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EIGHT YEARS PRESERVATION, MORPHOLOGICAL STABILITY AND PURITY OF *COCHLIOBOLUS SATIVUS*

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A B S T R A C T

While spot blotch and common root rot caused by the fungus *Cochliobolus sativus* are destructive diseases of wheat and barley which lead to significant yield losses globally, few publications have appeared on preservation which is important in understanding relationships of this pathogen with its host. The aim of this study was to preserve 32 isolates causing spot blotch and 22 isolates causing common root rot differing in their phenotypical characters in two different methods: the storage treatments 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. The viability, morphological alteration and contamination by other microorganisms were then verified: phenotypic characteristics were evaluated qualitatively at 6, 12, 18, 24, 30, 36, 60, 96 months. All *C. sativus* remained viable and the morphological alteration and contamination by bacteria or other fungi were not observed after 8 years of storage in cold water and by freezing. Neither time in storage nor isolate of two diseases was associated with a lack of viability. This study helps to retain viability and morphological stability for 55 isolates of *C. sativus* by the two tested storage methods for over 96 months.

Keywords: freezing, fungal contamination, morphological stability, viability, water storage.

INTRODUCTION

The preservation of plant pathogens in a stable condition is an essential aspect for systematics, epidemiology, pathogenesis and genetic studies; the main characteristics that need to be preserved are purity, viability, and genetic stability (Abd-Elsalam *et al.,* 2010). For example, fungal pathogens stored for long periods in ideal conditions could be used in studies of plant-pathogens interactions dealt with enhancing of host resistance by mineral nutrition to fungal invasion in small-grain creels (Sakr and Kurdali, 2023; Sakr and Mohamad, 2023). Preservation can be done usually by continuous subculture method and storage at 4°C, unfortunately fungal culture and medium are dried after short period and then new sub culturing is needed (Ryan and Smith, 2004). This method of preservation is time consuming and labor-intensive job. Also, physiological and molecular characteristics may be changed as well as the chances of contamination may also be increased (Ansari *et al.,* 2011). To overcome these problems, various long term methods have been investigated, even though results are varied.

The best preservation mediums for the storage for many years of numerous fungal isolates are accepted as lyophilization or cryopreservation in liquid nitrogen or in a mechanical deep freezer (Milosevic *et al.,* 2007; Homolka, 2013). Nevertheless, these methods require complex and expensive equipment; the mechanical deep freeze uses electricity, and liquid nitrogen must be replenished regularly. Therefore, simple, rapid, and inexpensive preservation techniques have been tried successfully for different fungal species as storage in sterile distilled water, silica gel, sand, and by freezing (Borman *et al.,* 2006; Arabi*et al.,* 2007; Salustiano *et al.,* 2008; Garcıa-Garcıa *et al.,* 2014). For all stock cultures, no single storage

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technique has been conducted efficiently due to the particular characteristic of each species (Ansari *et al.,* 2011). Indeed, the employed preservation method should be supplemented by a second storage method to increase the chance of retaining both viability and morphological stability for several years (Borman *et al.,* 2006). The choice of maintenance treatment depends on fungal species, availability of resources, and the final objective of the research (Abd-Elsalam *et al.,* 2010).

Spot blotch and common root rot caused by the fungus *Cochliobolus sativus* are destructive diseases of wheat and barley which lead to significant yield losses globally (Agrios, 2004). Due to its widespread distribution and often destructive effects *C. sativus* has been studied in a wide range of areas (Kumar *et al.,* 2020), and interest in this fungus continues. Studies on population genetics or fungicide resistance of this fungus are important to establish better control strategies. These studies are based on experiments using large numbers of field isolates, originating from laboratory fungal collections, which should be properly conserved. Since the variability of the fungus is well documented, a reliable preservation method of cultures is needed. Storage of causal agents of spot blotch disease has gained little attention. Methods currently used to preserve *C. sativus* use sterile water, sterile soil, and silica gel. Arabi *et al.* (2007) observed that viability and virulence were maintained for three spot blotch isolates for 2 years of storage in sand and silica gel, but a risk of mutation was found. Recently, Sakr (2020) demonstrated that *C. sativus* species causing spot blotch and common root rot is maintained in sterilized distilled water and by freezing successfully for up to 60 months, showing the integral maintenance of viability, purity and morphological characteristics. Sakr (2020) reported that cultures of *C. sativus* were viable in sterile distilled water at room temperature for 3 years. This simple and inexpensive method was also used to preserve *C. sativus* on filter paper disks carrying fungal mycelium at 4°C (Sakr, 2020). Viability and morphological stability was achieved of *C. sativus* upon preservation as a fungal suspension at 4°C and by freezing at -16°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 water and freezing storage effect on survival of *C. sativus* for longer storage periods. Here we extend this study to examine viability and morphological stability for the same isolates (previously evaluated for five years' period) after being stored for 96 months in sterile distilled water and freezing.

