SYMBIOTIC SEED GERMINATION OF *AERIDES MULTIFLORA* ROXB. – A STUDY *IN VITRO*

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Abstract

Aerides multiflora Roxb. is an epiphytic orchid well known for its beautiful and long lasting flowers. Poor natural regeneration and unregulated commercial collections have resulted in shrinkage of its natural populations. Present study was planned to isolate the mycorrhizal associates of *Aerides multiflora* and to investigate their role in inducing symbiotic seed germination *in vitro.* Surface sterilized root segments were cultured on Oat Meal Agar medium, and two fungal associates (AM301-AM302) were isolated based upon micro - morphological features (colony colour, hyphal characteristics, presence/absence of conidia and/ moniloid cells, *etc*.) and molecular characteristics (sequencing the internal transcribed spacer regions of ribosomal RNA gene). Seeds from undehisced yellowish green fruit were then co–cultured separately with both of these fungal isolates. Embryos swelled after two wks and the testa ruptured after three weeks in more than 74.28% seeds. Further morphogenetic changes, however, occurred only in seeds cultured with isolate AM301 (*Ceratobasidium* sp.). The fungal hyphae made entry inside seeds from their general surface (rarely through micropylar end), and colonized the embryonic cells. Protocorms developed after 8.00±0.40 wks. First leaf and root emerged after 12.50±0.60 and 15.40±0.49 wks respectively. Seedlings were transferred to greenhouse with 98.10±0.46% survival rate.

Introduction

ORCHIDS ARE well known for their beautiful foliage and attractive flowers of varying shapes and sizes. They produce a large number of seeds, but less than one percent of these germinate under natural conditions. For this, there is an obligatory requirement of a suitable mycorrhizal partner, which helps orchid host in a number of ways (Rasmussen, 1995). It is believed to augment carbohydrate nutrition by breaking down the complex organic compounds in the soil and facilitating their subsequent release in the plants (Hijner and Arditti, 1973). This includes carbon, phosphorus, nitrogen, water and vitamins (Hossain *et al*., 2013a). Role of mycorrhizal fungi in orchid seed germination was first indicated by Bernard (1909). It was later confirmed that the mycorrhiza not only promotes seed germination but also stimulates the growth and development of protocorms and seedlings (Aggarwal *et al*., 2012; Bertolini *et al*., 2011; Bhatti *et al*., 2016; Qua-Xia *et al*., 2014; Sathiyadash *et al*., 2013, 2014). Therefore, symbiotic seed germination signifies an efficient technique to investigate the orchid–fungus association and specificity (Stewart and Kane, 2007).

Aerides multiflora Roxb. is an ornamental orchid, which bears beautiful pink flowers arranged in long pendulous inflorescences. Poor natural regeneration and unregulated commercial collections have resulted in shrinkage of its natural populations throughout the Himalayan region (Vij *et al*., 2013). Destruction of its

natural habitats and cutting down of host trees for varied purposes have also added to its scarcity (Verma *et al*., 2009). An attempt was presently made to isolate the mycorrhizal associates of this species and to investigate their role in inducing symbiotic seed germination and seedling development *in vitro*.

Materials and Methods

Collection of Plant Material, and Isolation and Characterization of the Fungal Associates

Roots of a vigorously growing *Aerides multiflora* plant (Fig. 1A) were collected from Tihra town (31⁰46' N longitude, 76⁰40' E latitude, 1057 m) of Sarkaghat subdivision (District Mandi, Himachal Pradesh) during its active vegetative growth phase (mid June, 2013). They were rinsed with tap water and scrubbed with a soft brush to remove surface debris. Transverse sections of the roots were cut and stained with lactophenol cotton blue. Only those with fungal colonization were selected for fungal isolation, which was carried out following Currah *et al*. (1987) with slight modification. For this, roots were cut into approx. 15 mm long segments and washed with detergent (Teepol) under running tap water. Further surface sterilization was carried out under aseptic conditions in a laminar air flow cabinet. Root segments were initially dipped in 70% ethanol for 5 seconds and then treated with 0.1% solution of $HgCl_2$ for 3–4 min. They were thoroughly rinsed twice in sterile distilled water and their cut ends

