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# Establishment of *In-vitro* Culture of *Glomus clarum* using Vesicles

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## ABSTRACT

**Arbuscular mycorrhizal (AM) fungi are obligate symbionts belonging to the phylum Glomeromycota that enhance host plant growth through nutritional benefits. AM fungal propagules exist as spores, living hyphae, isolated vesicles, and mycorrhizal root segments. The present study reports establishing an *in vitro* culture of *Glomus clarum* using mature vesicles grown monoxenically with Ri T-DNA transformed *Cichorium intybus* L. (chicory) roots. Upon inoculation, 90% germination was recorded in vesicles after 36 h in the Modified Strullu and Romand (MSR) medium. Germinated vesicles were transferred to 9 cm diameter Petri plates (one vesicle/plate) containing 15 days of actively growing Ri T-DNA transformed chicory roots and incubated in the dark at 26°C. Sporulation was observed after five weeks of inoculation. The study suggests that isolated vesicles constitute an excellent source of inoculum for initiating successful *in vitro* culture of *G. clarum*.**

**Keywords:** Vesicles, *Glomus clarum*, Germination, Sporulation, *In vitro*, Chicory roots

## INTRODUCTION

AM fungi are obligate symbionts belonging to the phylum Glomeromycota (Brundrett and Tedersoo, 2018) that enhance host plant growth through nutritional benefits (Smith and Read, 2008; Smith and Smith, 2011). They are major components of the soil microbial population, forming a symbiotic association with most plant species. Despite their great economic potential, mass production of these fungi is restricted due to their obligate symbiotic nature and the cultural conditions employed. Earlier *in vitro* culture systems provided useful information about AM fungal spore ontogeny (Pawlowska *et al*., 1999) and sporulation dynamics without disturbing the symbionts (Declerck, 2001). These studies have greatly improved our understanding of AM fungal propagation and life cycles. Spores represent the quiescent life cycle stage in AM fungi. Therefore, their protein content must be sufficiently stable to achieve successful germination and early hyphal elongation (Strullu *et al.*, 1997). Many attempts to grow these organisms *in vitro* have been implemented, most results being obtained with spores as starting material (Mugnier and Mosse, 1987; St-Arnaud *et al.*, 1996). Besides spores, various forms of inocula, such as mycorrhizal root fragments (Strullu and Romand, 1986) and vesicles (Declerck *et al*., 1998), have been exploited to achieve monoxenic cultures of AM fungi. Gerdemann and Trappe (1974) noted that the intraradical vesicles in *Glomus fasciculatum* were identical to chlamydospores. Using pot experiments, Biermann and Lindermann (1983) suggested that the intraradical vesicles have higher inoculum potential than AM fungal spores. Vesicles can be easily isolated by lacerating heavily colonized roots. The present study reports the successful establishment of an *in vitro* culture of *G. clarum* using mature vesicles grown monoxenically with Ri T-DNA transformed *Cichorium intybus* L. roots.

## MATERIALS AND METHODS

The AM fungal species *Glomus clarum* Nicolson & Smith was initially isolated from the rhizosphere of a mangrove plant *Acanthus ilicifolius* L. from Terekhol estuary in North Goa (India) and propagated using sievings in pot cultures with sterile sand as substrate and *Solenostemon scutellarioides* (L.) Codd as host. Subsequently, *G. clarum* pure culture was raised and maintained. After mass multiplication, six weeks old *G. clarum* cultures were harvested to obtain the mature vesicular stage **(Figure 1a)**. Identification was carried out using the classical morphological spore characteristics and the relevant keys (Rodrigues and Muthukumar, 2009; Schenck and Perez, 1990). After taxonomic identification and purity assessment of the culture, *S. scutellarioides* roots were sampled for the presence of mature vesicles using a stereo-microscope (Olympus SZ61). The selected root segments containing mature vesicles were stored at 25°C before being used. Within 24 h after storage, each mycorrhizal root fragment was placed in a Petri plate containing sterile distilled water. This was followed by disinfection for 4 min in 2% w/v chloramines-T and then a 10 min bath in an antibiotic solution (Streptomycin sulfate 0.02% w/v and gentamycin sulfate 0.01% w/v). After each disinfection step, the root fragments were rinsed three times in sterile distilled water. Using fine, non-magnetic Dumont tweezers (110 mm length, tips 0.06 x 0.10 mm), the disinfected roots were gently lacerated to separate the vesicles under a stereo-microscope. The separated vesicles were surface sterilized using sodium hypochlorite (0.04% w/v) for 2 min. Vesicles found floating were discarded. This was followed by washing in

