Official publication of Pakistan Phytopathological Society
Pakistan Journal of Phytopathology
ISSN: 1019-763X (Print), 2305-0284 (Online)
http://www.pakps.com

LONG TERM STORAGE FOR FIVE IMPORTANT CEREAL PHYTOPATHOGENIC SPECIES

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A B S T R A C T

Fusarium head blight (FHB), spot blotch (SB) and common root rot (CRR) are consistently of the most important cereal diseases globally, but few publications have appeared on preservation. We have stored seventy FHB, SB and CRR fungal isolates of five species (*Fusarium culmorrum*, *F. equiiseti, F. solanii*, *F. verticilioides*, and *Cochliobollus sativus*) in sterile distilled water and freezing in two independent experiments for 36 months. Fungi were tested for viability, purity and morphological stability. The storage treatments included fungal inoculum consisting of spores and hyphae suspended in sterile distilled water at 4ºC and mycelial cultures on Petri-plates with potato dextrose agar (PDA) by freezing at -16ºC. The first experiment included seventy isolates was tested six times, after 6, 12, 18, 24, 30 and 36 months of storage. The second experiment with 16 isolates, out of the tested 70 isolates, was tested over periods from 1 to 36 months. The 70 three-year old water-stored and frozen cultures did grow when sub-cultured on PDA. Moreover, viable 16 cultures were recovered from the two storage methods having 100% revival at each time point for up to 3 years. All tested isolates were found to be pure, and maintained their original morphological features. Survival of cultures was not affected with preservation procedure, time in storage or taxonomic classification. The two techniques were found to be easy and reliable for preservation of important cereal fungi for up to 36 months. To our best knowledge, this is the first research reporting the possibility of preservation for FHB, SB and CRR causal agents by cold water and freezing for three years.

Keywords: freezing, viability, water storage

INTRODUCTION

Culture collections, as libraries persevering the vigor and genetic features of previously analyzed pathogens, are essential resources for further in-depth study in plant pathology and related disciplines as breeding programs and determining plant resistance against pathogens and fungicides (Kang *et al.,* 2006). In belief, by lowering the metabolism rate, these isolates can be stored for long period of time. Accumulation of mutation produced by biochemical along with morphological modifications can be prevent by this method. Not any storage technique has been universally practiced to all fungi due to the particular characteristic of each species (Ryan *et aI.,* 2000). For fungi, adoption of a single specific preservation method not only depend the success of the method but also the anticipated use of the organism, time, facilities and maintenance costs (Abd-Elsalam *et al.,* 2010). Fungal cultures were traditionally kept by routine sub-culturing. However, it is not appropriate for prolonged preservation because of strain drift (Karen *et al.,* 2004; Roy *et al.,* 2014). Over the years, numerous methods for the storage of fungal cultures have been developed in order to eliminate these disadvantages, even though results are varied (Abd-EIsaIam *et aI.,* 2010; Tariq et *aI*., 2015; Akhtar et *aI*., 2016).

Preservation of fungi under sterile water, a procedure described by Castellani seventy years ago, has been used to successfully store many diverse fungi up to several years (McGinnis *et aI.,* l974; EIIiott 2005; Boreman *et aI.,* 2006; Roy *et aI.,* 20l4). It is easy, rapid and economically technique reserved both viability along with morphology, physiology as well as genetic stability of the specific organism. Castellani's technique should be complemented by a 2nd storage technique to preserve fungal cultures for

Submitted: May 30, 2019
Revised: August 28, 2019
Accepted for Publication: September 02, 2019
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long time period (Borman *et al.,* 2006; Abd-Elsalam *et al.,* 2010). Storing fungal cultures by freezing in standard freezers (operating at -20°C and readily available in almost mycology laboratories) appears to be a back-up preservation method. In addition, this economical method has been yielded satisfactory results in maintaining survival of frozen cultures for many years (Legard and Chandler, 2000).

