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LONG TERM AND LEAST EXPENSIVE PRESERVATION METHODS FOR VARIOUS FUNGAL CULTURES

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A B S T R A C T

A comparative study was conducted on long-term fungal culture preservation methods. A total of eight species (112 isolates) of phytopathogenic fungi (*Alternaria alternata*, *Alternaria solani*, *Fusarium oxysporum* f. sp. *lentis*, *Fusarium oxysporum* f. sp*. capsici*, *Rhizoctonia solani*, *Lasiodiplodia theobroma*e, *Colletotrichum gloeosporioides* and *Curvularia lunata*) belonging to six genera were stored up to 2 years by using five preservation methods (silica gel, cereal grains, mineral oil, soil and distilled water) and their viability was tested periodically after 6, 12, 18 and 24 months. The morphological characters viz. colony diameter, color, texture, topography and sporulation was studied. Each plant pathogenic isolate was considered as viable if morphology of culture was same to the original culture and comparable with the identification keys documented for fungi. Fungal cultures preserved on silica gel had maintained the highest viability as compared to other methods overall. Preservation using soil, and cereal grains was recognized as good storage method. However distilled water and mineral oil storage of some of the preserved cultures showed the least viability after eighteen months. The highest revival percentages after 24 months of preservation for *A. alternata* and *A. solani* was by sterile soil (90% and 83.3% respectively), *F. oxysporum* f. sp. *lentis* and *F. oxysporum* f. sp*. capsici* (93.3% and 92.8% respectively), *L. theobromae* (77%) and *C. lunata* (63.6%) by silica gel preservation, *R. solani* (88.8%) and *C. gloeosporioides* (85%) by cereal grains. The above least expensive and long term methods for fungal preservation are beneficial for laboratories that have limited resources.

Keywords: Preservation methods, fungal culture, viability.

INTRODUCTION

Long term preservation of phytopathogenic fungi in a viable state plays a pivotal role for plant pathology, molecular and morphological identification, strategies development for new and re-emerging pathogens, breeding resistant plant and quarantine (Abd-Elsalam *et al*., 2010). Short term preservation of fungal cultures was difficult to maintain, as due to transferring of these isolates at regular intervals there might be a risk of losing their virulence, pathogenicity, cultural and biological characteristics and they also require special equipment and continuous attention (Smith & Onions, 1983b). Therefore, various practical and inexpensive long term methods were used for preserving fungal cultures (Eugenia *et al*., 2009).

No single preservation technique has been applied efficiently to all fungi. On silica gel beads fungal isolates have been preserved successfully for up to 11 years (Smith & Onions, 1983a). Phytopathogenic fungi were successfully stored for ten years by immersion in distilled water (Figueiredo & Pimentel, 1975). Subsequent reports have indicated success and wide application of the oil overlay method to preserve several different fungi (de Moraes Borba & Rodrigues, 2000). Many fungal species have been preserved for up to 10 years on cereal grains (Singleton *et al*., 1992). To preserve filamentous sporulating micro-organisms particularly those belonging to soilborne groups sterile sandy loam soil may be one of the most cost-efficient and practical ways (Booth, 1971).

The objective of the present study was comparison of viability, sporulating capability and suitability of different preservation methods employed for preservation of specific fungi.

MATERIALS AND METHODS

Studies on long term preservation methods for various phytopathogenic fungi were conducted in Fungal Plant

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Pathology Laboratory, Department of Plant Pathology, PMAS-Arid Agriculture University Rawalpindi, Pakistan. The viability, purity and morphology of the various pathogenic fungi were monitored after 6, 12, 18 and 24 months of storage.

Fungal growth medium: For the revival of cultures, Czapek dox agar medium comprising glucose: 20 g, KH_2PO_4: 0.5 g, $NaNO_3$: 2 g, $MgSO_4.7H_2O$: 0.5 g, yeast extract: 1 g, $FeSO_4.7H_2O$: 1%, agar: 20 g, distill water: 1000 mL was used and pH set at 7.3±0.2.

Preservation methods: For silica gel preservation, McCartney universal vials were filled with sterilized silica gel crystals (4g) and chilled before use. A spore suspension was prepared in cold skimmed milk (5% w/v) and with the help of pasture pipette 1 mL of suspension was added drop by drop in each vial. The vials were stored for 10 days at 25±2°C with the cap slightly loose until the silica gel was crystallized. Caps of the bottles were tightened up and stored at 4 °C (Perkins, 1962).

