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REGULAR ARTICLE

MAXIMIZING THE SHELF LIFE OF MONOXENICALLY PRODUCED CARRIER-BASED AM FUNGAL BIO-INOCULA FOR MAINTAINING ITS LONG-TERM VIABILITY

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi are often applied as bio-inoculants due to their plant growth promoting benefits. The objectives of the present work were to maximize the shelf life of monoxenically produced carrier-based AM fungal bio-inocula of *Rhizoglomus intraradices* and *Funneliformis mosseae*. Shelf life of *in vitro* produced inoculum was studied by assessing the infectivity potential of *in vitro* produced inocula in an optimum carrier formulation (vermiculite: cow dung powder: wood powder: wood ash in the ratio of 20:8:2:1) during storage at three different temperatures *viz.,* 4 °C, 25 °C and room temperature (RT). The re-germination potential of *in vitro* produced spores from carrier-based inocula to *in vitro* conditions was also examined. The *in vitro* produced inocula stored at 25 °C remained viable up to 6 mo in the organic carrier formulation. 100 % germination was recorded when the spores of both AM species were cultured back to *in vitro* conditions indicating high viability, and efficiency of the carrier formulation in maintaining vigour of *in vitro* produced propagules.

Keywords: *Rhizoglomus intraradices*, *Funneliformis mosseae*, Carrier-based bio-inocula, Shelf life

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are biotrophic symbionts that provide beneficial effects to a wide range of host plant species [1]. They represent a key link between soil and plants, and have gained a growing attention as ecosystem engineers and bio-inoculants [2-4]. Currently, AM fungal bio-inoculants are being used in the agricultural practices for reclamation of stress affected soils and also as an alternative for chemical fertilizers [5, 6]. Bio-inoculants are usually prepared as carrier-based inoculants containing effective micro-organisms [7-9]. AM fungal inoculum being commercially available in many forms [10-13]. Over the last decades, a considerable effort has been made to develop suitable carrier formulations of highly infective and efficient AM fungal propagules [3]. Rodrigues and Rodrigues [14] developed an optimum carrier formulation that is able to maintain high inoculum infectivity and efficiency of the *in vitro* produced isolates of *Rhizoglomus intraradices* and *Funneliformis mosseae*. Considering the carrier formulation (treatment 5 consisting of vermiculite, cow dung powder, wood powder, and wood ash in the ratio 20:8:2:1). The type of carrier materials used, their physico-chemical properties and the proportions incorporated proved that the organic carrier formulation prepared is suitable for mass production of *in vitro* produced AM fungal inocula. However, for any bio-inoculant to be used within agronomical practices, the carrier formulation should primarily retain the viability of a large population of the incorporated inoculant microorganism during long-term storage period [15]. A good carrier should also ensure sufficient shelf life of at least 2-3 mo at room temperature [16]. Non-availability of good quality and appropriate carrier materials can affect shelf life of the beneficial microbial inoculants. Drying process, moisture content, storage conditions plus storage temperature are also important determinants of the shelf life of microbial inoculants or formulations and can affect their activity pre-or post-application [17-21].

Efficient storage of bio-inoculants is a major constraint in the production of AM inoculants [22]. In general, fungi can be preserved and stored as per three procedures *viz.,* continuous growth method, drying by air or with silica gel, and reduction of available water in the cells, which results in suspension of metabolism [23]. Monoxenic cultivation of AM fungi is the best method for production of contaminant-free inocula [24]. However, sub-cultivation is being practiced for the maintenance [25-27] and contaminations risk is common problem [25]. Moreover, sub-cultivation is difficult or genetic variation may occur [28] and, genetic and physiological changes overtime cannot be prevented [29, 25, 30]. Thus, a method to maintain the viability, purity and stability of monoxenically produced AM fungal isolates over long-term storage periods is needed.

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The present study is aimed at maximizing the shelf life of monoxenically produced carrier-based AM fungal bio-inocula by assessment of infectivity potential of *in vitro* produced inocula in an optimum carrier formulation (vermiculite: cow dung powder: wood powder: wood ash in the ratio of 20:8:2:1) during storage at different temperatures (4 °C, 25 °C and room temperature), and by assessment of re-germination potential of *in vitro* produced spores from carrier-based inocula to *in vitro* conditions.

