Journal of Phytology 2026, 18: 47-55
https://doi.org/10.25081/jp.2026.v18.9928

Research Article

Endophytic association with *Fusarium oxysporum* enhances reintroduction survival of *Aenhenrya rotundifolia* (Blatt.) C.S. Kumar & F.N. Rasm.

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(Received: January 09, 2026;Revised: March 28, 2026;Accepted: April 01, 2026;Published: April 10, 2026)

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Abstract

Conservation efforts for the monotypic endangered jewel orchid, *Aenhenrya rotundifolia* requires a multifaceted approach. The present study outlines a dual strategy that combines an efficient *in vitro* propagation protocol with an investigation into the role of native endophytic fungi. The propagation phase of the study found that 1.0 mg/L *meta*-topolin was highly effective for inducing multiple shoots (74.3), while 0.5 mg/L Indole-3-Acetic Acid and 0.75 mg/L Naphthalene Acetic Acid were optimal for promoting shoot elongation (3.1 cm) and frequency of rooting (72.93%), respectively. Concurrently, four distinct endophytic fungi from the orchid's rhizome were isolated and identified as *Daldinia eschscholtzii*, *Fusarium oxysporum*, *Colletotrichum boninense*, and *Aspergillus sydowii*. To assess their potential symbiotic benefits, *in vitro* raised plantlets were co-cultured with *F. oxysporum,* which has resulted in a significant increase in survival rates of about 91.58%.

Keywords: Monotypic terrestrial orchid, *Fusarium oxysporum*, Endophytic fungi, Plant growth regulators, Symbiosis

Introduction

Most land plants, including orchids, form a mutualistic relationship with mycorrhizal fungi. In this relationship, plants provide fungi with carbon, and in return, the fungi supply essential soil nutrients such as nitrogen and phosphorus. This symbiosis is crucial for orchids, as it enhances seed germination and improves nutrient uptake, and promotes the accumulation of beneficial compounds throughout their life cycle (Nguyen *et al.,* 2020; Long *et al.,* 2022; Lee *et al.,* 2025). However, many orchids exhibit restricted distribution and declining population densities due to habitat degradation, the absence of pollinators, and increasing commercial demands (Tinoammini *et al.,* 2024). Among the orchid flora of India, 24 species of Jewel orchids are reported, belonging to seven genera: *Aenhenrya* Gopalan, *Anoectochilus* Blume, *Cheirostylis* Blume, *Goodyera* R. Br., *Odontochilus* Blume, *Rhomboda* Lindl., and *Zeuxine* Lindl. Of these, seven species are endemic to India (Bhattacharjee & Chowdhery, 2012).

Aenhenrya rotundifolia (Blatt.) C.S. Kumar & F.N. Rasm. is a monotypic, terrestrial jewel orchid native to the southern Western Ghats of India, particularly in Tamil Nadu and Kerala, India. It typically inhabits the shaded understories of evergreen forests characterised by high precipitation and favourable climatic conditions. This ornamental species is easily distinguished from other terrestrial orchids by its rounded foliage and delicate white blooms. Due to its highly restricted distribution and ecological vulnerability, the species is categorized as Critically Endangered (Lal *et al.,* 2025). Recent studies have highlighted its phytosociological interactions, potential shifts in habitat suitability under climate change, and the urgent need for comprehensive conservation efforts, including reforestation and *ex situ* propagation (Palaniyappan *et al.,* 2025). *In vitro* propagation technology represents an important approach for the conservation of endangered orchids through large-scale multiplication and *ex situ* preservation. Plants raised through plant tissue culture can be maintained outside their native habitats under controlled conditions and later reintroduced into the wild. Although micropropagation has been identified as an effective method for the mass production of *A. rotundifolia*, previous studies have reported a relatively low survival rate during plantlet acclimatisation, with only about 65% survival (Ahamed Sherif *et al.,* 2020).

