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CONSERVATION OF CEREAL FUNGI FOLLOWING DIFFERENT METHODS OF PRESERVATION FOR LONG TERMS

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ABSTRACT

Preservation of soil- and air-borne phytopathogenic fungi for extent durations of time is crucial so that analyses can be followed up any moment. The goal of the present research was to analyze storage methodologies in maintaining field isolates of Fusarium head blight (FHB), spot blotch (SB) and common root rot (CRR) diseases. Cereal fungal isolates were stored at four independent storage experiments (1) filter paper disks carrying fungal mycelium at 4°C, (2) fungal suspension at ambient temperature, (3) fungal suspension at 4°C and (4) mycelial cultures on Petri-dishes with potato dextrose agar by freezing at -16°C. The survival, absence of microbial contamination and morphological constancy of cultures were tested in periods ranging from 1 to 60 months. The isolates were recovered from the four preservation treatments having +% survival and maintaining their purity and morphological stability at each time point. Neither used storage technique, time in storage nor taxonomic classification was associated with a lack of viability, microbial contamination or morphology alternation. As far as we know, the present work is the first report highlighting the possibility of conservation for FHB, SB and CRR causal agents using filter paper disks stored in cold water for three years, fungal suspension at ambient temperature for 3 years, fungal suspension at 4°C in cold water and mycelial cultures by freezing for 5 years.

Keywords: cereal fungi, freezing, viability, water storage.

INTRODUCTION

The storage and keeping of phytopathogenic fungi in a viable yet constant situation for extent periods has always been crucial. Strains of these fungal pathogens can assist as standard for distinguishing of quarantine taxonomies. Such isolates are also essential for assessing disease resistance and for plant breeding strategies (Abd-Elsalam *et al.*, 2010). Continual sub-culture is the most common method of fungal isolate preservation in isolate collections but is not suitable for prolonged preservation because it is taking a lot time and vulnerable to contamination (Holden and Smith, 1992). Over the years, several methods for the storage of fungal cultures have been

assessed in order to reduce these disadvantages, even though results depend on the fungus group in question. Methods to date are based on lyophilization, cryopreservation, sterile water, silica gel, sand, and freezing (reviewed in Abd-Elsalam *et al.*, 2010). No singular method has been carried out effectively to all fungi due to the particular characteristics of each species.

Water storage method, described seventy years ago by Castellani, in addition to being simple and economically practical for small laboratories yields acceptable results for the keeping of most species of fungal pathogens. The temperature applied in water preservation method is varying, from 4°C to ambient temperature (McGinis *et al.*, 1974; Dioigo *et al.*, 2005; Eliot, 2005; Bormaan *et al.*, 2006; Bueno *et al.*, 2006; Guimaraes *et al.*, 2014; Roy *et al.*, 2014). In addition, fungal cultures can be kept frozen for long periods of time at -20°C in standard freezers (readily available in almost mycology laboratories) to -95°C by deep

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freezing to maintain viability. Unfortunately, the mechanical deep freezers that can store cultures at -70°C or -80°C are costly to buy, and depend on a constant feeding of electricity, or on a gasholder back-up method. Fusarium head blight (FHB), provoked by different *Fusarium* species and spot blotch (SB) and common root rot (CRR) caused by *Cochliobolus sativus* are consistently ones of the most important diseases of cereals worldwide (Agrios, 2004). However, a rarity of studies is available regarding the preservation in stable conditions for 10 years in silica gel, lyophilization and soil for some FHB species (Dhingara and Sinclair, 1985; WindeIs *et al.*, 1993; Milosevic *et al.*, 2007), and for 2 years in sand and silica gel for SB causal agents (Arabi *et al.*, 2007). Recently, FHB, SB and CRR isolates were stored in a constant status in the absence of any morphological changes and the microbial contamination of fungal culture can be reduced by freezing the culture at about -16°C up to 36 months (Sakr, 2018, 2019a). In addition, these stored FHB isolates in cold water or by freezing were used successfully in resistance and pathogenic studies in both barley- and wheat-FHB pathosystems (Sakr, 2019b, 2020a,b), indicating the importance of these storage methodologies in preserving fungal cultures in viable conditions (Sakr, 2018, 2019a). Thus, the maintaining of FHB, SB and CRR cultures in cold water or by freezing at -16°C for extended periods is required to ensure the viability of these storage methods. So far, studies with water method for long time preservation of cultures at ambient temperature to keep FHB, SB and CRR causative agents have not been reported. Furthermore, using mycelia submerged cultures on filter paper disks is considered as a back-up strategy to enhance the chances of keeping both survival and morphological constancy for extent periods (Roy *et al.*, 2014). Thus, there is a need to explore these easy, low cost and simple procedures in water storage at different temperatures and by freezing adequate in small mycological laboratories where the lyophilization and cryopreservation facilities are not necessarily available. The present study evaluates the viability, purity and morphological stability for several fungal cultures of five important cereal pathogens causing FHB, SB and CRR diseases at four independent experiments (1) filter paper disks carrying fungal mycelium at 4°C for three years, (2) fungal suspension at ambient temperature for three years, (3) fungal suspension at 4°C and (4) PDA plates containing culture can be stored upto five years at -16°C .

MATERIALS AND METHODS

Fungal isolates: Seventy fungal cultures sampled from naturally infected wheat and barley plants in different regions of Syria at the 2015 growing season of sixteen isolates from four species of Fusarium head blight, *Cochliobolus sativus* causing spot blotch (32) and common root rot (22) were used. Recently, the 16 fungal isolates were molecularly analyzed by random amplified polymorphic DNA (Sakr, unpublished data).

