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Long-term preservation in cold water keeps viability and pathogenicity of different *Fusarium* species causing head blight

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Abstract

To develop an effective control strategy to defeat Fusarium head blight (FHB) disease, fungal isolates must be stored for long term. We then evaluated the survival, morphological and pathogenic stability of four pathogens stored as fungal suspensions (spores and hyphae) in sterile distilled water at 4°C after 96 months of preservation. It was observed that the tested 16 FHB isolates were viable after the different time points of storage (6, 12, 18, 24, 30, 36, 60, 90 and 96 months) with no morphological alteration and contamination by bacteria or other fungi. Survival was completely independent of the duration of storage. In addition, all isolates did not lose the infection ability, which could be an indicator of keeping their pathogenicity. All tested *Fusarium* isolates maintained the similar level of pathogenicity that they had prior to preservation. In general, there were no impacts on *Fusarium* radial growth, morphological characteristics and pathogenicity for all FHB isolates after 8 years of storage in cold water. These findings suggest that Castellani's method is an inexpensive and easy technique for long-term storage of *F. culmorum*, *F. verticillioides*, *F. solani* and *F. equiseti*, and can be especially utilized in the creation of stock collections of limited resources.

Keywords: microbial contamination, morphological and pathogenic stability, viability, water storage.

Abbreviations: Area under disease progress curve (AUDPC); Fusarium head blight (FHB); potato dextrose agar (PDA); sterile distilled water (SDW).

1.Introduction

Fusarium genus which is one of the most important genera of plant pathogenic fungi was described for the first time over 200 years. Since that date, it is still in the spotlight of researchers worldwide, mostly due to a diverse array of *Fusarium* pathogens which are responsible for vascular wilt, damping-off, and root rot of devastating infections in many kinds of economically important plants [1]. Fusarium head blight (FHB) is one of the most important diseases of small grain cereals including wheat and barley throughout the world. To date, more than 20 species of *Fusarium* causing head blight have been identified globally [2]. The isolates and their pathogenicity were well identified and assessed [3]. In recent years, several FHB epidemics due to the impact of changes in cereal farming systems and global warming in Asia and Australia, Europe, North America, and South America have had a devastating economic influence on agriculture, indicating that it is a main threat to world grain production [4]. One of the major concerns related to head blight is the contamination of grains with mycotoxins which are harmful to humans and livestock [3]. As a result, significant amount of research work is conducted annually on FHB disease [4]. Features related to the adaptation, population dynamics, genetic diversity, biology, and host-pathogen interactions have been analyzed over time to establish better management policies [2, 3, 4]. Long-term storage methods to preserve *Fusarium* fungi are needed [5].

The best storage method should guarantee the fungal culture survival, purity, genotypic and phenotypic stability, maintaining the identical characters of the original culture [6]. For selecting a

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fungal storage technique, both the biological traits of the fungal species as well as the resources available in the laboratory must be taken into account [7]. Usually, the classical method of storing fungal culture can be conducted by constant subculture technique on nutrient medium [8]. Unfortunately, medium and fungal cultures are dried after short duration and this necessities frequent transfer onto fresh nutrient medium. Continuous subculture maintenance over a period of time is time consuming, could suffer modification due to continuous handling, and leads to change in physiological and molecular characteristics, pathogenicity and growth rate of the isolate [9]. The most popular means of preservation today are lyophilisation and liquid nitrogen storages which ensure long-term viability, purity and pathogenic stability and minimize genetic alterations of most large collections [10]. However, these both storage techniques are costly and require specialized equipment [6].