Based on this context, the present study aims to standardise a reproducible protocol for efficient micropropagation of *A. rotundifolia* using internodal explants on Modified Murashige and Skoog medium (MMS) with reduced nitrogen levels. The study also seeks to isolate and identify potential symbiotic endophytes from *in vivo* plants using morphological characters and molecular techniques. These symbionts were subsequently integrated into the hardening phase of micropropagated plantlets to enhance their survival rate.

Materials and Methods

Collection of plant material

Aenhenrya rotundifolia was collected in Srivilliputhur-Megamalai Tiger Reserve, Tamil Nadu, India. The specimens were identified and authenticated from the Botanical Survey of India, Southern Regional Centre, Coimbatore, India vide their letter reference number BSI/SRC/5/23/2022/TECH./373 dated 10.01.2022. The voucher specimen of the plant has been deposited in the Madras Herbarium at BSI (MH 178274). The name of the plant (*Aenhenrya rotundifolia*

(Blatt.) C.S. Kumar & F.N. Rasm.) has been checked online with http://www.theplantlist.org/tpl1.1/record/kew-3662. Mature plants, rhizome-containing plantlets of *A. rotundifolia* were collected from different altitudes within the Srivilliputhur-Megamalai Tiger Reserve (Figure 1a). These plantlets were then brought and maintained under sustainable conditions at the Department of Botany, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. The rhizome explants used for endophyte isolation and the internode explants used for the micropropagation procedure were sourced from these *in vivo*-maintained plants.

Micropropagation of A. rotundifolia

Initially internodes of *A. rotundifolia* were cut in to 1.5 cm in length. Then the internodal explants were rinsed under running tap water for 5 minutes, with a few drops of teepole solution, to remove surface debris. Inside a sterile laminar flow chamber, the explants were sterilized sequentially: first, with 50% alcohol for 45 seconds, followed by 2% sodium hypochlorite (NaOCl, w/v) for 3 minutes, and lastly with 0.01% (w/v) mercuric chloride ($HgCl_2$) for 2 minutes. Between each sterilization step, the explants were washed 3-5 times with double distilled water (ddH_2O) to eliminate any residual sterilants (Ahamed Sherif *et al.,* 2020).