Seventeen various species of *Fusarium* are involved in head blight of *Fusarium* development which is a serious disease of cereals including wheat and barley. Barley is commonly attacked by various diseases including common root rot and spot blotch caused by *Cochliobolus sativus.* FHB, SB and CRR are consistently of the most important diseases of cereals worldwide (Agrios 2004). There is a rareness of articles related the storage of the fungus of *Fusatium* head blight and spot blotch of barley. By the use of soil and silica gel various *Fusarium* head blight species can be stored for ten years in stable conditions (Dhingra and SincIair, l985; WindeIs *et aI.,* l993; MiIosevic *et aI.,* 2007). In sand and silica gel storage methods, viability and virulence were preserved for three SB isolates for 2 years, but a danger of mutation was noticed (Arebi *et aI.,* 2007). Recently, Sakr (2018) found that fungal isolates of FHB, SB and CRR diseases can retain viability by two storage methods (sterile distilled water and freezing) for over 12 months. The present study reports the viability, purity and morphological stability for 70 fungal cultures of five important cereal phytopathogenic species (*F. cuImorum*, *Fusarium equiseti, Fusarium verticiIIioides*, *Fusarium solani*, as well as *C. sativus*) after being stored for 36 months in two safe, easy and simple methods: distilled sterilized water at 39.2ºF (4ºC) and freezing at 3.2ºF (-16ºC).

MATERIALS AND METHODS

Fungal isolates and experimental designs: From naturally infected cereals including wheat and barley, 70 various fungal isolates were recovered from various wheat and barley growing areas of Syria in the year 2015 of five important cereal phytopathogenic species were included. Of these isolates, 16 were of Fusarium species causing head blight (*Fusarium culmorum*, *Fusarium equiseti, Fusarium solani*, and *Fusarium verticillioides*), 32 were of *Cochliobolus sativus* causing spot blotch and 22 were of *C. sativus* exhibiting common root rot. Table number 01 gives identification for these seventy fungal isolates which were used in the current study. For mycelial growth of the fungal isolates, the fungal culture were incubated in the PDA containing Petri plates having 9cm diameter at 22 ± 1ºC for ten days.

The viability, purity and morphological stability of fungal cultures viz. colony diameter, color, texture, topography and sporulation stored in sterile distilled water and by freezing were tested in two independent experiments. Each experiment dealt with the two storage methods and the storage period continued for up to 36 months. Even though 16 isolates, out of all tested cultures, were common to the two experiments, these individual isolates were selected depending on various morphological characteristics including color of the isolates as well as mycelial growth of the isolates. All tested species were included in the two experiments.

The first experiment with 70 isolates was tested six times, after 6, 12, 18, 24, 30 and 36 months of storage. The second experiment included 16 isolates was tested periodically. For water storage, tests were performed at monthly intervals for up to 36 months. Regarding freezing storage, analyses were carried out without interruption at three-month intervals up to three years. The two experiments were initiated at the same time.

Preservation in sterile distilled water: The fungal inoculum comprising of spores and fragments of hyphae, for seventy fungal isolates subjected to the first experiment and for 16 isolates for the second experiment, was dislodged by lightly stirring eight milliliters of distilled sterilized water on the aerial growth of actively growing colony in PDA containing Petri-dishes with the help of micropipette. The suspension of fungal isolates was transferred into sterilized glass tube in the laminar flow chamber in an aseptic condition and tightly wrapped with the help of parafilm to conserve moisture or prevent dehydration and then incubated at 4°C in an incubator.

To preserve the fungal culture for long time upto three years, 100 micro litters of the fungal suspension was taken from already incubated glass tubes with the help of pipette and transferred it to the PDA containing petri plates in the aseptic conditions in laminar flow chamber. These PDA containing petri plates were incubated in an incubator at 4ºC for fungal mycelial growth. Growth of mycelia showed the viability of the fungal culture.

Reservation by freezing: Hermetically closed and sealed Petri-dishes with PDA containing vigorously growing mycelial cultures, for 70 isolates from the first experiment

and for 16 isolates from the second experiment, were stored in the freezer at -16ºC without freezing module. For viability test after storage of three-month time period up to three years, the PDA containing frozen petri plates were taken out from incubator and allow them to thaw at about four degree centigrade for one day. Then cut a piece of about 5 mm from the perti plate with the help of sterilized inoculating blade and put it in fresh PDA containing Petri plate in an aseptic environment in the laminar flow chamber. These PDA containing petri plates were incubated in an incubator at 4ºC for fungal mycelial growth. Growth of mycelia showed the viability of the fungal culture.