MATERIALS AND METHODS

Fungal isolates: We used 55 fungal isolates of *Cochliobolus sativus* based on their unique morphological and pathological characteristics. These *C. sativus* isolates were recovered in 2015 from both: naturally infected barley leaves showing spot blotch symptoms (32 isolates) and barley plants showing common rot root symptoms (22 isolates) in different locations of Syria. Identification for 55 *C. sativus* isolates used in the current research was represented in Tables 1 and 2. The fungi were grown separately in 9 cm Petri dishes containing potato-dextrose agar (PDA, HiMedia Laboratories) for 10 days at 22 ± 1ºC in the dark to allow mycelial growth and sporulation.

Preservation in sterile distilled water: For 54 *C. sativus* isolates, the fungal suspension composed of spores and hyphae was prepared by gently agitating 10 ml of sterile distilled water on the surface of Petri dishes containing fungal cultures reaching suitable growth. In an aseptic chamber, the inoculation of sterile glass ampoules, hermetically closed and sealed with 2 cm Parafilm strips (Pechiny, Thomas Scientific) to prevent water evaporation, containing fungal suspension was conducted. Then, sterile glass ampoules were stored at 4°C.

After maintenance over 96 months, 100 µL of fungal suspension were seeded in Petri dishes with PDA and incubated in conditions mentioned above to allow mycelial growth. If the fungal cultures grew, they were recorded as viable.

Preservation by freezing: Methods for freezing assay were carried out as reported previously by Sakr (2018, 2019, 2020) to assess the effects of freezing on the viability and other criteria of the same isolates over 5 years of storage at -16°C. The samples of PDA-Petri dishes containing fungal cultures reaching suitable growth for 54 C. *sativus* isolates were frozen at -16°C. After preservation for 96 months, stored and frozen Petri dishes were thawed at 4°C for 24 hours. Five agar plugs (5 mm diameter) of the colony were cut of for each culture, then placed onto the surface of PDA Petri dishes and incubated in conditions

mentioned to allow mycelial growth for viability test. Isolates exhibiting survival of at least one out of five agar plugs were considered viable.

For the two maintenance methods, viable cultures were examined for morphological aspects (agreement of colony characteristics with the previously known identification) and contamination by bacteria or other fungi.

STATISTICAL ANALYSES

Experimental data were analyzed using DSAASTAT add-in version 2011.The Fisher's least significant difference test LSD test was used to compare storage data at P>0.05.

RESULTS

The experiment showed that 54 eight-year old water-stored at 4°Cand frozen cultures at -16°Cdid grow when sub-cultured on PDA and incubated at 22°C for 10 days (Tables 1 and 2). There was no loss of vitality or recovery ratio (100%) for two storage methods for 54 *C. sativus* isolates for the tested period (Tables 1 and 2). For each isolate, no significant differences at P>0.05 were indicated among storage times and storage methods (Tables 1 and 2). All tested isolates were found to be pure by showing the absence of contamination by bacteria or other fungi which harm the preservation of phytopathogenic colonies (Figures 1 and 2). Moreover, the viable cultures maintained their morphological features corresponded to the original description (Figures 1 and 2).

Table 1. Viability (%) of 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
C.S. 14 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 27 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 32 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 92 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 20 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 2 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 80 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 7 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 18 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 30 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 93 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 16 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 87 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 83 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 45 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 11 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 9 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 15 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 26 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 59 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 17 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 34 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 21 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 89 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 53 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 86 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 74 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 49 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 9 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a

C.S. 12 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 63 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 55 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 41 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 50 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 37 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 36 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 24 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 23 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 44 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 48 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 52 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 13 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 6 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 38 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 25 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 46 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 47 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 51 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 8 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 40 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 1 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 10 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 5 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 28 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a

According to the Fisher's LSD test, means among the storage times within a row followed by the same letter are not significantly different at P>0.05 for each isolate. Viability response of 54 fungal isolates over 6, 12, 18, 24, 30, 36, and 60 months was analyzed previously and cited by Sakr (2018, 2019, 2020), however, the viability response in the current study, was reanalyzed of 54 fungal isolates at the following different time points of storage (6, 12, 18, 24, 30, 36, and 60 months).