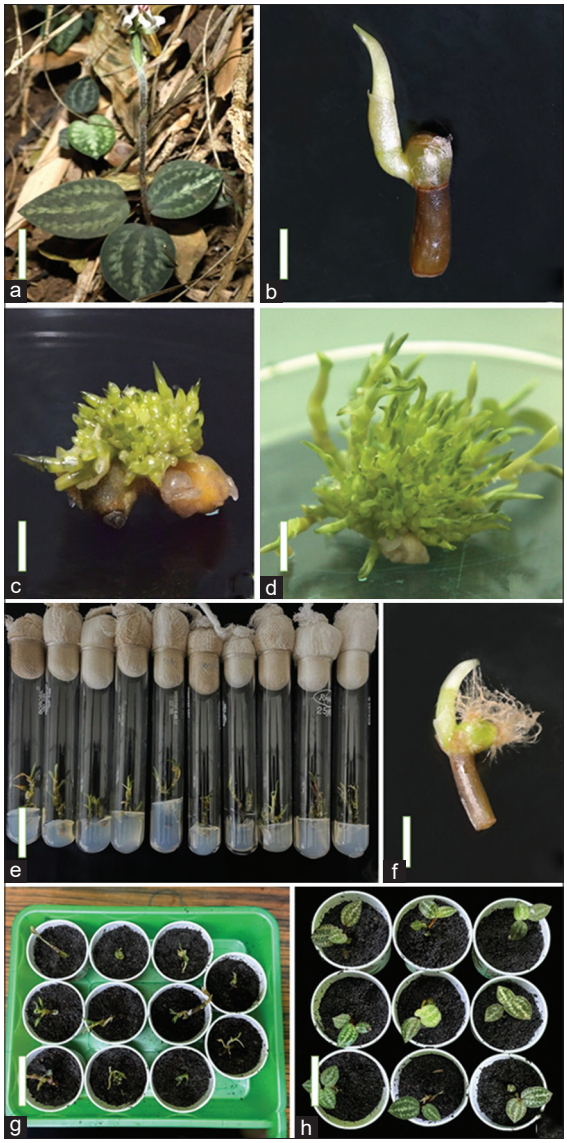

Figure 1: Micropropagation of *Aenhenrya rotundifolia* a) Growth habit of *Aenhenrya rotundifolia*, b) Initial shoot emergence after 7 days of inoculation, c) Multiple shoot formation from the same explant after 28 days of culture, d) Elongation and proliferation of shoots after 56 days of culture, e) Elongation of regenerated shoot, f) shoot showing root emergence, g) *In vitro* raised plantlets hardened in potting mixture and h) Hardened plants after 30 days of hardening. (Scale: a - 5 cm; b, c, d - 0.5 cm; e - 3 cm; f - 0.5 cm; g, h - 6 cm)

The process of plant tissue culture begins with internodal explants of 0.5 cm length, which are placed on Modified Murashige and Skoog medium (MMS) with reduced nitrogen salt (Tavares *et al.,* 2012) enriched with different plant growth regulators (PGRs) like 6-benzyladenine (BA), Thidiazuron (TDZ), *meta*-topolin (*m*T), 6-furfurylaminopurine (KN), and Zeatin (Zea) in the concentration ranging from 0.5-2.0 mg/L to induce multiple shoots. Once shoots develop, these cultures were transferred to fresh MMS medium containing auxins such as Indole-3-butyric acid (IBA), Indole-3-acetic acid (IAA), and α-Naphthalene acetic acid (NAA) and cytokinin 6-2-isopentyl adenine (2-ip) in the concentration range of 0.15-1.0 mg/L to promote shoot elongation and root formation. Finally, the rooted plantlets were hardened and acclimatized in a potting mixture of garden soil, sand, coconut coir, and vermicompost (4:2:2:1), facilitating their gradual transition from controlled *in vitro* conditions to natural growth environment.

Isolation of endophytic fungi

Thin, uniform transverse sections of the rhizomes were prepared, stained with 0.1% Lacto phenol cotton blue, and examined under a Labomed light microscope to detect the presence of pelotons in the cortical cells of *A. rotundifolia* and also the section was observed under a Scanning Electron Microscope (Avhad *et al.,* 2023). Further rhizome samples exhibiting pelotons were cleaned with running tap water to eliminate soil contaminants and subsequently rinsed multiple times with distilled water. The rhizomes were sectioned into 1-2 cm fragments and subjected to a series of surface sterilization procedures: immersion in 75% ethanol for 4 minutes, followed by 4 minutes in a 2.5% sodium hypochlorite solution, and a final 30-second immersion in 75% ethanol. Post-sterilization, the rhizome fragments were rinsed four times with sterile distilled water and dried using sterile filter paper.

Subsequently, cross-sectional slices of the sterilised roots, approximately 2 mm thick, were excised with a sterile surgical blade and placed on petri dishes containing Potato Dextrose Agar (PDA) medium for fungal isolation. Each petri dish received 3 to 5 explants, yielding 3 replicates per condition. The petri dishes were incubated in the dark at 25±2 °C for 7 days. Fungal hyphal tips originating from rhizome cells containing pelotons were then transferred to fresh PDA to establish pure fungal cultures (Ezeonuegbu *et al.,* 2022). Fungi isolated from the inner portions of the root sections were categorized as potential endophytic fungi.

Morphological identification of fungi

Morphologically distinct fungal colonies were identified by examining key characteristics, including colony shape, colour, surface texture, and growth rate. The shape and colour of the colonies, including any changes over time, were recorded. Surface texture was classified as smooth, wrinkled, powdery, or velvety. The types of conidial spores, observing their shape, size, and unique features, were also analysed. This comprehensive approach enabled precise fungal identification by integrating observations of colony and spore characteristics (Ma *et al.,* 2018; Jiang *et al.,* 2019; Sutthinon *et al.,* 2021).