For preservation on cereal grains, grains were immersed in water overnight supplemented with chloramphenicol (250 µg/mL). Water was decanted off and grains were autoclaved for two consecutive days at 121°C for an hour. Mycelia disk from the margins of actively growing cultures were transferred to McCartney vials having 4g cereal grains each, incubated at 25±2°C for 10 days and stored at -20°C (Sneh *et al.*, 1991). For preservation under oil, mineral oil was autoclaved twice on consecutive days for 20 min at 121°C. Two weeks old fungal cultures grown on agar slants were completely submerged into mineral oil. The slants were kept at room temperature in an upright position and oil level were checked periodically (Elliott, 2005).

For preservation in soil, McCartney vials were one-third filled with loam soil, sterilized, allowed to cool for some time and again sterilized. Sterile distilled water was added to the culture plate and surface of colony was harvested gently to produce mycelial and spore suspension. To each bottle of soil, 1mL of the suspension was added. Incubation was done for 10 days at 25±2°C (Atkinson, 1954).

For distilled water preservation, agar disks were removed aseptically from the developing edge of the colony and transferred to sterilized McCartney vials that were filled with 15 mL of sterile distilled water. Caps of the vials were screwed tightly and stored at room temperature between 20-25±2°C (Ellis, 1979).

RESULTS

The results for various fungi after 6, 12, 18 and 24 months using five preservation methods i.e silica gel (Figure 1), sterile soil (Figure 2), distilled water (Figure 3), Mineral oil (Figure 4) and cereal grains (Figure 5) were recorded (Table 1).

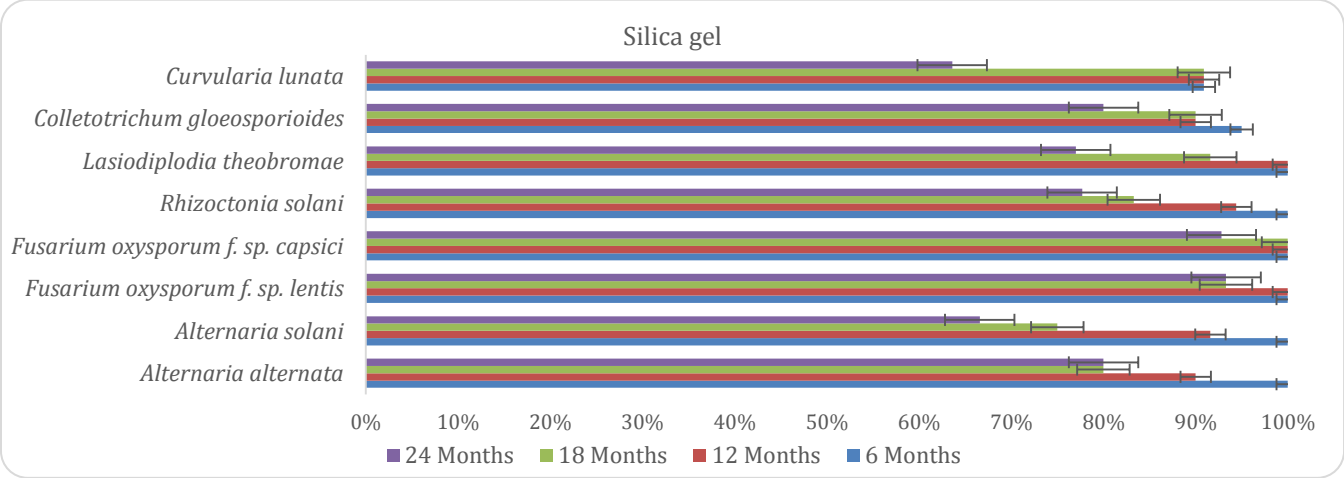

Figure 1. Percentage revival of various fungi by silica gel after 6, 12, 18 and 24 months of preservation.

The revival percentage after 24 months of preservation of *Alternaria* species (*A. alternata*, *A. solani*) had demonstrated best storage by using sterile soil (90% and 83.3% respectively), while preservation using silica gel and cereal grain were recognized as good storage alternatives. Mycelial growth and sporulating capacity of *Alternaria* sp. was less by using distilled water and mineral oil after 18 months of storage. Hyphal characteristics were observed for *Rhizoctonia solani* and best preservation of isolates was by cereal grains (88.8%) and sterile soil (83.3%) preservation followed by silica gel (77.7%), mineral oil (55.5%) and distilled water (55.5) storage.