Over 80 years ago, pieces and/or mycelium of fungal cultures and suspensions of fungal spores had been reserved submerged under large volumes of sterile distilled water (SDW) in sterile tubes and left at ambient temperature (25-28°C) [11]. Ideally, strains preserved in water should remain viable over prolonged durations but be dormant by slowing down fungal metabolism under restricted oxygen access in order to inhibit the accumulation of mutations that lead to biochemical and morphological modifications [10]. It is simple, easy and reported by several researchers as able of keeping survived strains of plant pathogenic fungi for durations of up to 20 years without loss or change of morphological traits and viability [5, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20]. However, few reports have studied the impact of Castellani's technique for long duration on pathogenicity of fungal strains [21, 22, 23, 24]. SDW method appears to be the most appropriate for small collections with little funding [6].

One study has shown that *Fusarium* species causing head blight were stored in sterilized distilled water successfully for up to 60 months, highlighting the integral preservation of viability, purity and morphological traits. Sakr [5] reported that cultures of *F. culmorum*, *F. verticillioides*, *F. solani* and *F. equiseti* were viable in SDW at room temperature for 3 years. This simple and inexpensive method was also used to preserve these four FHB species on filter paper disks carrying fungal mycelium at 4°C [5].Viability and morphological stability of *Fusarium* species was achieved upon preservation as a fungal suspension at 4°C for up to 60 months. In addition, to the best of our knowledge and based on information available in the scientific literature, there have been no studies to date regarding SDW storage effect on pathogenicity of FHB species. This work was aimed for the first time to evaluate the survival, pathogenic and morphological stability of FHB isolates of four *Fusarium* pathogens preseved as fungal suspensions (spores and hyphae) in SDW at 4°C for 8 years.

2. Materials and Methods

2. 1. Fungal isolates

The pathogens were originally isolated from wheat heads showing head blight disease symptoms through the 2015 growing season in Ghab Plain with a FHB history, one of the principal Syrian wheat production areas. Six *F. solani*, five *F. culmorum*, four *F. verticillioides* (synonym *F. moniliforme*) isolates and one *F. equiseti* isolate were monosporic-derived cultures of the original field-type isolates. We used 16 fungal isolates of four *Fusarium* pathogens based on their unique morphological and pathological characteristics [25]. On 9 cm-Petri dishes with potato dextrose agar (PDA, HiMedia, HiMedia Laboratories) with 13 mg/l kanamycin sulphate added after autoclaving, the isolates were morphologically identified with the aid of the Leslie and Summerell [26] manual on the basis of microscopic studies of the shape and size of macro- and micro-conidia, and were molecularly distinguished by random amplified polymorphic markers [27]. *Fusarium* monosporic-derived cultures were grown separately in Petri dishes in an incubator (JSPC, JS Research Inc., year of appliances: 2004) for 10 days at 22 ± 1ºC in the dark to allow mycelial growth and sporulation for testing cold water storage method. The first morphological and pathogenic characterization was carried out in the "day zero" and this was used as a standard for assessing the alternations in viability, purity, micro-morphology, macro-morphology and pathogenicity over 8 years of the experiment.

2.2. Preservation in SDW

FHB cultures with suitable growth were covered with 10 ml of SDW (autoclaved at 121°C for 15 minutes) and gently agitated to release fractions of hyphae and spores. The fungal suspension obtained in this manner was aseptically transferred to sterile

clear glass ampoules, which were then hermetically closed and sealed with 2 cm Parafilm strips (Pechiny, Thomas Scientific) to stop water evaporation, and then maintained at 4°C. The success of SDW storage system was assessed by monitoring the growth of the FHB fungi on freshly prepared PDA culture. After 6, 12, 18, 24, 30, 36, 60, 90 and 96 months of storage, fungal isolate' viability, morphological characteristics, and purity were evaluated, while pathogenic stability was evaluated after 96 months of storage.

For survival evaluation, the isolates were removed from storage at the specified periods; 100 µL of fungal suspension was collected under aseptic conditions with a micropipette from sterile sealed glass ampoules and placed into PDA Petri dishes and placed at the above-mentioned conditions to allow mycelial growth and sporulation. Survived cultures were tested for (1) morphological observation (agreement of presence/absence of chlamydospores, the shape and size of the macroconidia, and supporting structures of the microconidia and macroconidia with the previously known identification), (2) contamination by bacteria or other fungi that alter *Fusarium* cultures and (3) pathogenicity.

