Indian Journal of Agricultural Sciences **95** (4): 445–450, April 2025/Article
https://doi.org/10.56093/ijas.v95i4.149488

Evaluation of viability of arbuscular mycorrhiza fungi on wheat (*Triticum aestivum*) plant

SARITA[1], NARENDER SINGH[2], RAKESH KUMAR CHUGH[2], SUNIL KUMAR[3]*, SUMIT KUMAR AGGARWAL[4], HARMANJOT KAUR[4], KAILASH CHAND KUMAWAT[5] and SAJAD UN NABI[6]

Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana 125 004, India

Received: 12 March 2024; Accepted: 23 January 2025

ABSTRACT

Mycorrhiza is the most common type of symbiotic relationship that exists between fungi and plant roots. Arbuscular mycorrhizal fungi (AMF) plays significant role in plant development, improving soil structure, nutrient cycle and plant resilience to environmental challenges like drought and pathogens. The present study was carried out during 2020 and 2021 at Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana to evaluate the viability of different mycorrhizal fungi (*Glomus mosseae*, *G. fasciculatum*, *G. hoi* and *G. intraradices*) up to seven months after shoot removal. Mycorrhizal fungi were raised and maintained on wheat (*Triticum aestivum* L.) (variety WH-1105) roots in earthen pots and it was observed that the sporocarp population (4475) and mycorrhizal colonization (96%) were highest in *G. fasciculatum*. The results indicated that the significantly lowest viability was observed in *G. intraradices* (35.4%) and maximum in *G. mosseae* (40.1%) during 2020. During 2021, *G. intraradices* again demonstrated the lowest viability (32.9%), with *G. hoi* achieving the highest (39.2%). The viability of different mycorrhizal species was found inversely proportional to the period after shoot detachment. The findings emphasize the necessity for better inoculum management techniques to maintain AMF functionality in agricultural environments and the dependence of AMF viability on host association.

Keywords: Chlamydospores, *Glomus* spp., Mycorrhiza, Sterilized soil, Viability

Arbuscular mycorrhizal fungi (AMF) are crucial for ecosystem functioning due to their significant roles in nutrient cycling, enhancing soil structure, and supporting plant growth (Wang *et al.* 2024). AMF represent the most widespread form of mycorrhizal associations found in the world (Das *et al*. 2022). A mutual beneficial partnership has been established between higher plants roots along with a particular fungal group inhabiting the soil (Chen *et al*. 2023). AMF are strong soil-beneficial microorganisms abundant in forests, farms, grasslands etc. (Zhu *et al.* 2014, Saia and Jansa 2022). Opaque hyphae networks can form in rhizosphere root cortex cells and soil following AMF symbiosis with plants (Rani *et al*. 2018). These networks can enhance the efficiency of water and nutrient uptake by mobilizing essential nutrients, facilitate the host's direct or indirect absorption of soil mineral elements, regulate the host's metabolic processes, and encourage plant growth (Yang *et al.* 2021, Li *et al.* 2022, Ma *et al.* 2022). Furthermore, this effective symbiosis can assist host plants in surviving a during biotic and abiotic stresses, including diversity of soil borne phyto-pathogens, drought, salt and heavy metal pollution (Riaz *et al.* 2021, Ma *et al.* 2022, Weng *et al.* 2022).

Spores have been recognized as the most vigorous form of arbuscular mycorrhizal fungal propagules, and they play a role to the fungi's persistence, dispersal and growth. However, significance of these propagules and their survival can vary significantly among different fungal species and influenced by various environmental factors under changing climatic conditions. Moreover, the viability of inoculum can be influenced by the storage of AM fungal propagules in soil. Among these factors, temperature stands out as the most influential in shaping the dynamics of mycorrhizal symbiosis. Temperature affects critical processes in the AM fungal lifecycle, including spore germination, hyphal

[1]RNB Global University, Bikaner, Rajasthan; [2]Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana; [3]College of Dairy Science and Technology, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan; [4]ICAR-Indian Institute of Maize Research, Punjab Agricultural University, Ludhiana, Punjab; [5]Jacob Institute of Biotechnology and Bioengineering, Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj, Uttar Pradesh; [6]ICAR-Central Institute of Temperate Horticulture, Rangreth, Srinagar, Jammu and Kashmir.*Corresponding author email: jangirsunil90@gmail.com