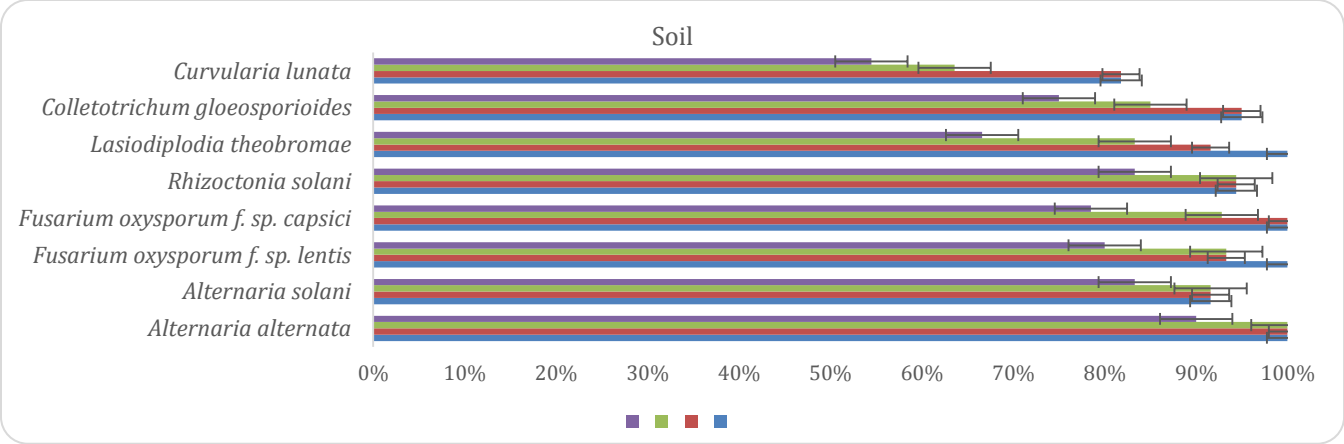

Figure 2. Percentage revival of various fungi by sterile soil after 6, 12, 18 and 24 months of preservation.

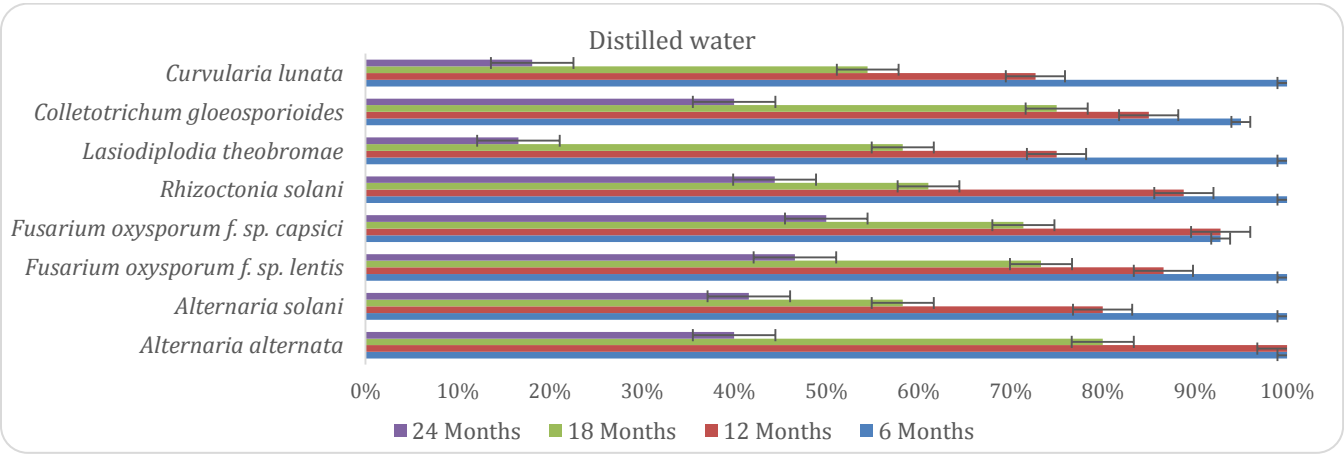

Figure 3. Percentage revival of various fungi by distilled water after 6, 12, 18 and 24 months of preservation.