From the two preservation techniques, viability of the fungal cultures was observed for different morphological characteristics including color of the colony, diameter of the colony, texture of the mycelial growth as well as spore formation and structure of spores.

STATISTICAL ANALYSES

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

RESULTS

The results of the 1st experiment showed hundred percent viability of the fungal culture which was stored at 4ºC in an incubator, when it was transferred on new PDA containing Petri plates in the aseptic environment (Table No. 01). In addition, storage techniques along with storage time have no significant impact on fungal isolates (Table No. 01). For the second experiment, viable 16 cultures of the five species were recovered from the two storage methods having 100% revival at each time point over periods from 1 up to 36 months (Tables No. 02 and 03). In addition, storage techniques along with storage time have no significant impact on fungal isolates (Table No. 02 and 03). All the fungal isolates showed significant purity when they were cultured in the aseptic conditions in the absence of microorganisms including other fungal species and bacteria. Moreover, the viable cultures maintained their morphological features corresponded to the original description (Figure 1).

Table 1. Viability (%) of 70 water-stored at 4ºC and frozen cultures at -16ºC of five cereal phytopathogenic species (*Fusarium equiseti, Fusarium culmorum*, *Fusarium solani*, *Fusarium verticillioides*, and *Cochliobolus sativus*) for 6, 12, 18, 24, 30 and 36 months (experiment 1)

Fungal isolates (identification)	Preservation methods											
	sterile water						freezing					
	6	12	18	24	30	36	6	12	18	24	30	36
F1 (*Fc*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F2 (*Fc*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F3 (*Fc*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F28 (*Fc*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F30 (*Fc*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F7 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F31 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F35 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F20 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F26 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F29 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F15 (*Fv*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F16 (*Fv*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F21 (*Fv*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F27 (*Fv*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F43 (*Fe*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 14 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 27 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 32 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 92 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 20 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 2 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 80 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a

C.S. 7 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 18 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 30 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 93 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 16 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 87 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 83 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 45 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 11 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 9 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 15 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 26 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 59 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 17 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 34 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 21 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 89 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 53 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 86 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 74 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 49 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 9 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 12 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 63 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 55 (SP)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 41 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 50 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 37 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 36 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 24 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 23 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 44 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 48 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 52 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 13(CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 6 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 38 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 25 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 46 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 47 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 51 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 8 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 40 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 1 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 10 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 5 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C.S. 28 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a

Fc: *Fusarium culmorrum*, *Fs*: *F. solanii*, *Fv*: *F. verticilioides*, *Fe*: *F. equseti*, SB: Spot blotch and CRR: common root rot. Rendering to the Fisher's LSD design, means among the storage times in each storage method within a row foIIowed by the same Ietter are not significantIy different at P>0.05 for each isolate. Rendering to the Fisher's LSD design, 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. Viability of all isolates for 12 and 30 months was presented by Sakr (2018, unpublished data).

Table 2. Viability (%) of 16 water-stored cultures at 4°C of five cereal phytopathogenic species (*Fusarium verticilioides*, *Fusarium culmorrum*, *Fusarium equiiseti, Fusarium solanii*, and *Cochliobolus sativus*) for 36 months (experiment 2)

Fungal isolates (identification)	Periods in months																																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
F1 (*Fc*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F2 (*Fc*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F30 (*Fc*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F26 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F29 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F35 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F15 (*Fv*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F21 (*Fv*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F27 (*Fv*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F43 (*Fe*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 9 (SB)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 55 (SB)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 87 (SB)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 1 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 37 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 50 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a

Fe: *Fusarium equiiseti*, *Fs*: *Fusarium solanii*, *Fc*: *Fusarium culmorrum*, *Fv*: *Fusarium verticilioides*, SB and CRR. Rendering to the Fisher's LSD design, means of the storage times which are not significantly different from each other at P>0.05

Table 3. Viability (%) of 16 frozen cultures at -16°C of five cereal phytopathogenic species (*Fusarium culmorum*, *F. verticillioides*, *F. solani*, *F. equiseti* and *Cochliobolus sativus*) for 36 months (experiment 2)