discarded. The segments were then inoculated on Oat Meal Agar (OMA) medium in 6"×1" test tubes. The cultures were incubated in the dark at 25±2°C until hyphae emerged out from the inoculated segments and spread on the medium. The mycelium of the isolated fungi was taken on glass slides for microscopic observation (colony color, hyphal characteristics, presence/ absence of conidia and/ or moniloid cells, *etc.*). Molecular characterization of the isolates was done by amplification and sequencing of the internal transcribed spacer (ITS) regions using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described earlier by White *et al.* (1990). The PCR recipe consisted of 11 µl of Go Taq Green Master Mix 2X (Promega Corporation, USA), 0.5 µl of both primers (10 pmol), 2 µl of DNA template and final volume was made to 25 µl with nuclease free water. This reaction mixture was subjected for PCR amplification using the following temperature profile: initial denaturation at 94^0C for 5 min. followed by 50 cycles which consisted of denaturation at 94^0C for 25 seconds. annealing at 57^0C for 27 seconds. and elongation at 72^0C for 30 sec. final elongation at 72^0C for 7 min. The PCR amplified products were sent for custom sequencing at Chromous Biotech, Banglore, India. After sequencing, the sequences were searched for similarity in the database available at National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) algorithm. A dendrogram was constructed using MEGA4 software (Saitou and Nei, 1987).

Symbiotic Seed Germination and Morphogenetic Changes

Seeds were procured from an undehisced yellowish green fruit (16 wks after pollination) of the same plant that previously yielded roots. To clean outer surface, it was scrubbed with detergent (Teepol) using a soft brush and washed under running tap water for 10 min. Further sterilization was done in laminar air flow cabinet by treatment with 0.1% $HgCl_2$ for 5 min with occasional hand-stirring. The fruit was washed with sterile distilled water thrice, dipped in absolute alcohol for 2 seconds and flamed. It was then placed on a sterilized filter paper in a 90 mm glass Petri dish and dissected longitudinally with the help of sterile surgical blade. Seeds were scooped out and distributed over the surface of a sterile filter paper (Whatman No. 1) strip (10×30 mm) resting on the surface of OMA medium dispensed in 6×1 inch test tubes. A PDA block (10 mm³) with isolated fungus was placed at the lower edge of this strip. Both fungal isolates, delimited initially on the basis of morphological characteristics, were tested for symbiotic seed germination *in vitro*. The test tubes were incubated at

25±2°C under continuous 12hr/12hr light/dark photoperiod.

Symbiotic germination and subsequent development was scored on a scale of 0-4 following Aggarwal *et al.* (2012) with slight modification. Stage 0: Swelling of embryo but no rupture of seed coat; Stage 1: Further swelling of embryo and rupture of seed coat; Stage 2: Development of polarity and formation of protocorm; Stage 3: Emergence of first leaf; and Stage 4: Differentiation of first root. Seeds that entered Stage 1 were considered germinated irrespective of further changes occurring in them. The data were collected in five replicates and the values were expressed as means. Results were analyzed using a completely random design and subjected to one–way analysis of variance (ANOVA), and post hoc tests were employed to detect the significant differences ($p \le 0.05$) among different treatments using SPSS 17.0 (SPSS Inc., USA).