2.3. Viability test

Viability was determined by inoculating each preserved culture onto PDA followed by incubation at 22±1ºC under darkness for 10 days at the different time points of storage up to 96 months. A mycelium colony formed from each FHB culture indicated that the culture was survived. In a non-survived, no fungal growth was observed.

2.4. Pathogenicity test

Pathogenicity tests of all stored isolates and controls (non-stored) were carried out on a barley cultivar, Arabi Aswad (AS), showing a higher level of quantitative resistance to FHB [25, 27], using the Petri-dish method to evaluate pathogenicity of FHB pathogens under *in vitro* conditions [25]. Sterilized AS seeds were inoculated with a suspension of conidia at 1×10^6 conidia per ml for 16 fungal isolates (non-stored and stored) in Petri-dishes with sterile double-layer filter paper (Filtrak, Thermo Fisher Scientific Inc.), and arranged in a complete randomized design with three replicates and the experiment was repeated twice. Under the fungal inoculum in the slanting Petri-dish, AS seeds were submerged. Then, they were immediately aligned on the filter paper with the embryo turned upwards. To ensure high relative humidity and low air movement, Petri-dishes were hermetically closed and sealed with 2 cm ParaFilm strips. Infected treatments were incubated at 22ºC under continuous darkness. The pathogenicity of all stored isolates and controls (non-stored) was evaluated by area under disease progress curve (AUDPC). It was measured as disease progress over 6 days post inoculation (dpi) and its value ranged from 0 (not aggressive) to 1 (very aggressive) and calculated from the percentage of healthy coleoptiles as a function of time (from 2 to 6 dpi).

2.5. Data analysis

Analysis of variance was used to evaluate the impact of storage on pathogenicity of a set of 16 fungal isolates of four *Fusarium* species using DSAASTAT add-in version 2011. The differences in the means were compared at $P<0.05$ using Student's t-test.

3. Results and Discussion

To guarantee that pathogen culture collections are not lost because of insufficiency of reliable and adequate storage techniques, it is crucial that protocols used to maintain and develop cultures in the collection are suitable for each pathogen [6, 8]. In spite of the unstable origin of some fungi, the final objective of any storage methodology should be to guarantee the long-term viability of a pathogen with a minimal amount of alteration to its genomic and physiological integrity [6, 8], while reducing resources required to store large collections. There are just a few studies on the storage of *Fusarium* pathogens causing head blight [5]. In addition, reports available in the literature do not use pathogenic techniques to assess the FHB isolate' features after their long-term storage [5]. Our results show that cold water storage is a viable method for long-term preservation of all analyzed isolates of *F. culmorum*, *F. verticillioides*, *F. solani* and *F. equiseti* causing no lack to viability or significant alterations to pathogenicity, requiring little funding of resources to frequently preserve isolates over 96 months period. Furthermore, all isolates preserved in a collection are utilized irregularly; therefore, having a storage technique that can preserve them with no routine handling is valuable.

A decision key for fungal pathogens that help in choosing the best preservation method was published three decades ago by Ryan *et al.* [28].

An investigation of this lead linked with FHB pathogens shows that while these fungi do not have motile spores and produce asexual structures in culture [1], then storage in water is recommended. Preservation of fractions of hyphae, asexual structures, and spores for *Fusarium* species, maintain fungal features that are similar to the parent isolate. A technique that permits for removal of material over time (such as SDW storage permits), while preservation isolates in the principal collection is highly beneficial [28], and mainly in culture collections of little resources and funding. In this research, the technique used to obtain SDW-stored *Fusarium* cultures can be carried out several times, using always the identical glass ampoule, till total utilization of fungal suspension. In addition, space occupied by the whole set of glass ampoules is very little. It has been previously shown that storage of mycelial cultures of *Fusarium* species causing head blight on Petri-dishes with PDA by freezing at -16°C can be successful after 5 years of storage, where all tested FHB isolates had high viability after that time [5]. Combining this supplemented technique with SDW storage could potentially enhances the chances of retaining both morphological stability and viability over long durations.