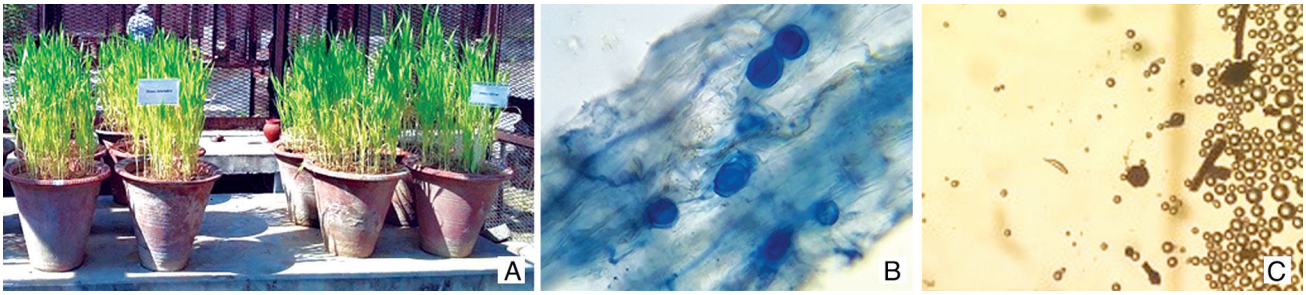

Fig. 1 (A) Wheat was grown in earthen pots for 90 days for multiplication of mycorrhiza; (B) Mycorrhizal colonization; (C) Mycorrhizal spores in soil samples.

development, root colonization, and sporulation (Weng *et al*. 2022). However, limited research data available regarding the long-term viability of spores stored for extended periods with different mycorrhizal species. The major objective of this study was to investigate the impact of extended storage on the viability of spores derived from different mycorrhizal species in shootless mycorrhizal plants.

MATERIALS AND METHODS

The present study was carried out during 2020 and 2021 at Chaudhary Charan Singh Haryana Agricultural University, Hisar (29º10'N, 75º46'E and altitude of 215.2 m amsl), Haryana.

Maintenance of mycorrhizal inoculum: The mycorrhizal inoculum used in the study was procured from the Centre for Mycorrhiza Culture Collection-The Energy and Resource Institute (CMCC-TERI) and maintained on wheat in earthen pots (top 22.5 cm and base 16.5 cm) of 5 kg capacity. Each pot was filled with 5 kg of sterilized river sand to create a medium for growth (Fig. 1). Mycorrhizal inoculum comprising 100 g soil, which contains roughly 450–500 chlamydospores and 10 g of root bits including root bits, was incorporated into top 5 cm of soil. Ten seeds of wheat (variety WH-1105) were sown in each pot, and four numbers of sets were maintained for the study. The research experiment was conducting using completely randomized design (CRD) statistical design. To provide essential nutrients, Hoagland's nutrient solution (Hoagland and Arnon 1950) (10 ml/pot) was administered every 30 days over a 90-day growth period. After 90 days, mycorrhizal colonization and sporocarp in soil was estimated. The plant's shoot part was clipped at soil level after 90 days, and the soil in the pots was allowed to dry naturally. The rootlets were divided into 1 cm segments by crumbling the dirt. This dirt served as an inoculum for mycorrhizal plants. The prepared inoculums were evaluated for its ability to sustain the viability of mycorrhizal fungi over a period of seven months without watering or nutrient supplementation.

AMF colonization: Roots were sainted by using the method described by Phillips and Hayman (1970) for estimation of AMF colonization.

Root staining procedure: The roots were sliced into one cm lengths, heated in 10% KOH for an hour at 90°C, cleaned with a fresh 10% KOH solution, and then submerged in alkaline hydrogen peroxide (H_2O_2) for half an hour. After that, it was acidified for 30 min with 5 N HCL and rinsed with distilled water to get rid of any extra H_2O_2. For five minutes, the roots were simmered in trypane blue in lactophenol (0.05%). These roots were placed in lactophenol for removal of excess dye and examine under microscope.

$$\text{Root colonization by AMF (\%)} = \frac{\text{Sum of all numerical ratings} \times 100}{\text{Total number of sample assessed} \times \text{Maximum scale}}$$

AMF root colonization rating scale: It ranges from 0, which indicates no mycorrhizal colonization (0 spores); 1 for 1–25 spores; 2 for 25–50 spores; 3 for 50–75 spores, and 4 for 75–90 spores (Jalali and Domsch 1975).