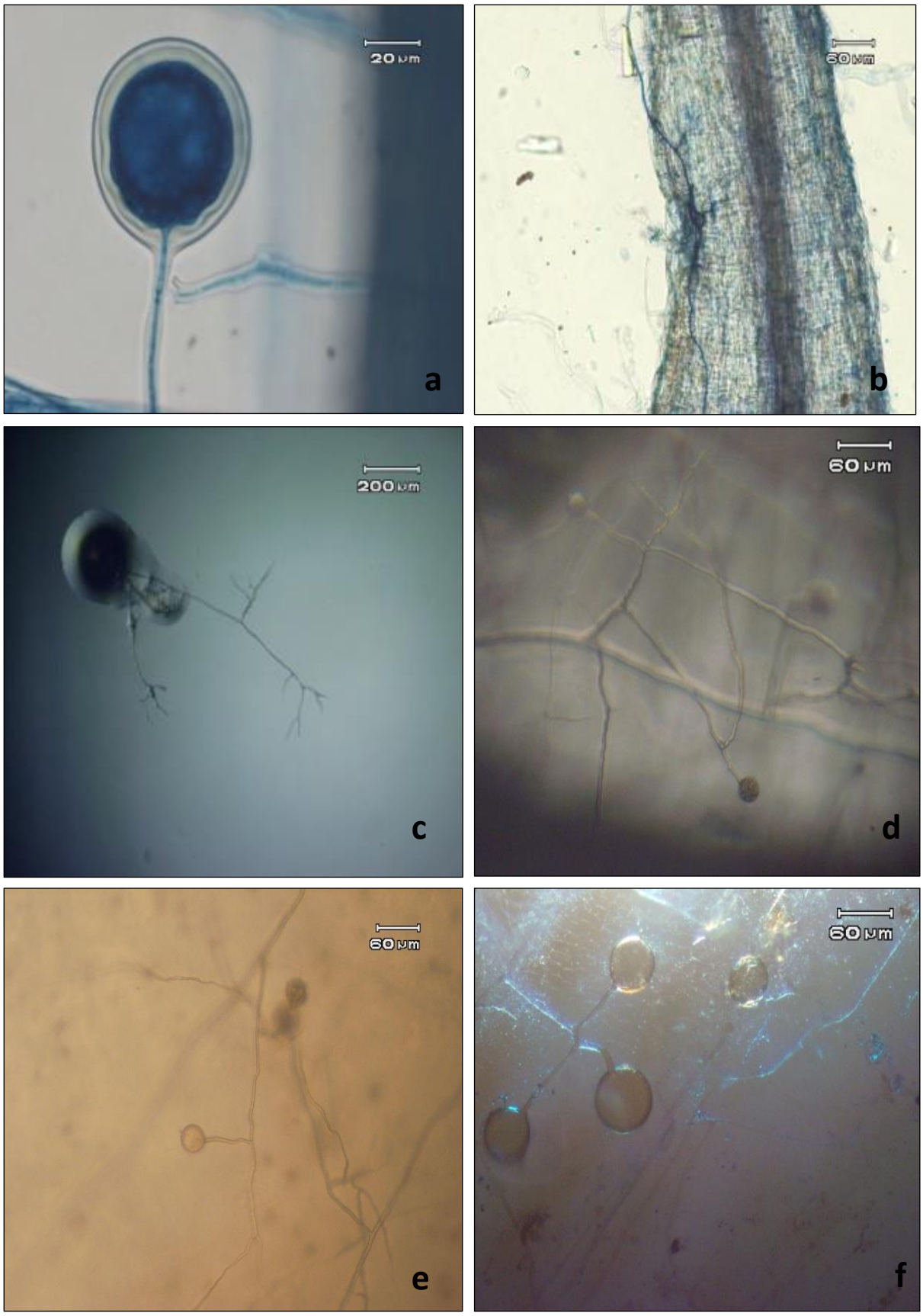


**Figure 1:** a, Vesicle stained with Trypan blue; b, Appresorium formation in transformed chicory roots; c, Germination of *Glomus clarum* and hyphal branching at right angles; d, Sporulation in *Glomus clarum*; e, Young spore; f, Cluster of spores.