Molecular identification

The total genomic DNA from the individual fungal colonies was extracted using a modified CTAB method. About 200 mg of each colony was used to isolate the genomic DNA and the isolated DNA was confirmed by 0.8% Agarose gel. Then the fungal DNA was amplified with the universal primers, Internal transcribed spacer ITS-1F (TCCGTAGGTGAACCTGCGG) and ITS-4R (TCCTCCGCTTATTGATATGC) were used to amplify the fungal DNA (Parthibhan & Ramasubbu, 2020). The PCR products were then confirmed on 1.2% agarose gel. The sequencing reaction was performed in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) using the Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA), and the experiment was carried out at the Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, Kerala.

Co-cultivation with an endophyte

In vitro-cultured *A. rotundifolia* plants were randomly selected for co-cultivation with endophytic fungi. Suitable growth substrates were prepared by mixing peat, humic soil, and coconut coir in a 1:1:1 ratio, then autoclaved. Pure endophytic fungi were inoculated into 200 mL potato dextrose broth (PDB) and incubated at 28 °C for seven days. After incubation, the fungal mycelia were collected by centrifugation at 3000 rpm for 10 minutes. The mycelia were resuspended in 200 mL of sterilized water and mixed with the substrate. For the control group, the potting substrate was mixed with 200 mL of sterilized water only. The selected plantlets were then planted in both the control and endophyte-amended substrates. All plants were maintained in a growth chamber at 25 °C with a 12-hour day/night photoperiod and 3500 lx light intensity for 20 days (Jiang *et al.,* 2019).

Results

Micropropagation

The plant growth experiment demonstrated significant variations in shoot development based on different concentrations of plant growth regulators. The highest response frequency of 91.27% was recorded in treatment 1.0 mg/L *meta*-topolin, also resulting in a maximum number of shoot buds of around 74.3 (Figure 1b, c & Table 1). Furthermore, shoot elongation was notably enhanced in the presence of Indole-3-Acetic Acid at a concentration of 0.5 mg/L, which achieved the maximum shoot length of 3.1 cm with a response frequency of 80.8% and an average number of 8.13 healthy elongated shoots. Additionally, the most significant rooting response was also observed when Naphthalene Acetic Acid was applied at a concentration of 0.75 mg/L (Table 2 & Figure 1d, e, f) where treatment recorded a response frequency of 72.93%. The initial survival rate of mycorrhizal-free hardened plants was found to be 68.15% (Figure 1g, h).

Microscopic observations peloton

The preliminary microscopic examinations of the rhizome sections of *A. rotundifolia* showed clear and visible free hyphae, which are tightly packed and coiled as pelotons near to the hairy roots. Fungal peloton was found to be dense, observed immediately after the hypodermal layer, and extended only up (5-7 rows) to the middle of the cortex. Each peloton in the cortical cells is highly interconnected with a large number of hyphal connections in the *A. rotundifolia* rhizome (Figure 2a, b).

Morphological identification

The fungal endophytes from *A. rotundifolia* were isolated on a PDA medium, showing visible growth within a week. Rhizome sections demonstrated fungal migration from cortical regions into the culture medium (Figure 2c, d). Each colony was sub-cultured to obtain pure cultures, which were initially classified into four types: AR-1, AR-2, AR-3, and AR-4. Subsequent morphological and spore analysis identified them as *Daldinia eschscholtzii* (AR-1), *Fusarium oxysporum* (AR-2), *Colletotrichum boninense* (AR-3) and *Aspergillus sydowii* (AR-4). The morphology and microscopic features of fungal endophytes are tabulated and represented in Table 3. *F. oxysporum* colonies were fast-growing and pink with felty aerial mycelium, solitary conidiophores, and phialidic conidiogenous cells producing large, hyaline, fusiform macroconidia and smaller, non-septate or one-septate microconidia (Figure 3a, b, c). *C. boninense* colonies were cream white and felted, with a cream to pink reverse, rare setae, and absent sclerotia; the conidia were straight, cylindrical with blunt ends, and a hilum-like protuberance at the base (Figure 3d, e, f). *A. sydowii* colonies were velvety, dark green to greyish, with a honey-brown reverse; the conidial heads were globose, with vesicles bearing hyaline phialides, and conidia were globose to subglobose (Figure 3g, h, i). The *D. eschscholtzii* colonies were white and felty on the surface, with a black-gray reverse, featuring septate hyphae and nodulisporium-like conidiophores (Figure 3j, k, l).