Sporocarp estimation in soil: Wet sieving and decantation techniques, as given by Gerdemann and Nicolson (1963), were used to estimate the number of sporocarp in soil. After thoroughly mixing the soil sample, 100 g of soil was suspended in a pan, and 1 L of water was added. Hold for 30 sec and then 20 mesh sieve was used to filter the suspension, and the filtrate was gathered in pan B. After stirring the B pan suspension by hand and letting it settle for a few seconds, it was run through a 60 mesh sieve. Pan C was used to collect the filtrate. Pan C suspension was run through a 100 mesh filter. Using a 100 mesh sieve, the most developed sporocarps were collected. After washing (get rid of extra dirt and other debris), the residue from a 100 mesh sieve was collected into a beaker. One milliliter of this solution was placed in a counting plate, and the sporocarp population in the soil was counted and inspected under a stereomicroscope.

Viability of different mycorrhizal fungi: The prepared inoculum of mycorrhizal fingi was filled in pots (top 22.5 cm and base 16.5 cm) and viability of mycorrhiza was evaluated at time interval of 0, 3, 4, 5, 6 and 7 months. The viability of mycorrhizal fungi was evaluated by extracting spores from a 100 g soil sample using the wet sieving and decantation method. The extracted spores were preserved in distilled water at 4°C. Viability was determined through an assay employing MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) as an indicator. Assay was prepared by using deionized water, a stock solution containing 0.5 mg MTT (3-(4,5-dimethylthiazol-yl)-2,5-diphenyl-2H-tetrazolium bromide)/ml. For several months, the stock solution can be kept in the dark area at 4˚C. A screw-cap

tube was filled with equal parts of the MTT stock liquid solution and an aqueous spore suspension (1 ml of each). After securing the cap tightly, the tube was incubated in the dark for 40 hours at 27˚C. After being removed, the spores were examined under a dark field stereomicroscope. MTT gave living spores a bright red stain. There were three replication of each treatment.

Data analysis: The data are presented as the means ± standard error of four replications. The statistical analysis of the experiment was performed using OPSTAT software (http://hau.ac.in). A two-way analysis of variance (ANOVA) was conducted to determine the significance of the factors affecting mycorrhizal viability at a 5% of significance level (P<0.05). Each treatment (*Glomus mosseae*, *G. fasciculatum*, *G. hoi* and *G. intraradices*) was replicated four times for each time period (0, 3, 4, 5, 6 and 7), resulting in a total 96 pots. For each pot 5 kg inoculum was used and spores were extracted from a 100 g soil sample from each pot in four replication was used for estimation of viability.

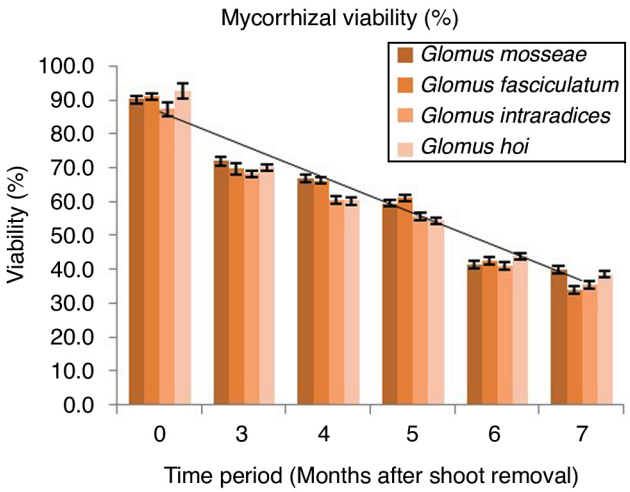

Fig. 2 Effect of viability of *Glomus mosseae, G. fasciculatum, G. hoi and G. intraradices* at variable times (means ± standard error of means) after shoot removal during 2020.

