collection preservation are costly, work-demanding and ineffective most of the times. The development of new forms of fungal storage for prolonged periods should be requested, principally in small labs to meet individual limitations and needs [3, 4, 5].

In spite of the two techniques, lyophilization and cryopreservation, have been largely employed for extended term storage, however, they are not necessarily available in all mycological facilities [11]. Taken into account, survival of two preservation techniques (water at 4 °C and freezing at -16 °C) for nine years were assessed for causal agents of major economic crucial diseases on wheat and barley. In this investigation, neither employed preservation technique, storage period nor taxonomic identification was linked with a decrease of survival, microbial contamination or morphology changes.

A decision-based key for selecting best preservation techniques suited for fungal isolates has been described by Ryan *et al.* [6]. A decision-based key has been innovated, which utilized questions linked

to fungal features and user economics and facilities to detect the most suitable technique for long-term storage of isolates. This key should simplify the decisions of microbiologists when considering storage of important fungal isolates [6]. An analysis of this key linked to the five cereal fungal pathogens included in the present work (*Fusarium culmorum*, *F. verticillioides*, *F. solani*, *F. equiseti* and *Cochliobolus sativus*) demonstrates the possibility in detecting which storage treatment to employ for these pathogens. The tested species do not have motile spores and easily produce asexual spores in culture medium [19], consequently, preservation in water and by freezing is recommended as effectively, economically, and suitable storage treatment, among the other available techniques [6]. Storage of asexual structures, fractions of hyphae, and spores for *Fusarium* spp. and *C. sativus* species, preserve fungal characters that are identical to the parent isolate as previously observed [22, 23, 24].

Revival of fungal cells has been reported as the most essential physiological state in mycological researches [5, 6]. In the current investigation, each fungal culture was considered viable if the fungal isolates did grow in the absence of microbial contamination at the same as that of the authentic isolate and if the morphological features of the colony corresponded the fungal identification certified for each culture. Our aseptic experiment showed that 70 nine-year old water-stored at 4°C and frozen cultures at -16°C for 16 FHB, SB and CRR diseases did grow when sub-cultured on PDA and incubated under darkness at 22°C for 10 days (Tables 1 and 2). There was no reuction of recovery or vitality ratio for two preservation techniques for 70 *Fusarium* spp. and *C. sativus* isolates for the tested period, i.e., 108 months (Tables 1 and 2). For each culture, no differences were detected among storage methods and preservation times (Tables 1 and 2). The conservation of mycelium for 70 fungal isolates in the two analyzed trials (fungal suspension at 4°C in cold water and mycelial cultures by freezing) assured a full revival for the analyzed periods at each time point. Our data are in accordance with these reported by Abd-Elsalam *et al.* [5] in which they found that prolonged term water and freezing preservation techniques encompassing mycelia submerged cultures, factions of mycelium hyphae and spores of fungi had high viability proportion. Richter [25] observes that saprotrophic fungi distinguished with sufficient suspension of spores and mycelia and good sporulating cultures had a higher survival in sterile cold water. High revival assessments were reported for plant pathogen isolates preserved in cold water and by freezing [16, 17, 18, 26].

Table 1. Viability of 16 *Fusarium* head blight isolates, 32 isolates for *Cochliobolus sativus* causing spot blotch and 22 isolates for *C. sativus* causing common root rot preserved in sterile distilled water at 4°C

Fungal isolates (identification)	**Assessment period (months)**								
	6	**12**	**18**	**24**	**30**	**36**	**60**	**96**	**108**
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*)	+	+	+	+	+	+	+	+	+
C.S. 14 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 27 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 32 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 92 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 20 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 2 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 80 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 7 (spot blotch)	+	+	+	+	+	+	+	+	+

C.S. 18 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 30 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 93 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 16 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 87 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 83 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 45 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 11 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 9 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 15 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 26 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 59 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 17 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 34 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 21 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 89 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 53 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 86 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 74 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 49 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 9 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 12 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 63 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 55 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 41 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 50 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 37 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 36 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 24 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 23 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 44 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 48 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 52 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 13 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 6 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 38 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 25 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 46 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 47 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 51 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 8 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 40 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 1 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 10 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 5 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 28 (common root rot)	+	+	+	+	+	+	+	+	+

Survival reaction of 16 fungal isolates for 6, 12, 18, 24, 30, 36, 60 and 96 months was tested previously and reported by Sakr [22, 23] and Sakr *et al.* [24], however, the survival reaction in the present work was reanalyzed of 70 fungal isolates at the following different time points of storage (6, 12, 18, 24, 30, 36, 60 and 96 months).
+: Presence of colony growth.