Fungal isolates (identification)	Periods in Days											
	90	180	270	360	450	540	630	720	810	900	990	1080
F1 (*Fc*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F2 (*Fc*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F30 (*Fc*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F26 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F29 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F35 (*Fs*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F15 (*Fv*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F21 (*Fv*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F27 (*Fv*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
F43 (*Fe*)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 9 (SB)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 55 (SB)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 87 (SB)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 1 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 37 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a
C. S. 50 (CRR)	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a	99.9a

Fe: *Fusarium equiiseti*, *Fs*: *Fusarium solanii*, *Fc*: *Fusarium culmorrum*, *Fv*: *Fusarium verticilioides*, SB and CRR. Rendering to the Fisher's LSD design, means of the storage times which are not significantly different from each other at P>0.05

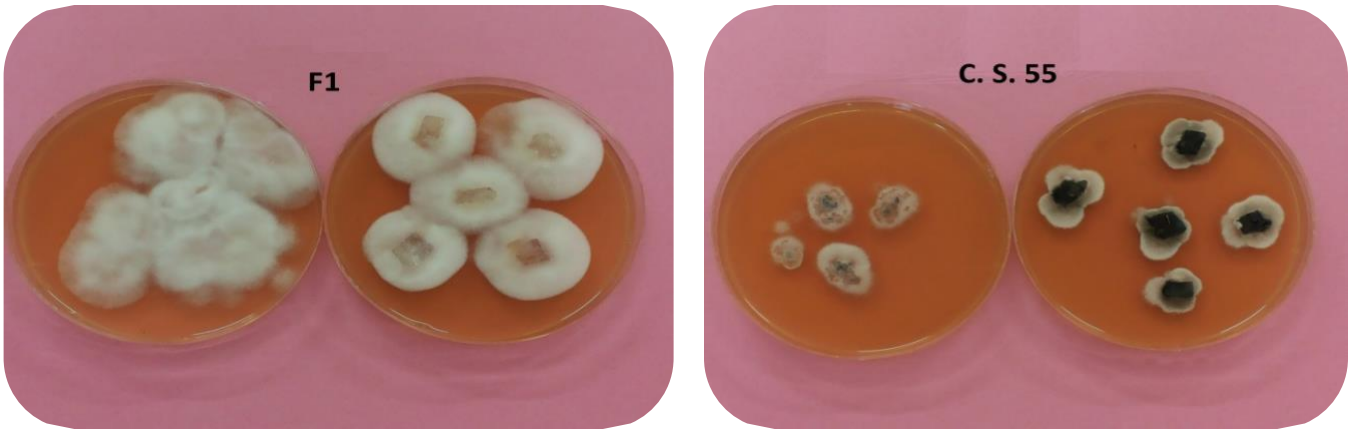

Figure 1. Revived cereal phytopathogenic cultures : F1 (*Fusarium culmorrum*) and C.S. 55 (*Cochlliobolus satiivus*) onto fresh medium recovered from cultures stored in distilled sterilized water at 4ºC (left side) and freezing at -16ºC (right side)

DISCUSSION

Available methods for plant pathogen storage collection are work-demanding, costly and inefficient most of the times. The development of novel forms of fungal maintenance for extended period should be thought for further fungal research including identification of the pathogen, growing of resistant germplasm and disease management (Abd-EIsaIam *et aI.,* 20l0). Ryan *et al.,* (2000) described the best techniques for the storage of fungal cultures. In this study, these described techniques were applied on different fungal isolates including *Fusarium solanii*, *Fusarium verticilioides*, *Fusarium equiiseti, Fusarium culmorrum*, and *Cochlliobolus satiivus* to test that which technique is best for any fundal isolate. These fungal species do not produce motile spores (sexual spores) but asexual spores were produced by these fungal isolates (Agrios, 2004), thus storage in water and by freezing are recommended as economical, effective and appropriate preservation techniques, among the other available methods (Ryan *et aI.,* 2000).