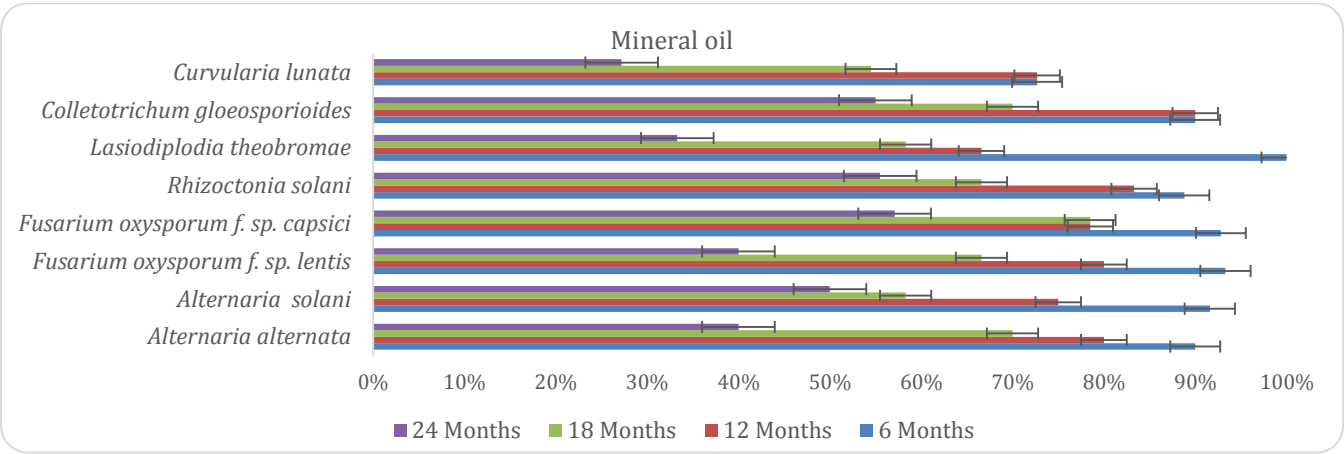

Figure 4. Percentage revival of various fungi by mineral oil after 6, 12, 18 and 24 months of preservation.

For *Fusarium oxysporum* isolates, silica gel and soil storage have achieved more stable growth while cereal grains, distilled water and mineral oil storage have achieved unstable and less mycelial growth on artificial medium. Best preservation of *Lasiodiplodia theobromae* was by silica gel (77%), followed by soil (66.6%), cereal grain (50%) and distilled water (40%). Preservation by mineral oil (16.6%) had shown stunted growth and recovery ratio was less. Conidia of *Lasiodiplodia theobromae* were not formed by using all the 5 preservation methods after 6 months of preservation. *Colletotrichum gloeosporioides* was revived successfully from cereal grains (85%), silica gel (80%) beads and soil (75%), while unstable growth was observed by mineral

oil (55%) and distilled water (40%) preservation. *Curvularia lunata* was preserved best by using silica gel and cereal grain method (63.6%). Good storage was achieved by using soil (54.5%), while preservation under mineral oil (27.2%) and distilled water (18.1%) had shown least growth rate, conidia formation and loss of certain valuable cultural characteristics.