Table 2. Viability of 16 *Fusarium* head blight isolates, 32 isolates for *Cochliobolus sativus* causing spot blotch and 22 isolates for *C. sativus* causing common root rot preserved by freezing at -16°C

Fungal isolates (identification)	**Assessment period (months)**								
	6	**12**	**18**	**24**	**30**	**36**	**60**	**96**	**108**
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*)	+	+	+	+	+	+	+	+	+
C.S. 14 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 27 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 32 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 92 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 20 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 2 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 80 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 7 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 18 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 30 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 93 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 16 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 87 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 83 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 45 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 11 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 9 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 15 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 26 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 59 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 17 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 34 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 21 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 89 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 53 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 86 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 74 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 49 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 9 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 12 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 63 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 55 (spot blotch)	+	+	+	+	+	+	+	+	+
C.S. 41 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 50 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 37 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 36 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 24 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 23 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 44 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 48 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 52 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 13 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 6 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 38 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 25 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 46 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 47 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 51 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 8 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 40 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 1 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 10 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 5 (common root rot)	+	+	+	+	+	+	+	+	+
C.S. 28 (common root rot)	+	+	+	+	+	+	+	+	+

Survival reaction of 16 fungal isolates for 6, 12, 18, 24, 30, 36, 60 and 96 months was tested previously and reported by Sakr [22, 23] and Sakr *et al.* [24], however, the survival reaction in the present work was reanalyzed of 70 fungal isolates at the following different time points of storage (6, 12, 18, 24, 30, 36, 60 and 96 months).

+: Presence of colony growth.

The obtained data showed that none of the two preservation techniques involved morphological modifications, either microscopic or macroscopic, in the several analyzed isolates of four *Fusarium* species and *C. sativus*. The viable cultures preserved their morphological characters corresponded to the original description (Figures 2 and 3). In addition, morphological colony features of *Gaeumannomyces graminis* var. *graminis* was not affected by water storage [16]. By freezing at −95°C, no clear morphological changes in strawberry pathogenic fungi maintained were observed [18].

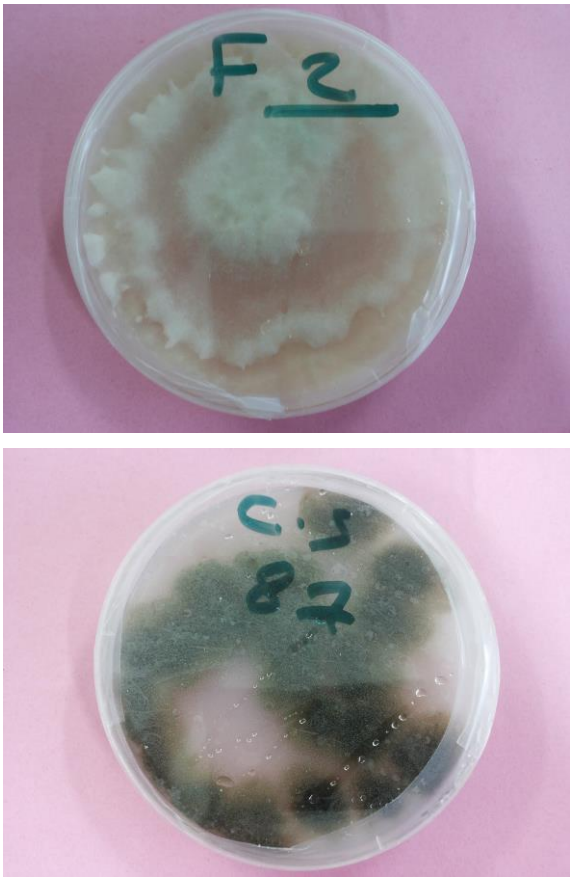

Figure 2. Survived *Fusarium* and *Cochliobolus* cultures of two isolates: F2 (F. culmorum) and C. S. 1 (common root rot) onto freshly prepared potato dextrose agar culture recovered from cultures preserved for 108 months in sterile distilled water at 4°C