Storage of filter paper disks carrying fungal mycelium in cold water (the first experiment)

The 70 fungal cultures were multiplied individually in Petri plates filled with 20 ml of potato dextrose agar (PDA) over 5-mm filter paper disks placed alongside the culture for 10 days at $22 \pm 1^{\circ}\text{C}$ under darkness to permit mycelial overgrowth of the disks. Then the filter paper disks were carefully and aseptically removed, and placed into sterile glass ampoules containing 8.0 ml of sterile distilled water. All ampoules were hermetically sealed and tightly wrapped with Parafilm strips. Finally, all ampoules were stored at 4°C .

For revival of the fungi, one culture laden filter paper disk was inoculated over fresh PDA Petri plates in an aseptic chamber and then incubated for 10 days for the fungal growth to be initiated. Viability tests were evaluated periodically after 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33 and 36 months.

Preservation in sterile distilled water at ambient temperature (the second experiment)

Ten fungal isolates of the five tested species, out of the 70 tested cultures, were chosen on the basis of morphological (mycelial growth rate, color, etc.) characteristics. The fungal suspension composing of spores and fragments of hyphae was harvested in softly flooding the cultures with 10 ml of sterile distilled water on the aerial growth of actively growing colony in PDA Petri plates using a micropipette. The resultant suspension was aseptically withdrawn and transferred to sterile glass ampoules, which were hermetically closed and tightly wrapped with 2 cm Parafilm strips to prevent dehydration. The suspension was kept at ambient temperature i.e., $22 \pm 2^{\circ}\text{C}$.

For survival of cultures during periods from 1 up to 36 months, 100 μL of fungal suspension was removed with a micropipette from sterile sealed glass ampoules under aseptic conditions. Fungal samples were grown in PDA Petri plates with and kept under continuous darkness for 10 days at $22 \pm 1^{\circ}\text{C}$ to allow mycelial growth.

Preservation in cold sterile distilled water (the third experiment)

For 70 isolates, the spores and fractions of hyphae were removed by softly scraping the airy growth with a pipette, and the resultant suspension was withdrawn and transferred to a sterile glass ampoules. The top of the ampoule was tightened to inhibit water evaporation. Then, sterile glass ampoules were kept at 4°C in two experiments initiated at two different dates.

After maintenance over 18 and 60 months, 300 µL of fungal suspension were grown in PDA Petri plates and kept in conditions the above-mentioned conditions to permit mycelial growth.

Preservation by freezing at -16°C (the fourth experiment)

The samples of PDA Petri plates including fungal cultures attaining appropriate growth for 70 isolates were frozen at -16°C in two experiments initiated at two different dates. After storage for up 18 and 60 months, isolates were thawed at 4°C for 24 hours. Five 5-mm agar plugs were cut from the margin for each isolate. Then, they were placed onto fresh media and kept under the above-mentioned conditions to permit mycelial growth. Samples showing revival of at least one out of five agar plugs were assessed viable.

For the four preservation treatments in independent experiments, survived cultures were tested for morphological observation (agreement of sporulation, colony, texture, color, diameter, topography features with the previously known identification) and contamination by bacteria or other fungi.

STATISTICAL ANALYSES

Data were performed using DSAASTAT add-in version 2011. For each isolate, treatment means among storage times and storage methods were compared using Fisher's LSD test at $P > 0.05$.

RESULTS

It became evident from the findings of four independent experiments (Tables 1 to 3) that all tested isolates grew with 100% revival when sub-cultured on PDA and incubated at 22°C for 10 days. There were no significant differences at $P > 0.05$ among storage times and storage methods for each isolate over the four experiments. None of the revived cultures were found to be contaminated by bacteria or other fungi (Figures 1 to 3). In general the isolates kept their separately distinguished morphological colony features.

DISCUSSION

The extended outstanding topic of "living specimen"

patterns for fungi will be resolved when it is proven that the life, properties and features of authentic cultures can be maintained (Smith and Onions, 1983; Nakasone *et al.*, 2004). Fungi are highly variable eukaryotic organisms with a slow growth velocity in culture media compared with most of the bacteria. Also, colonial preservation has been harmed probably with the contamination by bacteria or other fungi (Diogo *et al.*, 2005; Guimaraes *et al.*, 2014). The available methods for the mycology collection storage are work-demanding, costly and inefficient most of the times. The development of novel forms of fungal maintenance for extended period should be sought, especially in small facilities to meet individual needs and limitations (Diogo *et al.*, 2005; Abd-Elsalam *et al.*, 2010; Guimaraes *et al.*, 2014). Although the two procedures, cryopreservation and lyophilization, have been widely used for long term preservation, they are not necessarily available in all mycological laboratories (Borman *et al.*, 2006).

A decision-based key for choosing best storage methods suited for fungal cultures has been developed by Ryan *et al.*, (2000). A checking of this key related to the five cereal fungal species involved in this study (*Fusarium culmorum*, *F. verticillioides*, *F. solani*, *F. equiseti* and *Cochliobolus sativus*) clarifies the availability in determining which preservation method to use for these species. The analyzed fungi easily produce asexual spores in culture and do not have motile spores (Agrios, 2004), thus storage in water is recommended as economically, effectively and appropriate preservation technique, among the other available methods (Ryan *et al.*, 2000; Sakr, 2018, 2019).