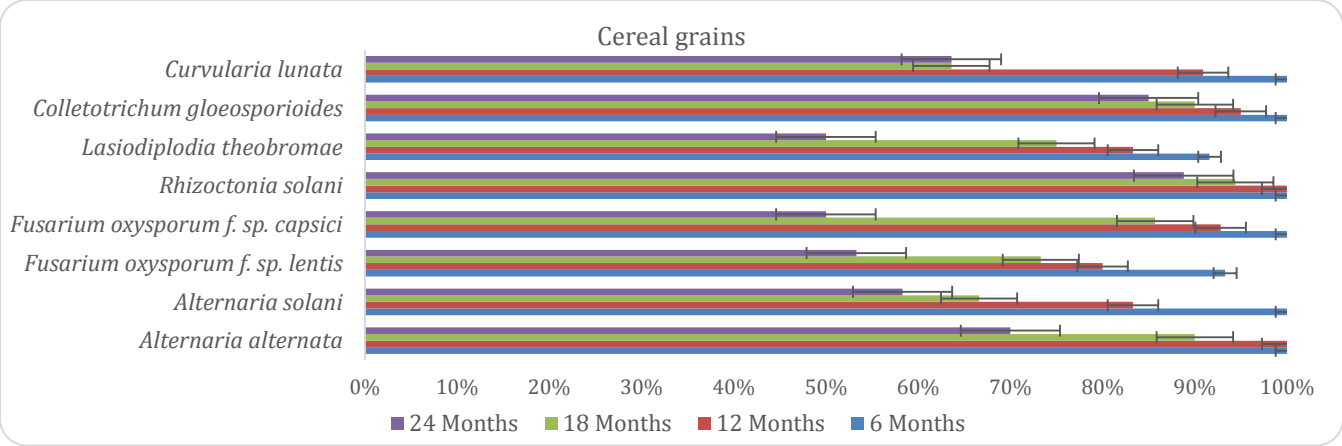

Figure 5. Percentage revival of various fungi by cereal grains after 6, 12, 18 and 24 months of preservation.

Fungi	Silica gel				Soil				Distilled water				Mineral oil				Cereal grains			
	6M*	12M	18M	24M	6M	12M	18M	24M	6M	12M	18M	24M	6 M	12M	18M	24M	6M	12M	18M	24M
A. alternata	100	90	80	80	100	100	100	90	100	100	80	40	90	80	70	50	100	100	90	70
A. solani	100	91.6	75	66.6	91.6	91.6	91.6	83.3	100	80	58.3	41.6	91.6	75	58.3	50	100	83.3	66.6	58.3
F. oxysporum f. sp. *lentis*	100	100	93.3	93.3	100	93.3	93.3	80	100	86.6	73.3	46.6	93.3	80	66.6	40	93.3	80	73.3	53.3
F. oxysporum f. sp. *capsici*	100	100	100	92.8	100	100	92.8	78.5	92.8	92.8	71.4	50	92.8	78.5	78.5	57.1	100	92.8	85.7	50
R. solani	100	94.4	83.3	77.7	94.4	94.4	94.4	83.3	100	88.8	61.1	44.4	88.8	83.3	66.6	55.5	100	100	94.4	88.8
L. theobromae	100	100	91.6	77	100	91.6	83.3	66.6	100	75	58.3	16.6	100	66.6	58.3	33.3	91.6	83.3	75	50
C. gloeosporioides	95	90	90	80	95	95	85	75	95	85	75	40	90	90	70	55	100	95	90	85
C. lunata	90.9	90.9	90.9	63.6	81.8	81.8	63.6	54.5	100	72.7	54.5	18.1	72.7	72.7	54.5	27.2	100	90.9	63.6	63.6

DISCUSSION

The preservation of fungal isolates in pure and viable state is of critical importance in plant pathology for morphological and molecular identification, pathogenicity and quarantine. In this study, five preservation techniques namely silica gel, mineral oil, cereal grains, soil and distilled storage were used to determine the suitability of the techniques for various fungi. For all the preservation methods evaluated, more consistent and stable growth was achieved by silica gel overall, preservation using sterile soil, cereal grains had also demonstrated the good results. While distilled water and mineral oil storage of some of the preserved cultures had revealed the least viability and loss of certain valuable cultural characteristics after twelve months of storage. Previous studies showed that,

due to intra specific variability there is no single preservation method that can be employed for all fungi (Ryan *et al.,* 2000). The selection of long term technique for preservation of plant pathogenic fungi depends upon resources, time and facilities (Manoharachary *et al.,* 2005). Long term storage of fungi under mineral oil overlay may have led to physiological and morphological changes such as sporulation capability of fungi being reduced, loss of virulence etc; as the fungi has covered by oil for long period of time (Silva *et al*., 1994). For preservation of cultures in sterile distill water, selection of good sporulating cultures and sufficient suspension consisting of spores and mycelia were the most important factors influencing survival of fungi in water over a longer period of time (McGinnis *et al*., 1974). The method of fungal preservation in sterile soil has been useful but also carries a risk of mutation which may result in loss of morphological characters and pathogenicity (Windels *et al*., 1993). Preservation on silica gel is trustworthy for maintaining genetic markers because silica gel prevents fungal metabolism and growth and reduces the chances of physiological and genetic changes (Windels *et al*., 1988).

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