Figure 3. Survived *Fusarium* and *Cochliobolus* cultures of two isolates: F21 *(F. verticillioides*) and C. S. 50 (common root rot) onto freshly prepared potato dextrose agar culture recovered from cultures preserved for 108 months by freezing at -16°C

Plant pathogens vary from each other in terms of resistance to storage conditions and methods. Storage conditions, i.e., temperature range influences on the revival of maintained fungi. At room temperature, Ko [27] exhibited that isolates of *Phytophthora palmivora*, *P. cinnamomi* and *P. parasitica* were found to be viable in water storage for 6 to 23 years. Some species of *Phytophthora* including *P. colocasia* and *P. infestans* which were stored at 5°C survived only 2-6 months in water while isolates of *P. parasitica* survived for one year [28]. Similar notifications was highlighted for *Gaeumannomyces graminis* in which room temperature was suitable for fungal viability and cooling was harmful in the recovery of *G. graminis* [16]. On the other hand, higher radial growth scores for revived *Botryosphaeria* cultures were clear in applications at the lower temperatures of 4ºC as compared to room temperature (approximately 20ºC) [29].

Our earlier reports [23, 24] confirmed that water preservation involved fungal suspension for all the tested FHB and *Cochliobolus sativus* isolates at room temperature was efficient for 100% viability of for 3 years, suggesting that *F. culmorum*, *F. verticillioides*, *F. solani*, *F. equiseti* and *Cochliobolus sativus* can actively persist on both: lower temperature or ambient temperature. Also, Naseri *et al.* [30] observed that temperature under controlled-environment conditions significantly influenced germination of, and hyphal growth from, ascospores of *Leptosphaeria maculans*, the causative agent of blackleg (phoma stem canker).

The pressures that cells are exposed to during freezing and thawing are well-reported [4]. When freezing is initiated in a dilute aqueous solution only a proportion of the water undergoes transition to ice and the gases and solutes in the residual aqueous solution become more concentrated [2, 10]. Cells in suspension are exposed to hypertonic solutions during freezing and the cellular morphology and survival are detected by the rate of cooling [2, 10]. Based on no effect of our tested conditions of freezing on culture survival and morphological characters of tested isolates, we can conclude that uncontrolled and rapid freezing conditions may be used for the storage of tested 70 fungal isolates of five pathogenic species. Pathogens' conservation is built on the reversible transition between an active vital state (biosis) and low activity state (hypobiosis) or an inactive (anabiosis). The low-temperature freezing method keeps the cell in an anabiosis state, while techniques such as stock in sterile or saline water, refrigerated sub-culturing, and refrigeration (4–10 °C) tend to keep the cell in a state of hypobiosis [5]. Our findings are in paralleled with findings reported by Legard and Chandler [18]. Regarding storage by freezing, genetic harm in fungal cultres could be resulted from the multiple freeze-thaw cycles of frozen cultures [18]. So, in this current study, frozen PDA Petri-dishes were utilized once as reported by Sakr [22] and Sakr *et al.* [24]. For frozen conidia of spot blotch cultures preserved at -20°C, Arabi *et al.* [12] found that isolate revival was not best preserved for 24 months. Great fungal spores (such those for *Fusarium spp.* and *C. sativus* employed in the current research) could be physically harmed and killed by freezing at -20°C, thus freezing treatments were regulated at -16°C in a freezer available in our laboratory [22, 24].

4. Conclusions

The highlighted findings can be accepted as encouraging for the storage of 70 isolates of five cereal pathogens in a suitable condition without any morphological modification by water and freezing methods for nine years. Our data propose that while storage in sterile distilled cold water at 4°C remains an inexpensive and easy technique for long-term maintaining of all analyzed fungal cultures, this procedure should be supplemented by a second storage technique as freezing at -16°C available in our facilities.

Compliance with Ethics Requirements. Author declares that he respects the journal's ethics requirements. Author declares that that he has no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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