MATERIALS AND METHODS

Assessment of infectivity potential of *in vitro* produced inocula in carrier formulation during storage at different temperatures

AM fungal inocula

The indigenous AM fungal isolates (*Rhizoglomus intraradices* and *Funneliformis mosseae*) obtained from Goa University Arbuscular Mycorrhizal Culture Collection (GUAMCC) were used for the present study. Monoxenic cultures were established by the association of disinfected spores [31] with transformed roots of chicory (*Cichorium intybus* L.) or linum (*Linum usitatissimum* L.) on modified Strullu–Romand (MSR) medium [27]. The Petri-plates were incubated in an inverted position in the dark at 27 °C. Sporulation in monoxenic cultures of *R. intraradices* was observed 18-20 d after association with transformed chicory roots and continued up to 7 mo. In monoxenic cultures of *F. mosseae*, sporulation was observed 15-20 d after association with transformed linum roots and continued up to 7 mo. The monoxenically cultured spores were isolated by solubilization of the MSR medium [32] and used for storage studies.

Storage of inocula

The inocula of the AM species *R. intraradices* and *F. mosseae* containing colonized transformed root fragments with extra-radical hyphae and spores was mixed along with optimum carrier formulation consisting of vermiculite, cow dung powder, wood powder and wood ash in proportion of 20:8:2:1 (64.51%: 25.80%: 6.45%: 3.22%) and stored at three different temperatures *viz.,* 4 °C, 25 °C and room temperature (28-30 °C) to assess the viability. Inocula of the two AM fungal species were stored separately in zip-loc polythene bags. Care was taken to ensure the absence of free moisture on the inside of the bags.

Infectivity tests

Infectivity tests of the inocula were carried out at the end of every 2nd month after storage for 2, 4, and 6 mo, by incorporating 10 g of the carrier-based inoculum containing approximately 1200 infective propagules into 500 g of sterilized sand in 15 cm plastic pots and using cuttings of *Plectranthus scutellarioides* (L.) R. Br. (Coleus) (Lamiaceae) as host plant. Each treatment consisted of six replicates. The pots were maintained for a period of 3 mo in the shade net of Department of Botany, Goa University, under natural conditions of light, temperature and humidity (4000-6000 lux light intensity, 32 °C/25 °C day/night, RH 80-90 %) for the establishment of AM symbiosis.

Processing of root segments for AM fungal colonization

Assessment of AM colonization in roots of *P. scutellarioides* was carried out by using Trypan blue staining technique after 3 mo of growth [33]. The infectivity of the stored inocula was ascertained by the same procedure at every 2nd month up to the 6th month.

Estimation of percent root colonization

Estimation of percent root colonization by AM fungi was carried out using root slide method [34].

Percent colonization = number of root segments colonized ÷ total number of root segments observed × 100

Statistical analysis

The experimental data was subjected to one-way analysis of variance (ANOVA) followed by Tukey post-Hoc pairwise comparison test. Statistical Package for Social Sciences (SPSS) (ver. 22.0 Armonk, NY: IBM Corp.) was used for all statistical analyses.

Re-germination potential of *in vitro* produced spores from carrier-based inocula to *in vitro* conditions

Extraction of AM fungal propagules

Extraction of AM propagules (spores and colonized root fragments) from the carrier formulation was carried out by wet sieving and decanting technique [35].

AM fungal propagule disinfection process

The disinfection process was modified from Mosse [36], Mertz *et al.* [37], Daniels and Menge [38], and Bécard and Fortin [31]. Isolated propagules were first rinsed twice with sterilized distilled water after which they were disinfected. The propagules were then disinfected with 250-400 µl sodium hypochlorite (NaClO) for 3-5 min. After disinfection, the propagules were rinsed three times with sterilized distilled water and treated with antibiotic solution (Streptomycin 0.02 %+Gentamycin 0.01 %) for 10 min.

Germination of disinfected propagules

Disinfected AM propagules were then inoculated on MSR media [27] minus sucrose and Petri plates were incubated in the dark at 27 °C in an inverted position.

RESULTS

Infectivity potential of *in vitro* prepared inocula in carrier formulation during storage

Upon storage the infectivity potential of *in vitro* prepared inocula of both the AM fungal species *viz., R. intraradices* and *F. mosseae* were observed to be optimum at 25 °C when compared to other storage temperatures (4 °C and room temperature) used in the study. AM fungal colonization levels ranged from 89-93% for *R. intraradices* and 85-90% for *F. mosseae* when stored at 25 °C indicating that the inocula did not lose infectivity potential even after 6 mo of storage. ANOVA comparing the effect of storage at the three different temperatures on percent colonization by *in vitro* prepared inocula in carrier formulation revealed that percent colonization was significantly greater in propagules stored at 25 °C (fig. 1, table 1).