Results and Discussion

Mycorrhizal Characterization

Transverse sections of the roots of *Aerides multiflora* revealed that their cortical cells were colonized with mycorrhizal fungi (Fig. 1B). Fungal presence inside orchid roots is a well known fact now, and according to Harley (1963), these plants are associated with mycorrhizal fungi at one or other stage of their life cycle under natural conditions. The mycorrhizal fungi are generally observed to colonize in that organ of the orchid plant which is in contact with soil or some other substrate (Bernard, 1899). Therefore, most of the fungal endophytes in orchids have been investigated from their roots (Roy *et al.*, 2009). Presently, the fungal colonization was noticed only in the outer and middle layers of the cortex. A number of root hairs were found protruding out of the epiblema layer, but none of them was found containing fungal hyphae (Fig. 1C). Therefore, fungal hyphae were believed to have entered into the root tissue directly through epiblema cells or probably, the fungus might have entered inside the tissue well before root collection. Literature studies also revealed that the fungus penetrate inside root either through root hairs, or directly through epiblema cells, or even through both of these structures (Burgeff, 1936; Muthukumar *et al.*, 2011; Nurfadilah *et al.*, 2013; Nurfadilah *et al.*, 2016; Sathiyadash *et al.*, 2012; Senthilkumar, 2001; Vij and Sharma, 1983). Earlier, the mycorrhizal associates were identified using micromorphological characteristics. But with the development of biotechnological tools, the molecular methods are now commonly employed for this (Hossain *et al.*, 2013a; Rasmussen, 1995). There are a number
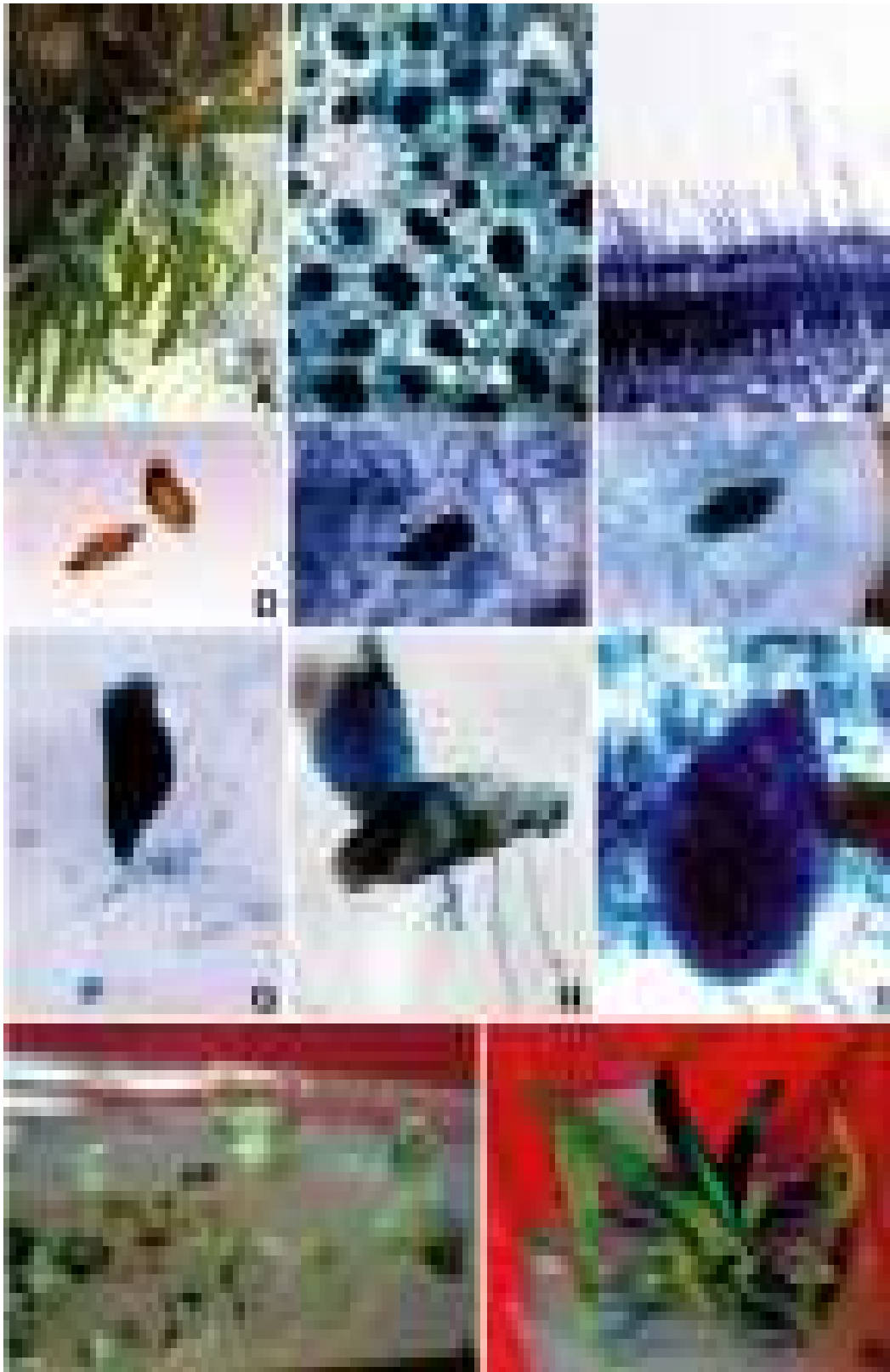
Fig. 1A-K. Symbiotic seed germination of *Aerides multiflora* Roxb.: A, Plants used as the source of roots; B, Root cortical cells colonized with mycorrhizal fungi; C, Root hairs protruding out of the epiblema layer; D, Seeds used for culturing; E-F, Hyphae penetrating inside seed from its general surface; G, Hyphae making entry from the micropylar end; H, Swelling of embryo and rupturing of seed coat; I, Fungus colonizing in the embryonic cells; J, Formation of protocorms; K, Well developed seedlings.