Culture survival is a common criterion of success for fungal storage methods. Cold SDW method was capable to keep *Fusarium* isolates recoverable (Table 1). All stored 16 FHB isolates were still survived and remained able to grow actively on freshly prepared PDA after SDW maintenance technique and times evaluated (Table 1). Overall, 100% of fungi were successfully cultured from stocks stored in SDW (Table 1). The micro and macro-morphological traits of all fungal isolates were consistent after storage on SDW compared to the original ones, and no contamination by bacteria or other fungi which damage the storage of *Fusarium* pathogen cultures was observed (Figure 1). identical viability estimates with no morphological modifications for fungi in water have been reported previously by Burdsall and Dorworthl [21] who preserved fungi for durations of 7 years with more than 90% survival, Qiangqiang *et al.* [15] who showed 89% survival for 78 isolates after 12 years of preservation, de Capriles *et al.* [13] who successfully cultured 90% of fungal isolates that had been preserved for 20 years, Borman *et al.* [10] who successfully revived 90% of fungal organisms for 21 years, and Neufeld and Oliveira [18] who preserved fungi to 11 years with more than 90% survival. In a study published by Borba *et al.* [14] who kept for 23 years four strains of *Sporothrix schenckii*, a medical species causing sporotrichosis, in SDW and all fungal strains were revived on Sabouraud glucose agar medium. According to the authors, fungal strains remained capable to develop with unchanged morphological features. Importantly, viability was completely independent of the period of preservation in our study, indicating that survival is possibly pre-determined by the age or state of the inoculum that was utilized to prepare the vial. Selection of sufficient *Fusarium* suspension composing of adequate amounts of pieces and several wefts of hyphae and spores suspended in SDW and actively growing mycelium is the most crucial component affecting viability in SDW storage for long durations [12]. In most fungi, the cultures remain in a stage of artificial dormancy as water decreases fungal metabolism to very low levels by preventing desiccation and diminishes gas exchange. Consequently, the culture survives when transferred to fresh medium besides avoiding the accumulation of mutations leading to biochemical and morphological changes [8].

The origin of the fungal species preserved in SDW may play a determined role for survival [6]. SDW medium has been utilized largely to preserve fungal cultures either at lower temperatures between 4°C and 5°C or at room temperature [5, 11, 20]. As observed in other water storage reports, preservation of the vials at ambient temperature have affirmed that storing the cultures at room temperature is more suitable for long term preservation than lower temperature. At room temperature, Ko [16] showed that cultures of *Phytophthora cinnamomi*, *P. parasitica* and *P. palmivora* were survived in SDW for 6 to 23 years. Some pathogens of *Phytophthora* involving *P. parasitica* which were stored at 5°C survived one year in water while isolates of *P. colocasia* and *P. infestans* survived only 2-6 months [17]. Comparable observation was showed for *Gaeumannomyces graminis* in which ambient temperature was appropriate for survival and refrigeration was deleterious in the survival of *G. graminis* [22]. On the other hand, higher radial growth rates for survived *Botryosphaeria* isolates were obvious in treatments at the lower temperatures of 4ºC relative to ambient temperature (approximately 20ºC) [19].

Table 1. Viability of 16 fungal isolates for four *Fusarium* head blight preserved in sterile distilled water at 4°C

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

Viability response of 16 fungal isolates over 6, 12, 18, 24, 30, 36, and 60 months was analyzed previously and cited by Sakr [5], however, the viability response in the current study, was reanalyzed of 16 fungal isolates at the following different time points of storage (6, 12, 18, 24, 30, 36, and 60 months). +: Presence of colony growth.