Viability of fungal cells has been noted as the *most* fundamental physiological state in mycological studies (Ryan *et al.*, 2000; Abd-Elsalam *et al.*, 2010). In the present work, each fungal isolate was assessed viable if the fungal cultures did grow in the absence of microbial contamination at the same as that of the authentic culture and if the morphological characteristics of the colony corresponded the fungal distinguishing certified for each isolate. The conservation of mycelium for 70 fungal cultures in the four tested independent experiments (filter paper disks stored in cold water, fungal suspension at ambient temperature, fungal suspension at 4°C in cold water and mycelial cultures by freezing) assured a total viability for the tested periods at each time point. Our findings are in parallel with these found by Abd-Elsalam *et al.*, (2010) in which they

observed that long term water and freezing storage methods incorporating mycelia submerged cultures, spores of fungi and fractions of mycelium hyphae had high revival proportion. High viability evaluations were found for plant pathogen cultures kept in water at ambient temperature, cold water and by freezing (Legard and Chandler, 2000; Elliott, 2005; Bueno *et al.*, 2006; Roy *et al.*, 2014). Indeed, Sakr (2018, 2019a) found that the viability of the same fungal isolates was +% for 3 years using fungal suspension kept in cold water and by freezing of fungal cultures.

Selection of good sporulating cultures: (a) over filter paper disks placed alongside the culture, (b) for preparing fungal suspensions kept at 4°C or ambient temperature (22 ± 2°C) and (c) kept by freezing at -16°C was the most crucial factor affecting revival of fungal pathogens in water or by freezing during our investigation. Our findings appear to assist Bunse and Steigleder's supposition (1991), since we observed the highest re-growth evaluations for frozen *F. culmorum*, *F. verticillioides*, *F. solani*, *F. equiseti* and *Cochliobolus sativus* isolates which produce abundant spores. This hypothesis can explain the better results when freezing agar was utilized as growth medium, because freezing agar is known to enhance the output of fungal spores (Bunse and Steigleder, 1991). In this research, the techniques used to obtain water stored fungal isolates can be performed several times, using always the same glass ampoule, until total use of filter paper disks and fungal suspension. Moreover, space occupied by the whole set of glass ampoules is minimal. Furthermore, the samples of PDA Petri plates including fungal cultures attaining appropriate growth can easily be stored and transported. Neither used storage technique, time in storage nor taxonomic classification was associated with a lack of viability, microbial contamination or morphology alternation.

No visible alternations in morphological characteristics, or contamination by bacteria or other fungi which damage the storage of plant pathogen cultures were observed in the recovered cultures for the four used techniques. Maintaining in water and by freezing did not seem to influence obvious morphological cultural features of the pathogen of *Poaceae* species *Gaeumannomyces graminis* var. *graminis* (Elliott, 2005) and strawberry pathogens (Legard and Chandler, 2000), and the same used 70 fungal isolates in this research kept as

a fungal suspension in sterile distilled water at 4°C and by freezing of fungal cultures at -16°C (Sakr, 2018, 2019a). On the other hand, Holden and Smith (1992) described problems of contamination in collections of fungi stored with the continuous sub-culturing method in PDA due to mites or other causes.

Longevity of water-stored cultures may be more or less efficient for an individual species or genus followed by its optimal storage condition. Regarding grapevine pathogenic fungi of genus *Botryosphaeria*, Baskarathevan *et al.*, (2009) observed a failure in maintaining mycelium plugs in water at room temperature. Either the greater respiratory activity of fungal species caused by higher temperature or the influence of day/night regular fluctuations for ambient temperature suppressed the cultural recovery. However, highly growth rates for revived isolates were shown in treatments at 4°C (Baskarathevan *et al.*, 2009). Also, cultures of medical species and 12 potentially toxigenic species of the genera *Aspergillus* and *Penicillium* were viable after stored in cold water (Diogo *et al.*, 2005; Guimaraes *et al.*, 2014). Notwithstanding, preservation of *G. graminis* var. *graminis* at ambient temperature was adequate for recovery due to the semitropical origin of this fungal species, and the worst treatments were in a cold water (Elliott, 2005). A wide range of fungi including human and plant pathogens retained viability by storage at room temperature as observed for 470 isolates of filamentous fungi, actinomycetes and yeasts belonging to 147 species from sixty six genera (McGinis *et al.*, 1974), while five hundred and ninety four isolates from one hundred and sixty species (de Capriles *et al.*, 1989), forty five species including one hundred and seventy nine isolates (Borman *et al.*, 2006) and two pathogenic fungi of rubber: *Corynespora cassiicola* and *Phytophthora meadii* (Roy *et al.*, 2014). More interesting, survival of soil-borne phytopathogenic species of the genera *Fusarium*, *Macrophomina*, *Rhizoctonia* and *Sclerotium* was achieved by water storage at 5°C and 28°C (Bueno *et al.*, 2006). It seems that refrigeration or keeping at ambient temperature enhances survival of stored water-cultures at 4°C or 22 ± 2°C of genera *Fusarium* and *Cochliobolus* used in the present study as observed previously by Sakr (2018, 2019a).