Molecular characterization and phylogeny

After morphological examination, fungal DNA was isolated and amplified using ITS primers for molecular identification. The sequences were blasted and submitted to NCBI, with the following lengths for the isolates:

Table 1: Response of internode explants cultured on MMS medium supplemented with cytokinins after 90 days of culture

PGR	Concentration (mg/L)	Frequency of response (%)	Average number of multiple shoots
6-benzyladenine (BA)	0.5	31.23±5.7^{n}	2.86±0.46^{q}
	0.7	36.5±6.13^{n}	2.74±0.70^{q}
	1.0	51.1±7.57^{lm}	8.10±0.41^{mnopq}
	1.5	58.97±0.76^{ijkl}	6.11±2.30^{nopq}
	1.7	55.7±3.70^{jkl}	3.31±1.72^{pq}
	2.0	45.7±1.28^{m}	3.9±1.39^{opq}
Thidiazuron (TDZ)	0.5	64.47±1.44^{fghij}	17.35±0.55^{hijk}
	0.7	74.83±2.71^{cde}	34.09±6.13^{cde}
	1.0	84.43±0.9^{8ab}	44.48±4.43^{b}
	1.5	86.8±1.55^{ab}	30.17±2.71^{def}
	1.7	77.53±1.54^{bcd}	21.46±1.9^{ghi}
	2.0	67.27±2.36^{efghi}	16.93±0.95^{hijkl}
***meta*-topolin (mT)**	0.5	69.2±4.72^{defgh}	20.57±1.12^{ghij}
	0.7	74.73±3.24^{cde}	37.05±4.72^{bcd}
	1.0	**91.27±3.66^{a}**	**74.34±6.21^{a}**
	1.5	88.77±1.9^{ab}	71.27±4.68^{ab}
	1.7	82.8±1.24^{abc}	40.81±4.14^{bc}
	2.0	82.43±3.74^{abc}	27.55±2.51^{efg}
6-furfurylaminopurine (KN)	0.5	46.03±1.82^{m}	8.91±1.21^{klmnopq}
	0.7	50.9±2.31^{lm}	11.97±1.25^{jklmnop}
	1.0	63.5±1.21^{fghijk}	17.23±0.65^{hijk}
	1.5	61.47±0.56^{hijk}	24.88±1.24^{fgh}
	1.7	56.87±0.93^{jkl}	14.34±1.51^{ijklmn}
	2.0	50.83±1.50^{lm}	8.44±0.98^{lmnopq}
Zeatin (Zea)	0.5	54.1±1.31^{klm}	12.23±0.52^{jklmno}
	0.7	61.06±1.12^{hijk}	16.48±2.10^{hijklm}
	1.0	72.06±1.22^{def}	21.92±0.9^{ghi}
	1.5	71.03±1.22^{defg}	14.93±2.1^{ijklm}
	1.7	66.47±0.61^{efghi}	10.62±1.18^{klmnopq}
	2.0	61.83±0.99^{ghijk}	5.9±0.18^{nopq}

Values represent means±S.E. Values followed by the same letter are not significantly different at p≤0.05 according to DMRT

Table 2: Response of developing plantlets on MMS medium supplemented with PGR after 30 days of culture