RESULTS AND DISCUSSION

In a soil sample from wheat sown pots, the sporocarp population was highest in *G. fasciculatum* (4475), then succeed by *G. mosseae* (4046), *G. intraradices* (3921) and lowest in *G. hoi* (2567) in 100 g soil and mycorrhizal colonization was also highest in *G. fasciculatum* (96), succeed by *G. mosseae* (94), *G. intraradices* (90) and minimum in *G. hoi* (86) was observed. The dominant AM fungi's mass production is determined by the host plant's specific kind and the duration of fungal infection. The host type has a greater impact on AM fungal early colonization and subsequent spore generation (Sharma *et al.* 2015). Mass multiplication of arbuscular mycorrhizal fungi was done on wheat. Similarly, Kadian *et al.* (2018) experimented on wheat, barley, chickpea and jowar). *G. fasciculatum*, *G. reticulatum*, *G. fragilistatum*, *G. citricolla, G. macrocarpum*, *G. globiforum*, *G. mosseae*, *Acaulospora laevis*, *Scutellospora pellicida*, *Scutellospora auriglobosa and Scutellospora calspora* were multiplied on *Eleusina coracana* (Wankhede 2020).

To assess spores' potential as propagules, their viability must be evaluated. Germination tests are commonly used to evaluate spore viability and methods using the vital stain MTT (Minkosse *et al.* 2023). Vishwakarma and Chahar (2024) evaluated effect of benotonite and temperature on viability of arbuscular mycorrhiza fungi (*G. intraradices*) spore used in biofertilizer and viability assed by using MTT stain. Similarly MTT stain was used for assessment of viability of mycorrhizal spores (Singh and Chahar 2021).

Viability of mycorrhizal spp. during year 2020: Viability of spores depicted in the Fig. 2 constantly decreased over time after shoot removal. This decline in viability had a detrimental impact on various mycorrhizal species, including *G. mosseae, G. fasciculatum*, *G. hoi* and *G. intraradices*, when they were separated from their host plants. After shoot removal (7 months), significantly lowest viability was observed in *G. intraradices* (35.4%) while the highest was recorded in *G. mosseae* (40.1%). Among the different mycorrhizal spp. maximum viability was observed in *G. hoi* (92.6%) followed by *G. fasciculatum* (91.0%), *G. mosseae* (90.1%) and minimum in *G. intraradices* (87.3%) at 0 months of shoot removal. The effect of shoot removal on the viability of different mycorrhizal species can be attributed to the disruption of the symbiotic association among plants and AMF. These depend on the host plant for photosynthetically produced carbohydrates, they lose their main supply of carbon when the plant is absent. Over time, the fungi's viability declines as a result of this carbon constraint, which makes it harder for them to sustain their metabolic processes (Hodge *et al.* 2001, Smith and Read 2008).

After three months of shoot removal, *G. mosseae* (71.9%) had the highest and statistically significant mycorrhizal spp. viability, followed by *G. hoi* (70.0%), *G. fasciculatum* (69.6%) and minimum in *G. intraradices* (68.2%) was observed. Among all the different mycorrhizal species maximum viability was observed in *G. mosseae* (66.8%) followed by *G. fasciculatum* (66.2% and 61.2%) at 4 and 5 months after shoot removal respectively. Highest viability was found in *G. hoi* (60.3% and 54.3%) at 4 and 5 months after shoot removal respectively. Among all the different mycorrhizal spp. maximum viability was recorded in *G. hoi* (43.9%), followed by *G. fasciculatum* (42.6%), *G. mosseae* (41.3%) and the minimum was found in *G. intraradices* (41.2%) after 6 months of shoot removal.