Re-germination potential of *in vitro* produced spores from carrier-based inocula to *in vitro* conditions

It was observed that spores of *R. intraradices* germinated 2-10 d while spores of *F. mosseae* germinated 10-15 d, after plating (fig. 2 a-d), and 100% germination was recorded indicating that spores of both the AM species retained viability to re-germinate when cultured back using *in vitro* conditions.

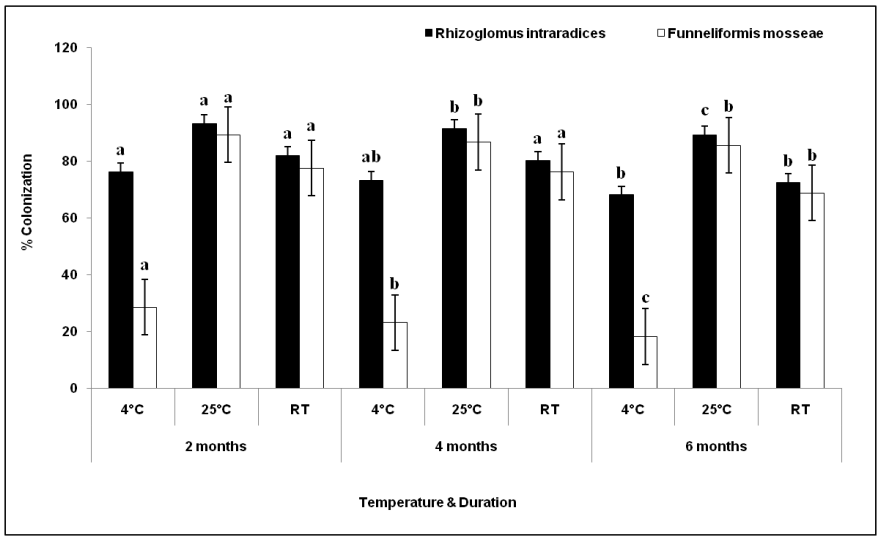

Fig. 1: Percent root colonization in *Plectranthus scutellarioides* (L.) R. Br. by *in vitro* produced AM fungal inocula stored at different temperatures

Legend: All values are means of six replicates±standard deviation. Bars not sharing the same letters are significantly different ($P \leq 0.05$). RT = Room Temperature (28-30 °C)

Table 1: Analysis of variance for percent colonization of *Plectranthus scutellarioides* (L.) R. Br. inoculated with *in vitro* produced AM fungal inocula stored at different temperatures

Temperature			*Rhizoglomus intraradices*				*Funneliformis mosseae*			
	Source	***df***	***SS***	***MS***	***F***	***P***	***SS***	***MS***	***F***	***P***
4 °C	Between	2	203.473	101.736	5.971	0.02*	319.062	159.531	4.455	0.03*
	Within	15	218.938	17.039			537.104	35.807		
	Total	17	422.411				856.167			
25 °C	Between	2	49.681	24.840	18.849	0.00**	43.942	21.971	16.118	0.00**
	Within	15	19.768	1.318			20.447	1.363		
	Total	17	69.449				64.388			
RT	Between	2	311.924	155.962	16.967	0.00**	265.577	132.789	5.324	0.01**
	Within	15	137.881	9.192			374.130	24.942		
	Total	17	449.805				639.708			

Legend: *df* degrees of freedom, *SS* sum of squares, *MS* mean square; **Significant at $P \leq 0.01$ and $P \leq 0.05$, *Significant at $P \leq 0.05$; RT = Room Temperature (28-30 °C).