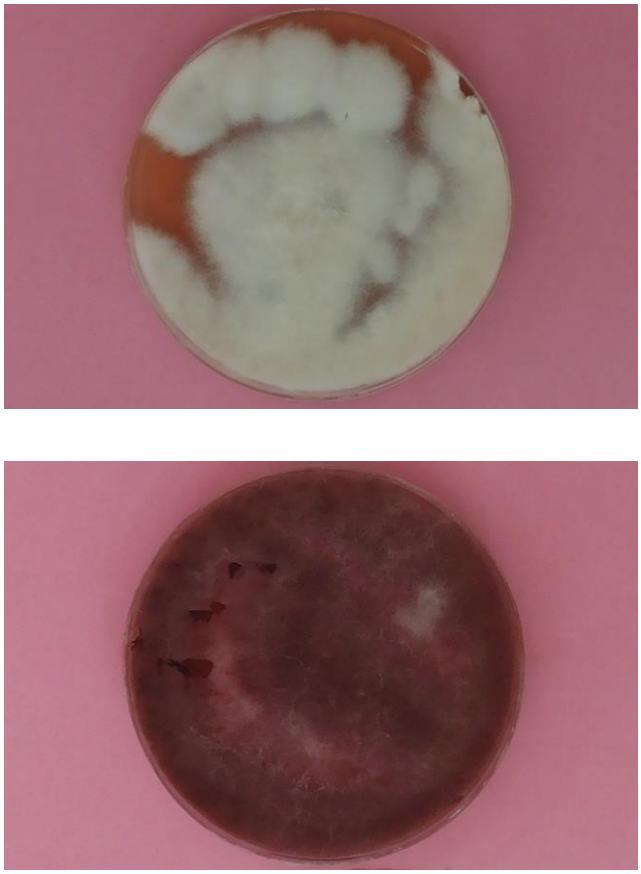


Figure 1. Survived *Fusarium* cultures of two isolates: F2 (*F. culmorum*) and F29 (*F. solani*) onto freshly prepared potato dextrose agar culture recovered from cultures preserved for 96 months in sterile distilled water at 4°C

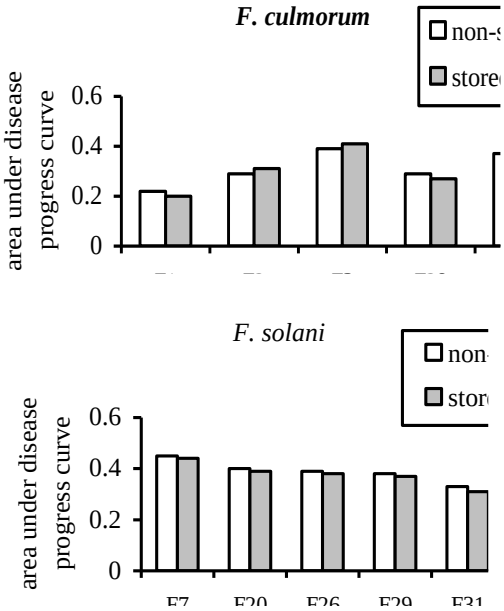
Our previous study [5] confirmed that SDW included fungal suspension all the tested FHB isolates at ambient temperature was effective for 100% survival of for 3 years, suggesting that *F. culmorum*, *F. verticillioides*, *F. solani* and *F. equiseti* can actively survive on both: room temperature or lower temperature. However, drying-up and contamination of the mycelial suspension were observed in glass ampoules preserved at room temperature under our experimental conditions (Sakr unpublished data). Perhaps, the

airtightness of the tube is influenced by fluctuations in the environmental temperature or the analyzed *Fusarium* species do not survive for long durations at higher temperatures i.e., 22 ± 2ºC, this indicates that low temperature is appropriate for SDW storage of FHB pathogens.