Irrespective of mycorrhizal species, significantly highest viability was documented in *G. mosseae* (61.6%), followed by *G. fasciculatum* (60.8%), *G. hoi* (60.0%) and minimum in *G. intraradices* (58.0%). Irrespective of different observation periods (0, 3, 4, 5, 6, and 7 months after shoot removal), highest viability was recorded at 0 months of shoot removal (90.3%). The spore germination rate of AMF and other filamentous fungus was lowered when the inoculants were kept at room temperature for an

extended period of time (Liu *et al.* 2024). Production and dormancy of spores are crucial factors for ensuring long term survival and persistence of arbuscular mycorrhiza in any given environment (Varga *et al.* 2015). The concept of spore dormancy in soil is instrumental in sustaining the long term presence of spore reservoirs. Dormant spores, as per their definition, do not initiate germination, despite being subjected to the same physical and chemical circumstances that enable the germination and hyphal growth of apparently identical non-dormant spores from the same species (Juge *et al*. 2002). Dead spores that seem normal may last for a long time in soil with harsh treatments such as soil fumigation (Menge 2018).

Viability of mycorrhizal species during year 2021: The viability of mycorrhizal species demonstrated steady declines over time after the removal of the shoots, according to data on viability in the year 2021 (Fig. 3). Mycorrhizal species showed that viability of different mycorrhizal species was decreased over time after shoot removal. At 0 month of shoot removal, *G. mosseae* (94.2%) had the highest viability among all mycorrhizal species, followed by *G. hoi* (91.7%), while *G. intraradices* had the lowest vitality (90.0%). The exceptional viability and consistent outcomes can be ascribed to the *in vitro* production of spores, conducted under controlled environmental conditions and without any growth limitations. Likewise, Marleau *et al.* (2011) reported viability at 15 days of age, spore primordia testing positive with majority, and this level of viability remained unchanged during the spore maturation time of 15–90 days old. Among mycorrhizal species, maximum and significantly highest viability was recorded in *G. mosseae* (73.1%) followed by *G. hoi* (72.3%), *G. fasciculatum* (70.8%) and the minimum were recorded in *G. intraradices* (70.3%) at 3 months after shoot removal. Similarly, four months after shoot removal *G. mosseae* (63.6%) showed the highest viability while *G. fasciculatum* (60.9%) had the lowest viability among all the mycorrhizal species.

Among different mycorrhizal species maximum viability 60.1% and 45.6% was observed in *G. mosseae* at 5 and 6 months after shoot removal respectively. Lowest viability was seen in *G. hoi* (56.2% at 5 months after shoot removal) and *G. intraradices* (41.9% at 6 months after shoot removal). Among all the different mycorrhizal species, maximum viability was observed in *G. mosseae* (66.8%) followed by *G. fasciculatum* (66.2% and 61.2 at 4 and 5 months after shoot removal respectively) and the minimum was observed in *G. hoi* (60.3% and 54.3% at 4 and 5 months after shoot removal, respectively). Irrespective of mycorrhizal species significantly highest viability was recorded in *G. mosseae* (62.3%), followed by *G. hoi* (61.1%), *G. fasciculatum* (60.1%) and the minimum being in *G. intraradices* (59.2%). After shoot removal (7 months) maximum and significantly lowest viability was recorded in *G. intraradices* (35.4%) and highest in *G. mosseae* (40.1%). The viability of fungal spores is not very accurate due to the reduced viability and tendency of the spores (Shankar 2021). After shoot removal (7 months), maximum and

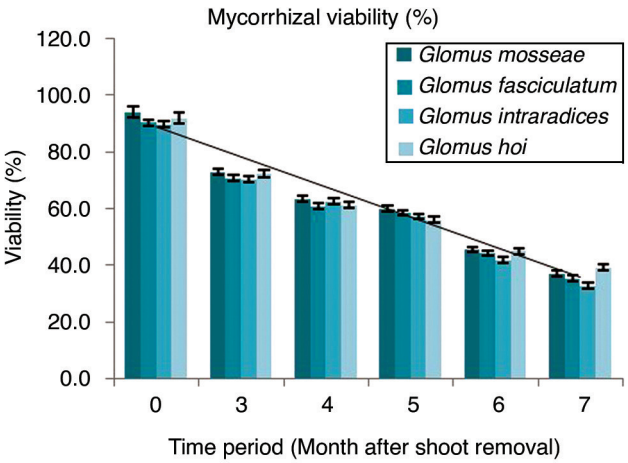

Fig. 3 Effect of viability of *Glomus mosseae, G. fasciculatum, G. hoi and G. intraradices* at variable times (means ± standard error of means) after shoot removal during 2021.