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). The 70 water-stored and frozen cultures did grow when sub-cultured on PDA (Table no. 1). Moreover, viable 16 cultures were recovered from the two storage methods having 100% revival at each time point for up to 3 years (Tables 2 and 3). The results of this study were highly correlated with the findings of Abd-EIsalam *et aI.,* (20l0) in which he elaborate that the mycelial fragments and spores of fungus have high viability ratio in the long term storage techniques. Indeed, Sakr (2018) found that the viability of the same fungal isolates was 100% for 1-year. Results highlighted that survival of 70 fungal cultures was not affected with preservation procedure (storage in water and by freezing), time in storage (prolonged periods from 1 up to 36 months) or taxonomic classification (five cereal phytopathogenic species of genera *Fusarium* and *Cochliobolus*) (Tables 1, 2 and 3).

All tested isolates were found to be pure, and maintained their original morphological features by the two preservation methods (Figure 1). Furthermore, Sakr (2018) reported that morphological alternation and pathogen contamination were not detected. Meanwhile the morphological characteristics including colony color and growth of the mycelium were not negatively affected while storage at 4ºC and freezing at -16ºC (Legard and Chandler 2000; Elliott, 2005).

In the present study, it is clear that the selection of actively growing mycelium and sufficient fungal suspension consisting of adequate amounts of spores and pieces and several wefts of hyphae suspended in sterile distilled water were the most significant aspects enhancing revitalization in water for extended time periods (McGinis *et aI.,* l974). While conducting this experiment the amount of water for the preparation of fungal suspension was copied from the previous studies of McGinis *et aI.,* (l974) who stated that the fungal culture can be survive in this medium for up to five years. The amount of high inoculum in this medium may enhance the ability of the viability was also tested in this experiment. This medium has the ability to lower down the growth of fungal mycelium for long term storage, when the culture applied on fresh PDA containing Petri plate the mycelium grow with all morphological

characteristics (Roy *et al.,* 2014). In this research, the technique used to obtain stored fungal isolates can be performed several times, using always the same glass ampoule, until total use of fungal suspension. Moreover, space occupied by the whole set of glass ampoules is minimal.

Almost all the metabolic activities are lower down at the temperature of -70ºC, ice reformation and physical and biological processes may affect the survival of cells even below this temperature (Jong l989). Various modules of freezing and thawing are required for the survival and the changes in the morphological features including growth and color of the hyphae (Morris *et aI.,* l988). This is very important to know about the specific information about these fungal species. While in our observation the survival of stored cultures with and without modules were excellent in the storage conditions. The results of our experiments have resemblance with the studies of Legerd and ChandIer (2000) in which they work on pathogenic fungus of strawberry. In this study, multiple freezing modules were used which could cause genetic damage to the mycelial culture frozen on PDA containing Petri Plates (Legerd and ChandIer, 2000). While the preservation of *Fusarium* sopt blotch culture was not performed better after 24 months of storage in frozen conidia technique (Arabii *et aI.,* 2007). Large fungal frictions (such as F. sp. And C. *satives* used in this research) fall into the entire process of cryopreservation, and the loss of hydration cannot be overstated. In addition, the formation of ice crystals physically destroys and destroys a large number of spores of similar size (Kairen *et aI*., 2004). Ishikewa *et aI*. (2000) reported that membrane deformation not only causes accumulation damage, but also causes freezing-induced hydration processes and osmotic contraction of cellular tissues, but also to physiological development of cellular tissue. Deformation Due to these potential difficulties, the temperature of the frozen process was adjusted to -16ºC in our study.

To our best knowledge, this is the first research reporting the possibility of preservation for FHB, SB and CRR causal agents by cold water and freezing for three years. The two employed revival techniques are less disordered than that for Fusarium head blight and spot blotch fungal cultures stored in silica gel, sand and lyophilization mediums (Dhinegra and SincIair l985; WindeIs *et aI.,* l993; MiIosevic *et aI.,* 2007). Taking into account the results from this study and the 'decision based key', it is argued that the economically most important pathogens of cereals could be successfully stored under sterile water and by freezing.

ACKNOWLEDGEMENTS

At the end special thanks goes to the HOD of Agriculture department and DG of AECS for their sublime love and support throughout this experiment.

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