Table 2. Viability (%) of 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
C.S. 14 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 27 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 32 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 92 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 20 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 2 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 80 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 7 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 18 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 30 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 93 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a

C.S. 16 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 87 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 83 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 45 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 11 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 9 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 15 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 26 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 59 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 17 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 34 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 21 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 89 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 53 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 86 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 74 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 49 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 9 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 12 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 63 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 55 (spot blotch)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 41 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 50 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 37 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 36 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 24 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 23 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 44 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 48 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 52 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 13 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 6 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 38 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 25 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 46 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 47 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 51 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 8 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 40 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 1 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 10 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 5 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a
C.S. 28 (common root rot)	100a	100a	100a	100a	100a	100a	100a	100a

Viability response of 54 fungal isolates over 6, 12, 18, 24, 30, 36, and 60 months was analyzed previously and cited by Sakr (2018, 2019, 2020), however, the viability response in the current study, was reanalyzed of 54 fungal isolates at the following different time points of storage (6, 12, 18, 24, 30, 36, and 60 months).

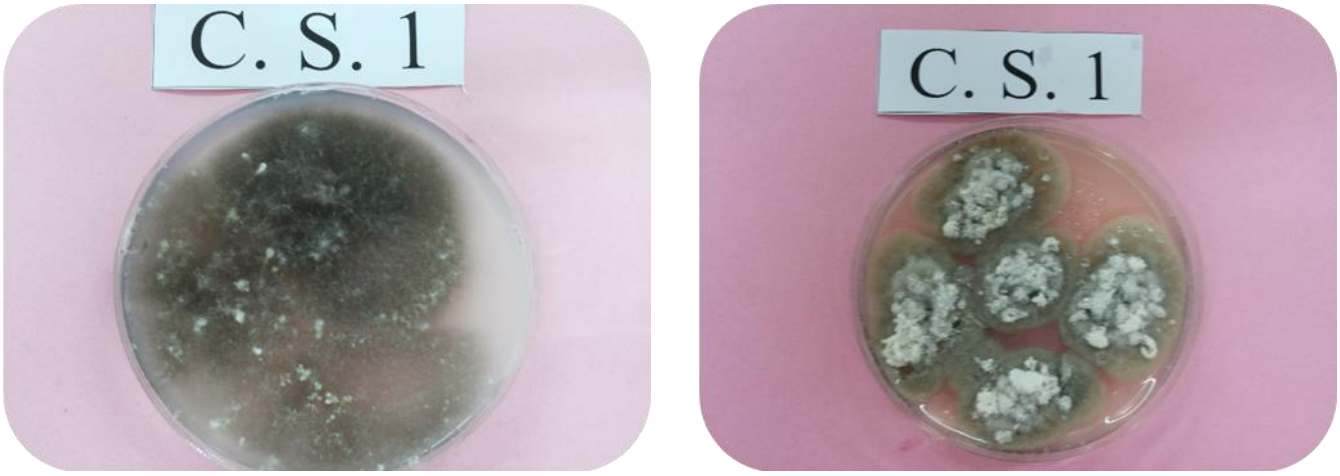

Figure 1. Fungal cultures of an isolate, C.S. 1 (*Cochliobolus sativus*) causing common rot root, on Petri dish with potato-dextrose agar obtained from cultures preserved in sterile distilled water at 4°C (left side) and freezing at -16°C (right side) for 96 months.