significantly lowest viability was recorded in *G. intraradices* and the highest was observed in *G. mosseae.* Since AMF spore viability decreases with time and fresh spores must be continuously formed in testing laboratories, through the long term culture of arbuscular mycorrhiza isolates. This research would be very relevant to long term applicability of the test guidelines (Mallmann 2020). Wagner *et al.* (2001) studied the effect of conditions of storage on *Glomus claroideum*, and the inoculants were stored at 4°C and 24°C for 272 weeks. They observed that spore viability reduced exponentially with storage time, and refrigeration at 4°C significantly increased the amount and viability of spores. Pepe *et al.* (2018) found that, the longevity and usefulness of *Funneliformis mosseae* and *Rhizoglomus irregular* are limited by host plant viability. Farhad *et al.* (2024) also demonstrated that MTT stain for estimation of the viability of fungal spores of *G. mosseae*, *Gigaspora margarita*, *Paraglomus brasilianum*, *G. aggregatum*, *G. clarum*, *G. deserticola*, *G. monosporus*, *G. etunicatum* and *G. intraradices*. Similarly, Kalousi *et al*. (2024) used MTT stain for the estimation of viability. An and Hendrix (1988) conducted study and found that spores from pot cultures, but, sometimes we found that the higher viability, up to 80 per cent in fresh cultures of *G. macrocarpum* was achieved, at the same time as the viability of spores stored for 5 years was 50 per cent. After a month of storageThe spore viability of the AMF biofertilizer prototype using spore carrier media volcanic sand and zeolite was high, reaching 91.78% and 90.67% (Rai *et al.* 2023). Similarly highest viability was recorded 85–100% after one month of storage (Rai *et al.* 2022). Meier and Charvat (1993) found that, the viability of *G. mosseae* cultures (fresh and stored) to be about 50%. According to An and Hendrix (1988), viability of fresh pot cultures perhaps higher but eventually drops to roughly 50%. Proper storage conditions like temperature (Wagner *et al.* 2001, Liu *et al.* 2024), light (Juge *et al.* 2002) and preservation methods (Lalaymia *et al.* 2014) can allow the spore to maintain their viability for

extended period. Consequently, it would seem that unless severe soil treatments like fumigation (methyl bromide) have been used, both native and applied AMF were non-detectable (Bendavid-Val *et al.* 1997). High temperatures cause decreased spore viability and eventually die (Setlow and Christie 2021).

The viability of different mycorrhizal species was decreased over time after shoot removal. AMF are obligate biotrophs, their metabolic processes are supported by photosynthetically produced carbohydrates from their host plants. The fungi's carbon supply is effected when shoots are removed, which eventually leads to decline in their viability over time because of energy depletion (Smith and Read 2008). While Kytoviita and Vestberg (2020) found that arbuscular mycorrhizal spores remain viable for years without a host, likely due to their rich lipid reserves and protective role of unsaturated fatty acids. This resilience may vary according to species (Sturmer *et al.* 2018) and environmental conditions (Kytoviita 2005). Klironomos (2000) observed that, greenhouse and laboratory study give evidence that little host plant specificity in host plant and AMF during pot culture. However, ecological specificity in the field can be significant, resulting in habitat-specific AMF host plant combinations (Hazard *et al.* 2013, Francini *et al.* 2014). This decline in spore viability of different mycorrhizal species, highlighting the importance of maintaining a symbiotic relationship with host plants for their survival and propagation. Among the tested species, *G. mosseae* showed the highest viability, while *G. intraradices* exhibited the lowest viability. The highest viability was recorded at 0 months of shoot removal, with *G. mosseae* having the maximum viability during the first year (2020) and *G. hoi* having the highest viability during the second year (2021).

The finding indicated that the viability of mycorrhizal species is inversely proportional to the time period after shoot detachment. There was an inverse relationship between mycorrhizal species viability and the time elapsed after shoot removal. This underscores the importance of further research into the mechanisms underlying the decline in viability and how it might impact ecosystem dynamics and plant-mycorrhizal interactions. Understanding these interactions is crucial for maintaining the health and functionality of ecosystems as well as for optimizing agricultural and horticultural practices that rely on mycorrhizal symbiosis.

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