Table 1. Viability (%) of 70 water-stored cultures at 4°C by two methods: filter paper disks containing mycelia and fungal suspension for five cereal phytopathogenic species (*Fusarium culmorum*, *F. verticillioides*, *F. solani*, *F. equiseti* and *Cochliobolus sativus*) for 36 months (first experiment)

| Fungal isolates (identification) | Preservation method | | | | | | | | | | | | Fungal isolates (identification) | Preservation method | | | | | | | | | | | |
|----------------------------------|---------------------|---|---|----|----|----|----|----|----|----|----|----|----------------------------------|---------------------|---|---|----|----|----|----|----|----|----|----|----|
| | Filter paper disks | | | | | | | | | | | | | Filter paper disks | | | | | | | | | | | |
| | Periods in months | | | | | | | | | | | | | Periods in months | | | | | | | | | | | |
| | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 | 27 | 30 | 33 | 36 | | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 | 27 | 30 | 33 | 36 |
| F1 (<i>Fc</i>) | | | | | | +a | | | | | | | C.S. 59 (SP) | | | | | | | | | | +a | | |
| F2 (<i>Fc</i>) | | | | | | +a | | | | | | | C.S. 17 (SP) | | | | | | | | | | +a | | |
| F3 (<i>Fc</i>) | | | | | | +a | | | | | | | C.S. 34 (SP) | | | | | | | | | | +a | | |
| F28 (<i>Fc</i>) | | | | | | +a | | | | | | | C.S. 21 (SP) | | | | | | | | | | +a | | |
| F30 (<i>Fc</i>) | | | | | | +a | | | | | | | C.S. 89 (SP) | | | | | | | | | | +a | | |
| F7 (<i>Fs</i>) | | | | | | +a | | | | | | | C.S. 53 (SP) | | | | | | | | | | +a | | |
| F20 (<i>Fs</i>) | | | | | | +a | | | | | | | C.S. 86 (SP) | | | | | | | | | | +a | | |
| F26 (<i>Fs</i>) | | | | | | +a | | | | | | | C.S. 74 (SP) | | | | | | | | | | +a | | |
| F29 (<i>Fs</i>) | | | | | | +a | | | | | | | C.S. 49 (SP) | | | | | | | | | | +a | | |
| F31 (<i>Fs</i>) | | | | | | +a | | | | | | | C.S. 9 (SP) | | | | | | | | | | +a | | |
| F35 (<i>Fs</i>) | | | | | | +a | | | | | | | C.S. 12 (SP) | | | | | | | | | | +a | | |
| F15 (<i>Fv</i>) | | | | | | +a | | | | | | | C.S. 63 (SP) | | | | | | | | | | +a | | |
| F16 (<i>Fv</i>) | | | | | | +a | | | | | | | C.S. 55 (SP) | | | | | | | | | | +a | | |
| F21 (<i>Fv</i>) | | | | | | +a | | | | | | | C.S. 41 (CRR) | | | | | | | | | | +a | | |
| F27 (<i>Fv</i>) | | | | | | +a | | | | | | | C.S. 50 (CRR) | | | | | | | | | | +a | | |
| F43 (<i>Fe</i>) | | | | | | +a | | | | | | | C.S. 37 (CRR) | | | | | | | | | | +a | | |
| C.S. 14 (SP) | | | | | | +a | | | | | | | C.S. 36 (CRR) | | | | | | | | | | +a | | |
| C.S. 27 (SP) | | | | | | +a | | | | | | | C.S. 24 (CRR) | | | | | | | | | | +a | | |
| C.S. 32 (SP) | | | | | | +a | | | | | | | C.S. 23 (CRR) | | | | | | | | | | +a | | |
| C.S. 92 (SP) | | | | | | +a | | | | | | | C.S. 44 (CRR) | | | | | | | | | | +a | | |
| C.S. 20 (SP) | | | | | | +a | | | | | | | C.S. 48 (CRR) | | | | | | | | | | +a | | |
| C.S. 2 (SP) | | | | | | +a | | | | | | | C.S. 52 (CRR) | | | | | | | | | | +a | | |
| C.S. 80 (SP) | | | | | | +a | | | | | | | C.S. 13(CRR) | | | | | | | | | | +a | | |
| C.S. 7 (SP) | | | | | | +a | | | | | | | C.S. 6 (CRR) | | | | | | | | | | +a | | |
| C.S. 18 (SP) | | | | | | +a | | | | | | | C.S. 38 (CRR) | | | | | | | | | | +a | | |
| C.S. 30 (SP) | | | | | | +a | | | | | | | C.S. 25 (CRR) | | | | | | | | | | +a | | |
| C.S. 93 (SP) | | | | | | +a | | | | | | | C.S. 46 (CRR) | | | | | | | | | | +a | | |
| C.S. 16 (SP) | | | | | | +a | | | | | | | C.S. 47 (CRR) | | | | | | | | | | +a | | |
| C.S. 87 (SP) | | | | | | +a | | | | | | | C.S. 51 (CRR) | | | | | | | | | | +a | | |
| C.S. 83 (SP) | | | | | | +a | | | | | | | C.S. 8 (CRR) | | | | | | | | | | +a | | |
| C.S. 45 (SP) | | | | | | +a | | | | | | | C.S. 40 (CRR) | | | | | | | | | | +a | | |
| C.S. 11 (SP) | | | | | | +a | | | | | | | C.S. 1 (CRR) | | | | | | | | | | +a | | |
| C.S. 9 (SP) | | | | | | +a | | | | | | | C.S. 10 (CRR) | | | | | | | | | | +a | | |
| C.S. 15 (SP) | | | | | | +a | | | | | | | C.S. 5 (CRR) | | | | | | | | | | +a | | |
| C.S. 26 (SP) | | | | | | +a | | | | | | | C.S. 28 (CRR) | | | | | | | | | | +a | | |

Fc: *Fusarium culmorum*, *Fs*: *F. solani*, *Fv*: *F. verticillioides*, *Fe*: *F. equiseti*, SB: Spot blotch and CRR: common root rot, + = mycelial growth expressed by 100%. According to the Fisher's LSD test, means among the storage times in each storage method within a row followed by the same letter are not significantly different at P>0.05 for each isolate.