of such reports available in the literature (Ding *et al.*, 2014; Hoang *et al.*, 2017; Hossain *et al.*, 2013a; Ma *et al.*, 2015). Presently, two fungi were isolated from the roots which fall under two groups based upon colony characteristics and micromorphological studies. In Group I (isolates AM301) the young colonies were cottony white and the mycelium growth followed a pattern of concentric zonation. The hyphae were septate and binucleate or multinucleate. They were branched at nearly right angles. Under Group II (isolate AM302) the colonies were light brown (initially grayish) with abundant aerial mycelium. The hyphae were septate, binucleate and irregularly branched. Both of these fungi were also characterized by using ITS sequencing method. The isolate AM301 (Accession No. KR149123) showed 98% similarity with *Ceratobasidum* sp. (Accession No. JX913820), and the isolate AM302 (Accession No. KU323895) showed 99% similarity with *Lasiodiplodia theobromae* (Accession No. KR260802) of NCBI Data Bank (Figs. 2, 3).

It is worth mentioning here that more than two hundred orchid genera have been investigated for their endophytic fungal diversity (Ma *et al.*, 2015), and the fungal endophytes isolated from these mainly belong to Basidiomycetes (*Ceratobasidium, Ceratorhiza, Epulorhiza, Mycena, Rhizoctonia, Sebacina, Thanatephorus, Tulasnella, etc.*) and Ascomycetes (*Alternaria, Bionectria, Cladosporium, Cochliobolus, Fusarium, Trichoderma, Xylaria,* etc.) genera (Alexander and Hadley, 1983; Bhatti *et al.*, 2016; Chen *et al.*, 2011; Downie, 1940, 1957; Dugger, 1915; Hossain *et al.*, 2013a,b; Ma *et al.*, 2015; Parmeter *et al.*, 1967; Rasmussen, 2002; Sathiyadash *et al.*, 2012, 2014; Warcup and Talbot, 1966). Interestingly, nearly one hundred species of non–mycorrhizal endophytic fungi (*i.e. Alternaria, Cercospora, Lasiodiplodia, Phyllosticta*) were also found associated with orchids (Salifah *et al.*, 2011; Sawmya *et al.*, 2013). Since the procedure of root surface sterilization has a significant influence on the fungal associates obtained, it is possible that some surface contaminants could be mistakenly identified as orchid mycorrhizal endophytes (Salifah *et al.*, 2011;

Sommer *et al.*, 2012). Similarly, a non-mycorrhizal endophyte (*Lasiodiplodia theobromae*) has been isolated presently.