Based on the findings involved in the present research, SDW preservation did not seem to influence pathogenicity of the 16 FHB isolates, even after 8 years (Figure 2). All isolates preserved their overall pathogenicity assessed in terms of the AUDPC on a moderately resistant barley cultivar, showing that no lack of pathogenic ability was observed (Figure 2). Similarly, there were no impacts on pathogenicity for *Harpophora maydis* and *Gaeumannomyces graminis* strains upon SDW storage for 2 and 10 years, respectively [22, 24].

In contrast, there was decrease in pathogenicity of the *Phytophthora* cultures after preservation in SDW for more than 5 years relative to the original growth [23].

It appears that SDW technique does not lead to alterations in physiological features and chromosome mutations that decrease pathogenicity; cold water do not permit to diverse patterns of intermingling of the nuclei and hence no modification in physiological expression is observed [29, 30]. The main recovery mechanism of the *Phytophthora* species during preservation was production of thick spherical chlamydospores [23]. However, additional studies are required to clarify the mechanisms which keep *Fusarium* pathogenicity following SDW storage for prolonged duration. It is mainly accepted that pathogenicity is a character easily lost by fungal isolates in axenic medium [8]. Previous studies have reported a reduction in the pathogenicity of fungal isolates proportional to an increase in storage time [29, 30]. In spite of the exact cause of this decrease is not known, it may be linked to prolonged duration of fungal inactivity or loss of a host stimulus for development. The only tool of recovering pathogenicity of fungal pathogens is by consecutive passages through the host plant [23]. The differences in the origin of fungal species in stress suffered during the reactivation and retention time could be determined to understand the pathogenic capacity to restore over prolonged durations [22].



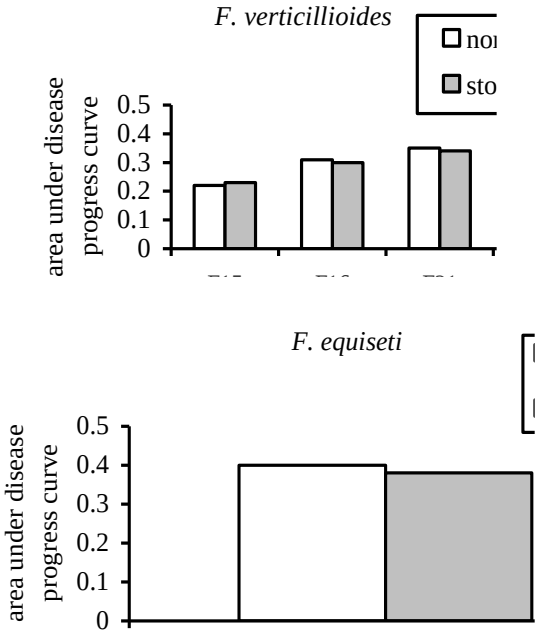


Figure 2. Pathogenicity rating, measured by area under disease progress curve (AUDPC) under *in vitro* conditions, of non-stored and stored isolates in sterile distilled water at 4°C and of four Fusarium head blight on barley cultivar Arabi Aswad, AS. According to the Student's t-test, means followed by the same letter for each fungal isolate are not significantly different at $P<0.05$. In the current study, the disease response of all tested non-stored fungal isolates was reanalyzed for AUDPC on AS; however, AUDPC of fungi was analyzed previously and cited by Sakr [25]

4. Conclusion

The present report shows for the first time the influence of the method of introduction of the isolates into the cold SDW on the pathogenic and morphological features of *Fusarium* pathogens preserved for up to 8 years. The morphological traits, purity, and pathogenic integrity of *F. culmorum*, *F. verticillioides*, *F. solani* and *F. equiseti* monosporic cultures were maintained by storage in SDW at 4°C, showing that this technique was more effective to store these fungal species. This system necessity little storage space and provides an rapid, easy, and inexpensive way of preservation a large fungal culture collection and creation of stock collections of limited resources and funding. We will continue this research to definitively determine the exact length of time *Fusarium* cultures in SDW at lower temperatures can be preserved.

Compliance with Ethics Requirements. Author declares that he respects the journal's ethics requirements. Author declares 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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