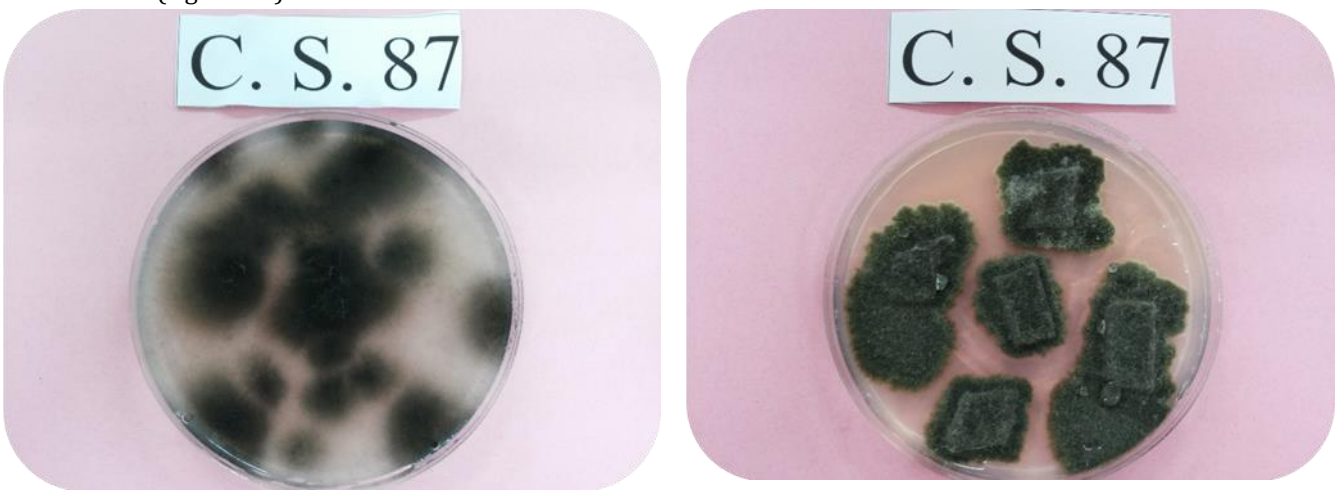

Figure 2. Fungal cultures of an isolate, C.S. 87 (*Cochliobolus sativus*) causing spot blotch, on Petri dish with potato-dextrose agar obtained from cultures preserved in sterile distilled water at 4°C (left side) and freezing at -16°C (right side) for 96 months.

DISCUSSION

The preservation of fungal isolates as reference stocks for ongoing research requires that the stored cultures remain viable for long time periods without any morphological or physiological alterations (Abd-Elsalam *et al.,* 2010). Plant pathogens differ from each other in terms of resistance to preservation conditions and techniques. Preservation conditions, i.e., temperature range effects on the viability of preserved fungi. At room temperature, Ko (2003) showed that cultures of *Phytophthora cinnamomi, P. parasitica* and *P. palmivora* were survived in water storage 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 (Sutton *et al.,* 2007). 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* (Elliott, 2005). 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) (Baskarathevan *et al.,* 2009). Our previous study (Sakr,

2020) 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. In addition, Naseri *et al.* (2008) found that temperature significantly affected germination of, and hyphal growth from, ascospores of *Leptosphaeria maculans,* which causes blackleg (phoma stem canker), in the controlled-environment conditions. The techniques, in turn, differ mainly in relation to the physiological or vital state that maintains the cells, the time of preservation that they allow and the type of equipment and labor specialization required for their realization (Smith and Onions, 1983). More economical plant pathogen preservation techniques should be tested due to expensive procedures of cryopreservation and lyophilization methods which are not necessarily available in all mycology laboratories (Milosevic *et al.,* 2007; Homolka, 2013). In this study, two preservation techniques namely sterile distilled water and freezing storage were used to determine the suitability of the techniques for causal agents of spot blotch and common root rot for 96 months. Indeed, Sakr (2020) tested the viability of the same isolates for the two storage methods used in the current research for a period of five years.

Before 30 years later, a decision key was published by Ryan *et al.* (2000) for fungal pathogens that help in choosing the best preservation method. A decision-based key has been devised, which uses questions related to fungal characters and user facilities and economics to determine the most appropriate method for long-term preservation of cultures. This key should facilitate the decisions of microbiologists when considering preservation of important fungal cultures (Ryan *et al.,* 2000). An investigation of this lead linked with *C. sativus* causing spot blotch and common root rot shows that while these fungi do not have motile spores and produce asexual structures in culture (Agrios, 2004), then storage in water and by freezing is recommended. Preservation of fractions of hyphae, asexual structures, and spores for *C. sativus* species, maintain fungal features that are similar to the parent isolate. A technique that permits for removal of material over time (such as storage in water and by freezing permits), while preservation isolates in the principal collection is highly beneficial (Ryan *et al.,* 2000), and mainly in culture collections of little resources and funding.