Symbiotic Seed Germination and Seedling Development

Owing to the production of dust seeds, the orchid requires mycorrhizal fungi, which provide carbon and mineral resources to stimulate the protocorms as well as seedling development in nature (Arditti, 1967; Clements, 1988; Rasmussen, 1995). Presently, for the *in vitro* symbiotic germination of *Aerides multiflora*, seeds from yellowish green and undehisced capsules were used (Fig. 1D). They were co-cultured with both (AM301, AM302) of the fungal isolates on OMA medium, which is most commonly used substrate for symbiotic germination experiments (Clydesdale, 1994; Rasmussen, 1995). Various morphogenetic changes recorded during the course of present investigation have been summarized in table 1 and presented below in detail.

Initial swelling of embryos (stage 0) was observed after 2.10 ± 0.22 - 2.20 ± 0.17 wks of co-culturing. Time taken to reach this stage varied significantly in case of different fungal isolates. It was, however, not significantly different from the control in both cases (Table 1). The highest seed germination percentage (87.00 ± 1.32) was noticed in cultures inoculated with *Ceratobasidium* sp. (isolate AM301), whereas it was lowest (74.28 ± 1.10) under control. As many as 78.66 ± 2.10 percent seeds germinated when co-cultured with *Lasiodiplodia theobromae* (isolate AM302). The fungal hyphae of both of these fungi surrounded the seeds and penetrated inside from their general surface (Fig. 1E-F). In a few cases, hyphae also made entry from the micropylar end (Fig. 1G). These modes of fungal entry are well documented in literature (Aggarwal and Zettler, 2010; Bhatti *et al.*, 2016; Chen *et al.*, 2014; Hossain *et al.*, 2013a; Rasmussen, 2002; Salifah *et al.*, 2011; Sathiyadash *et al.*, 2014). The seeds swelled and their thin coats ruptured (stage 1) after 3.15 ± 0.28 wks in AM301 cultures (Fig. 1H). The time taken to reach this

Table 1. *In vitro* symbiotic germination and successive morphogenetic stages of *Aerides multiflora* seeds co-cultured with two fungal isolates on Oat Meal Agar medium.

Mycobiont	Per cent seed germination	Time taken in wks for onset of				
		Stage 0	Stage 1	Stage 2	Stage 3	Stage 4
AM301	87.00 ± 1.32^c	2.10 ± 0.22^a	3.15 ± 0.28^a	8.00 ± 0.40^a	12.50 ± 0.60^a	15.40 ± 0.49^a
AM302	78.66 ± 2.10^b	2.20 ± 0.17^b	3.61 ± 0.22^{ab}	—	—	—
—	74.28 ± 1.10^a	2.18 ± 0.12^{ab}	4.58 ± 0.13^b	—	—	—

Data are shown as mean ± standard deviation. Values in a column with the same superscripts are not significantly different at $p \leq 0.05$.
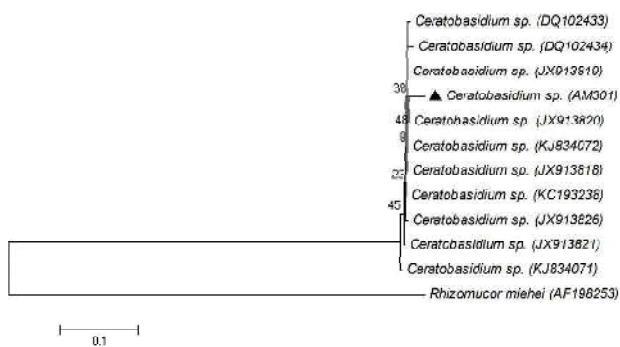
Fig. 2. A dendrogram constructed employing neighbor–joining method by alignment of ITS 1 and ITS4 regions of rRNA gene with *Rhizomucor miehei* out–group. The numbers in the branches are percentage bootstrap value (out of 1000 trials) as indicated. Accession numbers followed by names represent sequences from Gene Bank. Name preceded by a triangle represents sequence obtained presently.