Table 2. Viability (%) of 16 water-stored cultures at ambient temperature ($22 \pm 2^\circ\text{C}$) five cereal phytopathogenic species (*Fusarium culmorum*, *F. verticillioides*, *F. solani*, *F. equiseti* and *Cochliobolus sativus*) for 36 months (second experiment)

| Fungal isolates (identification) | Sterile water at $22 \pm 2^\circ\text{C}$ | | | | | | | | | | | | | | | | | |
|-------------------------------------|-------------------------------------------|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|
| | Periods in months | | | | | | | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
| F1 (<i>Fc</i>) | | | | | | | | | | | | | | | | | | +a |
| F2 (<i>Fc</i>) | | | | | | | | | | | | | | | | | | +a |
| F30 (<i>Fc</i>) | | | | | | | | | | | | | | | | | | +a |
| F26 (<i>Fs</i>) | | | | | | | | | | | | | | | | | | +a |
| F29 (<i>Fs</i>) | | | | | | | | | | | | | | | | | | +a |
| F35 (<i>Fs</i>) | | | | | | | | | | | | | | | | | | +a |
| F15 (<i>Fv</i>) | | | | | | | | | | | | | | | | | | +a |
| F21 (<i>Fv</i>) | | | | | | | | | | | | | | | | | | +a |
| F27 (<i>Fv</i>) | | | | | | | | | | | | | | | | | | +a |
| F43 (<i>Fe</i>) | | | | | | | | | | | | | | | | | | +a |
| C.S. 9 (SP) | | | | | | | | | | | | | | | | | | +a |
| C.S. 59 (SP) | | | | | | | | | | | | | | | | | | +a |
| C.S. 87 (SP) | | | | | | | | | | | | | | | | | | +a |
| C.S. 1 (CRR) | | | | | | | | | | | | | | | | | | +a |
| C.S. 37 (CRR) | | | | | | | | | | | | | | | | | | +a |
| C.S. 50 (CRR) | | | | | | | | | | | | | | | | | | +a |

| Fungal isolates (identification) | Sterile water at $22 \pm 2^\circ\text{C}$ | | | | | | | | | | | | | | | | | |
|-------------------------------------|-------------------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| | Periods in months | | | | | | | | | | | | | | | | | |
| | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 |
| F1 (<i>Fc</i>) | | | | | | | | | | | | | | | | | | +a |
| F2 (<i>Fc</i>) | | | | | | | | | | | | | | | | | | +a |
| F30 (<i>Fc</i>) | | | | | | | | | | | | | | | | | | +a |
| F26 (<i>Fs</i>) | | | | | | | | | | | | | | | | | | +a |
| F29 (<i>Fs</i>) | | | | | | | | | | | | | | | | | | +a |
| F35 (<i>Fs</i>) | | | | | | | | | | | | | | | | | | +a |
| F15 (<i>Fv</i>) | | | | | | | | | | | | | | | | | | +a |
| F21 (<i>Fv</i>) | | | | | | | | | | | | | | | | | | +a |
| F27 (<i>Fv</i>) | | | | | | | | | | | | | | | | | | +a |
| F43 (<i>Fe</i>) | | | | | | | | | | | | | | | | | | +a |
| C.S. 9 (SP) | | | | | | | | | | | | | | | | | | +a |
| C.S. 59 (SP) | | | | | | | | | | | | | | | | | | +a |
| C.S. 87 (SP) | | | | | | | | | | | | | | | | | | +a |
| C.S. 1 (CRR) | | | | | | | | | | | | | | | | | | +a |
| C.S. 37 (CRR) | | | | | | | | | | | | | | | | | | +a |
| C.S. 50 (CRR) | | | | | | | | | | | | | | | | | | +a |

Fc: *Fusarium culmorum*, *Fs*: *F. solani*, *Fv*: *F. verticillioides*, *Fe*: *F. equiseti*, SB: Spot blotch and CRR: common root rot, + = mycelial growth expressed by 100%. According to the Fisher's LSD test, means among the storage times in each storage method within a row followed by the same letter are not significantly different at $P > 0.05$ for each isolate.

Table 3. Viability (%) of 70 water-stored at 4°C and frozen cultures at -16°C of five cereal phytopathogenic species (*Fusarium culmorum*, *F. verticillioides*, *F. solani*, *F. equiseti* and *Cochliobolus sativus*) for 18 and 60 months (third and fourth experiment)