The best preservation method is considered to be the one where no growth and reproduction can take place, but where all the structural and functional characteristics are retained (Abd-Elsalam *et al.,* 2010). It is known that repeated sub-cultures can lead to changes in some of the characteristics of fungi, such as a decrease on the ability to sporulate (Nakasone *et al.,* 2004), and some fungal species have their sporulation ability decreased when stored by freezing, when compared to storage in medium at 4°C (Mota *et al.,* 2003). For the two evaluated preservation techniques, more consistent and stable growth was achieved by two tested storage methods. The principal criterion for a successful recovery of fungal isolates was the ability to survive the preservation process. Neither time in storage nor isolate of two diseases was associated with a lack of viability. Over 96 months, 54 *C. sativus* isolates were tested in six months intervals for both water storage and freezing techniques. Richter (2008) reports that saprotrophic fungi characterized with good sporulating cultures and sufficient suspension of spores and mycelia had a higher viability in sterile cold water. Abd-Elsalam *et al.* (2010) noted that storage by freezing incorporating fungal spores and mycelium had high viability rates. Although some studies showed that freezing can lead to a decrease in spore viability, either by structural damage in spores, due to the formation of ice crystals on the freezing process prior to lyophilization (Nakasone *et al.,* 2004), or in the drying process (Horaczek and Viernstein, 2004), all *C. sativus* isolates seemed to tolerate this kind of preservation. This is true for some fungi, i.e., *Puccinia striiformis* f. sp. *tritici* causing stripe rust on cereals, a study (Naseri and Sharifi, 2019) showed fungal spore death at 0°C or lower temperatures -5°C, -10°C. Indeed, Sakr (2020) found that the viability of the same fungal isolates was 100%.

The obtained results showed that none of the two preservation methods induced morphological alterations, either macroscopic or microscopic, in the different tested isolates of *C. sativus*. Also, morphological colony characteristics of *Gaeumannomyces graminis* var. *graminis* was not influenced by water storage (Elliott, 2005). Moreover, Legard and Chandler (2000) observed no clear morphological changes in strawberry pathogenic fungi maintained by freezing at −95°C. Indeed, Sakr (2020) found that morphological alteration

and pathogen contamination were not detected for the same fungal isolates.

The stresses that cells are exposed to during freezing and thawing are well-defined (Abd-Elsalam *et al.,* 2010). 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 (Nakasone *et al.,* 2004; Homolka, 2013). Cells in suspension are exposed to hypertonic solutions during freezing and the cellular morphology and viability are determined by the rate of cooling (Nakasone *et al.,* 2004; Homolka, 2013). Based on no influence of tested conditions of freezing on culture viability and morphological aspects of analyzed isolates, we can argue that rapid and uncontrolled freezing conditions may be applied for the storage of tested 54 fungal cultures. Pathogens' conservation is based on the reversible transition between an active vital state (biosis) and an inactive (anabiosis) or low activity state (hypobiosis). The low-temperature freezing technique keeps the cell in an anabiosis state, while methods such as refrigerated sub-culturing, stock in sterile or saline water and refrigeration (4–10 °C) tend to keep the cell in a state of hypobiosis (Abd-Elsalam *et al.,* 2010). Our results agree with data observed by Legard and Chandler (2000) and Sakr (2020). Regarding maintenance by freezing, genetic damage in fungal isolates could be resulted from the multiple freeze-thaw cycles of frozen cultures (Legard and Chandler, 2000). So, in this current study, frozen PDA Petri dishes were used once as described by Sakr (2020). For frozen conidia of spot blotch isolates stored at -20°C, Arabi *et al.* (2007) found that culture viability was not best maintained for 2 years. Big fungal spores (such those for *C. sativus* used in this study) could be physically damaged and killed by freezing at -20°C, thus freezing processes were regulated at -16°C in a freezer available in our laboratory (Sakr, 2020).

To conclude, the presented results can be accepted as encouraging for the preservation of 54 isolates of *C. sativus* in a suitable condition without any morphological alterations by water and freezing methods for 8 years. Our finding suggests that while preservation in sterile distilled water at 4°C remains an easy and inexpensive method for long-term preservation of *C. sativus* causing spot blotch and common rot root, this technique should be supplemented by a second preservation method as freezing at -16°C.

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Jalal Al. Attar	:	Helping in statistics