stage was 3.61±0.22 and 4.58±0.13 wks in case of AM302 and under control, respectively. Further morphogenetic changes were observed only in case of seeds co-cultured with isolate AM301. Isolate AM302 and control successfully invoke germination in seeds, but they ceased to develop beyond spherule stage. Salifah *et al.* (2011) isolated six species of *Fusarium* from roots of *Grammatophyllum speciosum* but not all of them were able to induce germination in its seeds. Literature studies revealed that such fungi might have some different functional consequences during some other life stage(s) of orchid plant (Aldana *et al.*, 2013; Hamilton *et al.*, 2012; Ma *et al.*, 2015; Mahdieh *et al.*, 2014; Singh *et al.*, 2011; Suryanarayanan *et al.*, 2012). The fungus started to colonize the embryonic cells and later formed loose networks (pelotons) therein. Such seeds subsequently entered stage 2 (protocorm) after 8.00±0.40 wks (Fig. 1I). Once the protocorm developed chlorophyll (9.80±0.14 wks), they were subcultured on
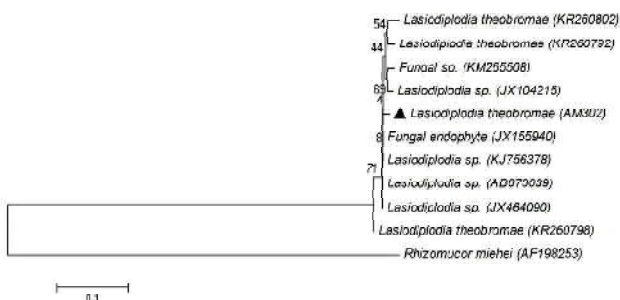

Fig. 3. A dendrogram constructed employing neighbor–joining method by alignment of ITS 1 and ITS4 regions of rRNA gene with *Rhizomucor miehei* out–group. The numbers in the branches are percentage bootstrap value (out of 1000 trials) as indicated. Accession numbers followed by names represent sequences from Gene Bank. Name preceded by a triangle represents sequence obtained presently.

PDA medium without any fungal partner because they became less dependent on the fungal partner (Fig. 1J). The protocorms differentiated first leaf after 12.50±0.60 wks and first root after 15.40±0.49 wks of co-culturing. Subsequently, they developed into seedlings after 18.10±1.10 wks (Fig. 1K). Aggarwal and Zettler (2010) recorded cent percent seed germination and seedling development of *Dactylorhiza hatagirea* seeds with fungus (*Ceratobasidium* sp.) isolated from its own roots. Earlier, Rasmussen (1995) utilized both *Ceratobasidium* and *Tulasnella* to successfully germinate seeds *D. majalis*, but only the *Tulasnella* strains prompted further seedling development. Sathiyadash *et al.* (2014) successfully germinated the seeds of *Coelogyne nervosa* by co-culturing with *Epulorhiza* sp. isolated for the roots of *Eulophia epidendrea*. Yamamoto *et al.* (2017) studied the successful symbiotic germination of *Bletilla striata* with *Tulasnella* sp. Therefore, the symbiotic seed germination signifies an efficient technique to look into the orchid-fungus relationship and specificity in a precise manner.

Park *et al.* (2013) recommended that seedlings or plantlets should have 2-3 leaves and 2-3 roots for their better hardening. In present study, the *in vitro* raised seedlings with 2-3 leaves and 1-2 roots were potted in 6 cm diameter clay or plastic pots containing bark, charcoal, and brick pieces in the ratio of 1:1:1. The survival rate of these *in vitro* raised seedlings was very high (98.10±0.46 percent) when transferred to the greenhouse. This is in line with earlier investigations that symbiotically raised seedlings exhibit higher survival rate during lab to land transfer (Bhatti *et al.*, 2016; Clements, 1988; Zettler *et al.*, 2007). Symbiotically raised seedlings are easy to harden because of the presence of mycorrhizal endophytes in their roots that help in supplying the requisite nutrients in the newer environment (Johnson *et al.*, 2007; Muna *et al.*, 2016).

Acknowledgement

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