| Fungal Isolates(identification) | Preservation methods | | | | Fungal Isolates(identification) | Preservation methods | | | |
|---------------------------------|----------------------|----|-----------------|----|---------------------------------|----------------------|----|-----------------|----|
| | sterile water | | freezing | | | sterile water | | freezing | |
| | 18 | 60 | 18 | 60 | | 18 | 60 | 18 | 60 |
| F1 (<i>Fc</i>) | +a ^a | | +a ^a | | C.S. 59 (SP) | +a ^a | | +a ^a | |
| F2 (<i>Fc</i>) | +a ^a | | +a ^a | | C.S. 17 (SP) | +a ^a | | +a ^a | |
| F3 (<i>Fc</i>) | +a ^a | | +a ^a | | C.S. 34 (SP) | +a ^a | | +a ^a | |
| F28 (<i>Fc</i>) | +a ^a | | +a ^a | | C.S. 21 (SP) | +a ^a | | +a ^a | |
| F30 (<i>Fc</i>) | +a ^a | | +a ^a | | C.S. 89 (SP) | +a ^a | | +a ^a | |
| F7 (<i>Fs</i>) | +a ^a | | +a ^a | | C.S. 53 (SP) | +a ^a | | +a ^a | |
| F31 (<i>Fs</i>) | +a ^a | | +a ^a | | C.S. 86 (SP) | +a ^a | | +a ^a | |
| F35 (<i>Fs</i>) | +a ^a | | +a ^a | | C.S. 74 (SP) | +a ^a | | +a ^a | |
| F20 (<i>Fs</i>) | +a ^a | | +a ^a | | C.S. 49 (SP) | +a ^a | | +a ^a | |
| F26 (<i>Fs</i>) | +a ^a | | +a ^a | | C.S. 9 (SP) | +a ^a | | +a ^a | |
| F29 (<i>Fs</i>) | +a ^a | | +a ^a | | C.S. 12 (SP) | +a ^a | | +a ^a | |
| F15 (<i>Fv</i>) | +a ^a | | +a ^a | | C.S. 63 (SP) | +a ^a | | +a ^a | |
| F16 (<i>Fv</i>) | +a ^a | | +a ^a | | C.S. 55 (SP) | +a ^a | | +a ^a | |
| F21 (<i>Fv</i>) | +a ^a | | +a ^a | | C.S. 41 (CRR) | +a ^a | | +a ^a | |
| F27 (<i>Fv</i>) | +a ^a | | +a ^a | | C.S. 50 (CRR) | +a ^a | | +a ^a | |
| F43 (<i>Fe</i>) | +a ^a | | +a ^a | | C.S. 37 (CRR) | +a ^a | | +a ^a | |
| C.S. 14 (SP) | +a ^a | | +a ^a | | C.S. 36 (CRR) | +a ^a | | +a ^a | |
| C.S. 27 (SP) | +a ^a | | +a ^a | | C.S. 24 (CRR) | +a ^a | | +a ^a | |
| C.S. 32 (SP) | +a ^a | | +a ^a | | C.S. 23 (CRR) | +a ^a | | +a ^a | |
| C.S. 92 (SP) | +a ^a | | +a ^a | | C.S. 44 (CRR) | +a ^a | | +a ^a | |
| C.S. 20 (SP) | +a ^a | | +a ^a | | C.S. 48 (CRR) | +a ^a | | +a ^a | |
| C.S. 2 (SP) | +a ^a | | +a ^a | | C.S. 52 (CRR) | +a ^a | | +a ^a | |
| C.S. 80 (SP) | +a ^a | | +a ^a | | C.S. 13(CRR) | +a ^a | | +a ^a | |
| C.S. 7 (SP) | +a ^a | | +a ^a | | C.S. 6 (CRR) | +a ^a | | +a ^a | |
| C.S. 18 (SP) | +a ^a | | +a ^a | | C.S. 38 (CRR) | +a ^a | | +a ^a | |
| C.S. 30 (SP) | +a ^a | | +a ^a | | C.S. 25 (CRR) | +a ^a | | +a ^a | |
| C.S. 93 (SP) | +a ^a | | +a ^a | | C.S. 46 (CRR) | +a ^a | | +a ^a | |
| C.S. 16 (SP) | +a ^a | | +a ^a | | C.S. 47 (CRR) | +a ^a | | +a ^a | |
| C.S. 87 (SP) | +a ^a | | +a ^a | | C.S. 51 (CRR) | +a ^a | | +a ^a | |
| C.S. 83 (SP) | +a ^a | | +a ^a | | C.S. 8 (CRR) | +a ^a | | +a ^a | |
| C.S. 45 (SP) | +a ^a | | +a ^a | | C.S. 40 (CRR) | +a ^a | | +a ^a | |
| C.S. 11 (SP) | +a ^a | | +a ^a | | C.S. 1 (CRR) | +a ^a | | +a ^a | |
| C.S. 9 (SP) | +a ^a | | +a ^a | | C.S. 10 (CRR) | +a ^a | | +a ^a | |
| C.S. 15 (SP) | +a ^a | | +a ^a | | C.S. 5 (CRR) | +a ^a | | +a ^a | |
| C.S. 26 (SP) | +a ^a | | +a ^a | | C.S. 28 (CRR) | +a ^a | | +a ^a | |

Fc: *Fusarium culmorum*, *Fs*: *F. solani*, *Fv*: *F. verticillioides*, *Fe*: *F. equiseti*, SB: Spot blotch and CRR: common root rot, + = mycelial growth expressed by 100%. According to the Fisher's LSD test, means among the storage times in each storage method within a row followed by the same letter are not significantly different at P>0.05 for each isolate.

According to the Fisher's LSD test, means between the two storage methods (sterile water and freezing) between the two columns for each storage time followed by the same superscript letter are not significantly different at $P > 0.05$ for each isolate.