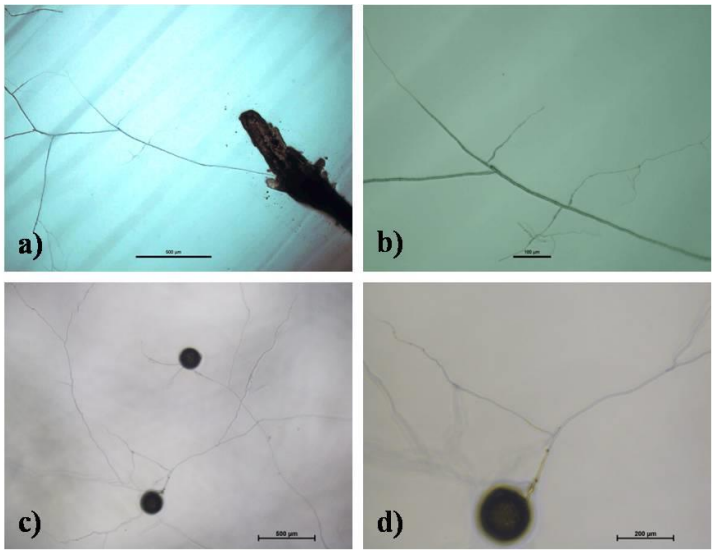

Fig. 2: Re-germination potential of *in vitro* produced spores from carrier-based inocula to *in vitro* conditions. a) *In vitro* germination of colonized root fragment of *Rhizoglomus intraradices*; b) Enlarged view of branching of hypha emerging from colonized root fragment of *Rhizoglomus intraradices*; c) *In vitro* spore germination of *Funneliformis mosseae*; d) Enlarged view of *in vitro* spore germination of *Funneliformis mosseae*

DISCUSSION

In the present study, *in vitro* prepared inocula in carrier formulation were tested for their infectivity potential during storage. Upon storage the infectivity potential of *in vitro* prepared inocula of both the AM fungal species *viz., R. intraradices* and *F. mosseae* were observed to be optimum at 25 °C compared to other storage temperatures used in the study. AM fungal colonization levels of 89-93% in *R. intraradices* and 85-90% in *F. mosseae* were recorded at 25 °C indicating that the inocula did not lose infectivity potential even after 6 mo of storage. Louis and Lim [39] showed that the germination ability of a tropical isolate of *R. clarum* was enhanced after 3-6 mo of dry storage at 25-30 °C. Kuszala and Gianinazzi-Pearson [40] preserved propagules of AM fungal isolates belonging to *Glomus*, *Acaulospora*, *Gigaspora*, *Scutellospora* extracted from 6-11 mo old pot cultures, in osmosed water at 4 °C, at ambient temperature, at+27 °C or at+37 °C. AM fungal propagules are commonly stored at 4-5 °C in dried pot culture soil [41]. At the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM), most of the AM fungal propagules produced in pot cultures are stored in dried soil or substrate at 4 °C for different time periods depending on the genus. A number of studies have demonstrated that the preservation of AM fungal propagules in soil, saturated salt solutions, alginate beads and osmosed water by drying or cold storage was optimum at 4 °C [42-47]. This was, however, contrary to our findings. Our results however indicate that 25 °C was optimum storage temperature for the carrier-based AM fungal inocula which can be considered as a feasible option.

It has been reported that the higher maintenance of viability of AM fungal inocula in substrates containing vermiculite due to better aeration and water retention capacity [48]. The viability and efficiency of the inocula can be maintained for several months at 20-25 °C, but the inocula should be kept in its packaging and must be partially dried [49]. It is important to stress that the inocula must be dried before storage [50, 51]. The viability of inocula will decrease under extended storage under humid substrate [52]. Earlier reports showed that the sources of inoculum stored in moistened vermiculite maintained its infective potential only for 2 mo [53, 54].

The AM species used in our study *viz., R. intraradices* and *F. mosseae* occur in a wide range of biomes and adapted to specific environment, and hence may exhibit viability in a range of temperatures. Our study, revealed that spores of *R. intraradices* germinated 2-10 d while spores of *F. mosseae* germinated 10-15 d after plating, with no spore dormancy indicating that spores of both the AM species were viable and re-germinating when cultured back to *in vitro* conditions as well as signifying the efficacy of the carrier formulation in maintaining the vigour of *in vitro* produced spores.

CONCLUSION

The results obtained in our study are very promising from the point of view of a possible commercial production of AM fungal inoculants. The *in vitro* produced inocula stored at 25 °C remained viable up to 6 mo in the organic carrier formulation composed of vermiculite, cow dung powder, wood powder and wood ash, therefore being recommended for use as bio-inoculants. However, further experiments may be needed to test the suitability of the carrier formulation developed for long-term preservation and reproducibility in other AM fungal species.

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