sterile distilled water five times and vigorously shaking the Petri plate for five minutes each time. Using a micropipette, separated vesicles were transferred to a Petri plate containing a 2% (w/v) ambistriyn-s solution and stored for 16 h at 4°C. MSR medium solidified with 5% clarigel and adjusted to pH 5.5 was used as substrate. Excised Ri T-DNA transformed chicory roots were used as a host for *G. clarum*. Following germination, vesicles were transferred to Petri plates containing actively growing 15-day-old Ri T-DNA transformed chicory roots. The germinated vesicle was placed within 1mm of the roots, with care taken to ensure they touched the substrate. The cultures were then incubated in an inverted position in the dark at 26°C. Observation of hyphal growth after transfer to the Petri plates containing actively growing Ri T-DNA transformed chicory roots was recorded every 12 h. Root colonization was estimated using the method of Phillips and Hayman (1970).

## RESULTS AND DISCUSSION

In the present study, vesicle germination commenced after 36 h of inoculation and recorded 90% germination. An earlier study by Desouza and Berbara (1999) reported germination in *G. clarum* after seven days using spores as inoculum. Vesicles are known to act as a source of reserves, with higher inoculation potential than other AM propagules, such as spores and hyphae (Mosse, 1988; Strullu and Romand, 1986). Being juvenile stage, the vesicle is rich in energy sources and nuclei, has fewer wall laminations, and hence germination is more rapid than in spores. Germinating vesicles produced germ tubes that grew through the subtending hyphae. The formation of appressoria and characteristic right-angled branching pattern of hyphae was observed in the present study **(Figure 1b and c)**. In earlier studies (David and Douds, 2002; Declerck *et al.*, 1998), the Ri-TDNA roots were placed close to germinating spores. However, in the present study, germinated vesicles were transferred into Petri plates containing 15 days old actively growing roots. Specific compounds from root exudates help improve mass inoculum production (Chabot, 1992). Strullu *et al*. (1997) hypothesized that the growth-promoting substances derived from host roots accumulate in intraradical structures like vesicles, allowing independent growth. Contact between the fungus and transformed roots occurred on the fourth day after transferring the germinating vesicles in the MSR medium. After initial root colonization, extensive development of extra-matrical hyphae was observed. The mycelial growth pattern of *G. clarum*, consisted of long non-septate hyphae growing on the medium. Colonization (60-70%) was observed in the roots. However, no arbuscules and vesicles were recorded. Fortin *et al*. (2002) suggested that due to the absence of photosynthetic activity, the addition of sucrose in the medium is known to modify the biochemistry of plant-fungal interaction. This might explain the absence of arbuscules and vesicles in Ri T-DNA transformed chicory roots, despite abundant hyphal colonization.

Sporulation was initiated after five weeks, while Desouza and Berbara (1999), using the spore of *G. clarum* as inoculum, reported sporulation after three months. Rodrigues and Rodrigues (2012) reported sporulation after four months of hyphal penetration. Upon sub-culturing the colonized transformed root fragments from the starter culture, sporulation was achieved after 20 days of culturing. St-Arnaud (1996) reported sporulation from hyphae emerging from spores after three months in *G. intraradices*. Declerck *et al*. (1998) suggested that mycorrhizal root segments containing vesicles exhibit higher inoculum potential than the spores. However, using root segments as inoculum, one cannot pinpoint the exact source of germination as intraradical hyphae are also known to be a potential source of inoculum and play a vital role in completing the life cycle. New spores were seen extending from the sporulating hyphae. They mainly appeared in terminal or intercalary position **(Figure 1d and e)** or more often in clusters containing 1-3 spores **(Figure 1f)**. The spores resulting from *in vitro* sporulation were globose, hyaline to creamish in their juvenile stage, becoming yellowish-brown at maturity with numerous lipid inclusions. The average size of the spores at maturity ranged from 100 to 140 µm. This technique can be exploited for the genus *Acaulospora* as, to date, only one species, *viz.*, *A. rehmii* (David and Douds, 2002), has been successfully cultured using transformed roots. This is attributed to their poor germination and sporulation ability. The study revealed that the isolated vesicle constitutes an excellent source of inoculum for successful *in vitro* culture and for enhancing the mass production of different AM fungal species.

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