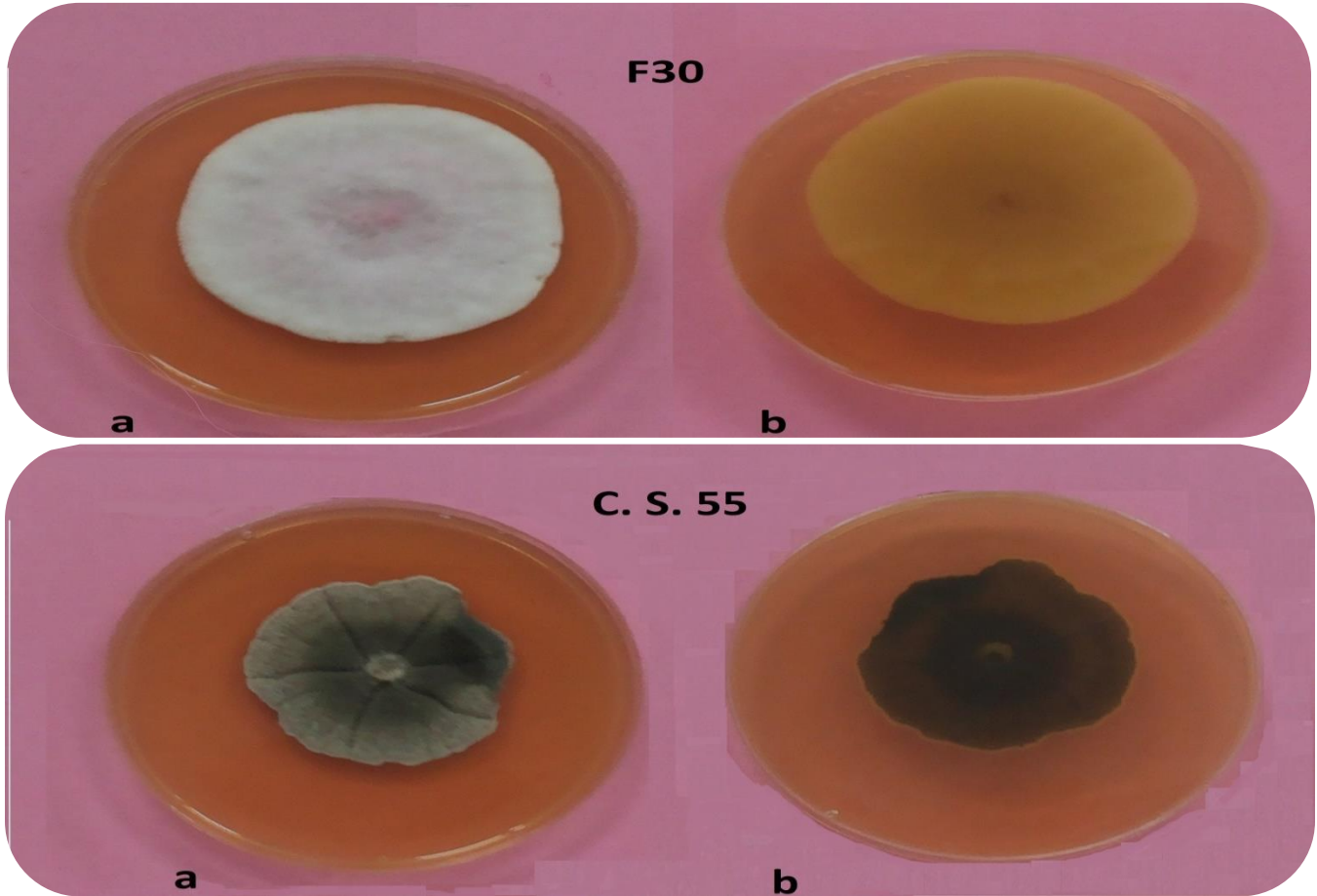


Figure 1. Revived fungal cultures of two isolates: F30 (*Fusarium culmorum*) and C.S. 55 (*Cochliobolus sativus*) onto fresh medium recovered from filter paper disks stored in sterile distilled water at 4°C. (a) Cultivation of fungal cultures growing in the PDA Petri-dish. (b) The bottom of the PDA Petri-dish with filter paper disks covered by mycelium (first experiment)

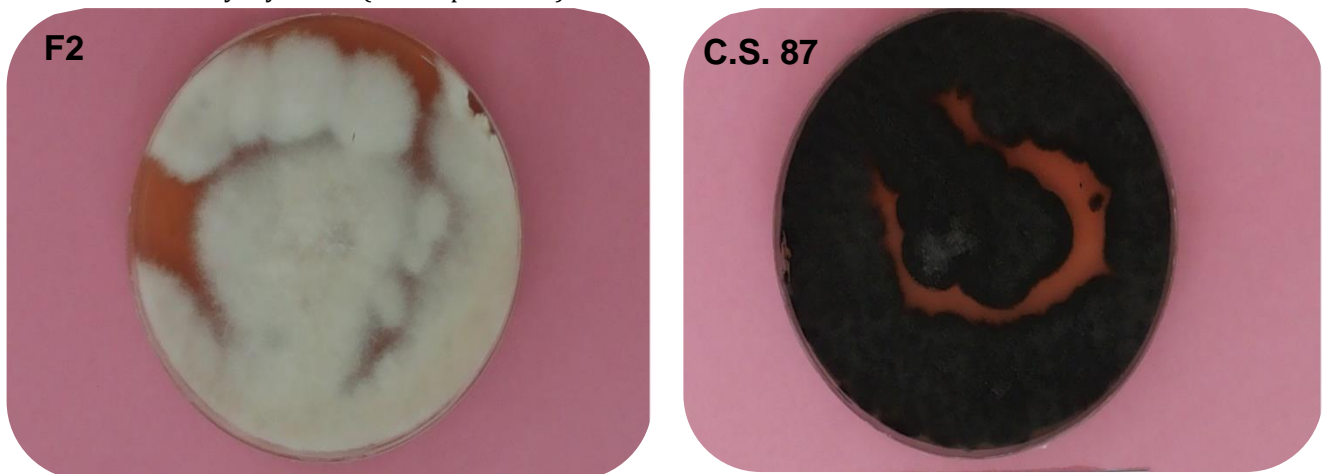


Figure 2. Revived fungal cultures of two isolates: F2 (*Fusarium culmorum*) and C.S. 87 (*Cochliobolus sativus*) onto fresh medium recovered from fungal suspension stored at ambient temperature ($22 \pm 2^\circ\text{C}$) (second experiment)

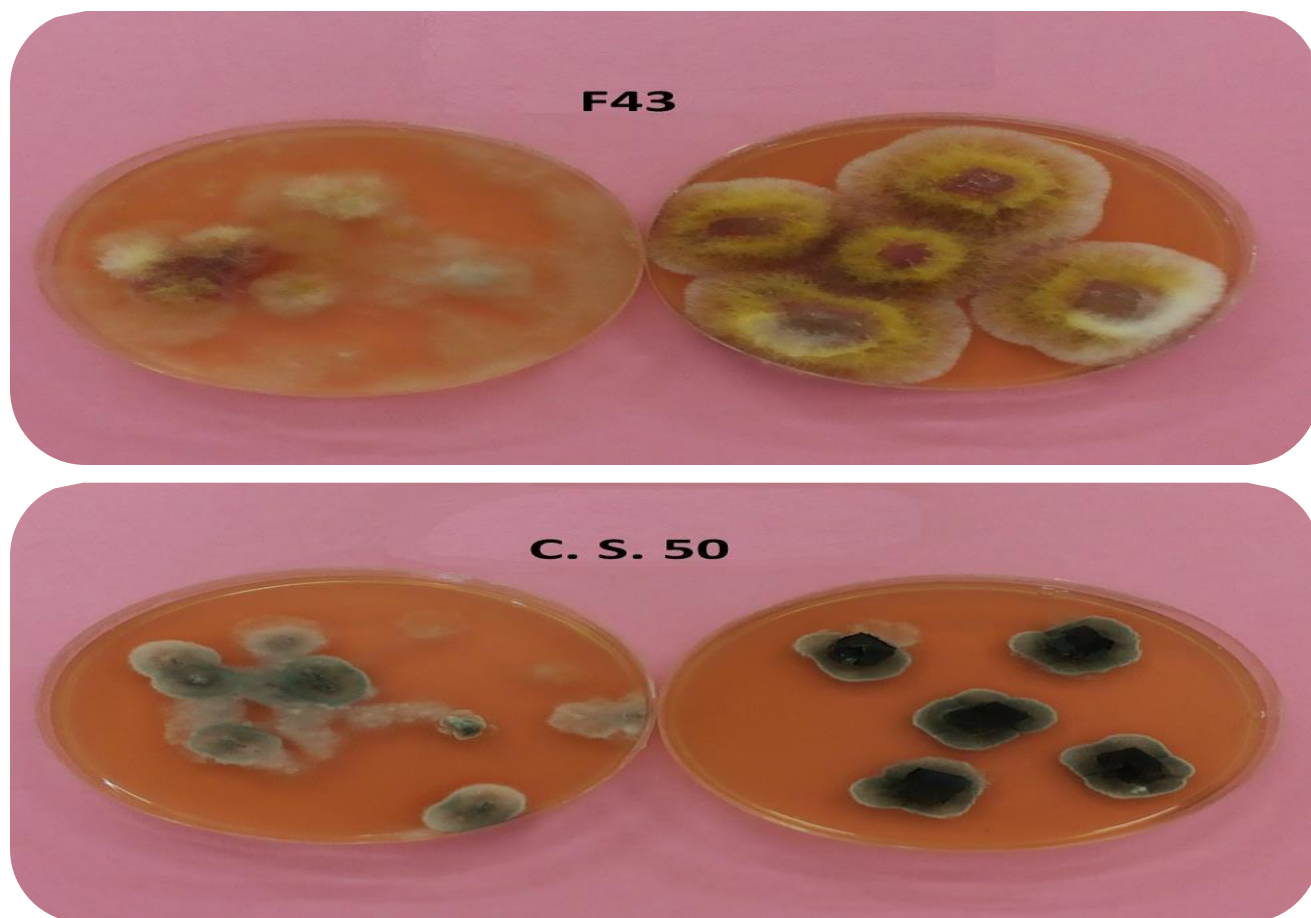


Figure 3. Revived fungal cultures of two isolates: F43 (*Fusarium equiseti*) and C.S. 50 (*Cochliobolus sativus*) onto fresh medium recovered from from cultures preserved in sterile distilled water at 4°C (left side) and freezing at -16°C (right side)

As far as we know, the present work is the first report highlighting the potential of conservation for FHB, SB and CRR fungi in a viable state using filter paper disks stored in cold water for three years, fungal suspension at ambient temperature for 3 years, fungal suspension at 4°C in cold water and mycelial cultures by freezing for 5 years. The presented four used techniques of preservation fungal isolates have the following • • interests: (1) the risk of contamination with bacteria, other fungi or mites is eliminated, (2) the specimens can readily be maintained and transferred, (3) the morphology of the cultures was not changed so that these can be utilized directly as references or for illustration, and (4) all revived cultures were identical with the corresponding storage isolates about the macro- and microscopical characters of the cultures and fungus cells. It is argued that these easy, low cost and simple procedures were adequate in small mycological laboratories where the lyophilization and cryopreservation facilities are not necessary available.

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