PGR	Concentration (mg/L)	Frequency of response (%)	Average number of shoots	Average shoot length (cm)	Frequency of rooting (%)
Indole-3-butyric acid (IBA)	0.15	23.6±0.89^{j}	2.46±0.20^{h}	0.6±0.05^{l}	21.5±0.37^{o}
	0.25	32.03±0.28^{i}	3.4±0.25^{fg}	0.76±0.03^{kl}	24.5±0.55^{no}
	0.5	36.56±0.59^{ghi}	4.03±0.08^{def}	1.13±0.12^{ijk}	36.6±0.87^{i}
	0.75	41.5±0.46^{efg}	4.33±0.14^{de}	1.8±0.17^{efg}	56.36±1.58^{d}
	1.0	33.26±0.86^{i}	3.93±0.23^{ef}	1.67±0.08^{fgh}	43.46±0.73^{fg}
Indole-3-acetic acid (IAA)	0.15	41.53±0.75^{efg}	4.7±0.26^{d}	1.27±0.29^{hij}	27.83±0.84^{lm}
	0.25	53.46±0.75^{d}	5.86±0.17^{c}	2.23±0.29^{cde}	36.03±1.48^{i}
	0.5	**80.8±4.39^{a}**	**8.13±0.49^{a}**	**3.1±0.057^{a}**	43.83±0.92^{fg}
	0.75	76±2.37^{b}	7.93±0.21^{ab}	2.73±0.12^{ab}	47.6±0.7^{e}
	1.0	64.5±2.25^{c}	6.7±0.32^{b}	2.8±0.11^{ab}	41.1±0.79^{gh}
a-naphthalene acetic acid (NAA)	0.15	38.73±2.52^{fgh}	4.03±0.12^{def}	1±0.15^{jkl}	35.33±1.50^{ij}
	0.25	44.4±1.32^{e}	4.2±0.05^{de}	1.2±0.05^{ijk}	46.03±1.53^{ef}
	0.5	54.7±1.41^{d}	5.36±0.17^{c}	1.67±0.08^{fgh}	61.96±1.41^{c}
	0.75	46.03±1.35^{e}	4.5±0.15^{de}	2.2±0.05^{de}	**72.93±0.73^{a}**
	1.0	41.6±0.47^{efg}	3.96±0.14^{ef}	2.06±0.14^{def}	65.1±1.65^{b}
6-2-isopentyl adenine (2iP)	0.15	27.53±0.67^{j}	2.96±0.12^{gh}	1.5±0.17^{ghi}	26.76±1.01^{mn}
	0.25	33.13±0.70^{i}	3.2±0.15^{g}	2.23±0.08^{cde}	30.66±0.82^{kl}
	0.5	38.16±0.79^{fgh}	3.83±0.03^{ef}	2.3±0.117^{bcd}	32.4±1.35^{jk}
	0.75	43.10±0.97^{ef}	4.1±0.05^{de}	2.66±0.08^{abc}	38.4±0.45^{hi}
	1.0	34.8±0.89^{hi}	3.86±0.17^{ef}	2.06±0.21^{def}	31.9±0.90^{k}

Values represent means±S.E. Values followed by the same letter are not significantly different at p≤0.05 according to DMRT

AR-1 (491 bp) identified as *D. eschscholtzii*, AR-2 (560 bp) as *F. oxysporum*, AR-3 (812 bp) as *C. boninense*, and AR-4 (546 bp) as *A. sydowii* (Table 4). A phylogenetic tree was constructed using MEGA X software, incorporating a final dataset of 24 nucleotide sequences. This dataset included pairs of the same species and four species from the same genus. The phylogenetic tree revealed four distinct clades with high bootstrap support (Figure 4), where the numbers next to the tree branches represent the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test.

Growth-promoting effect of F. oxysporum

After 20 days of incubation, *A. rotundifolia* co-cultivated with *F. oxysporum* exhibited a significant

increase in height compared to the control group, which was inoculated with sterilized water instead of fungi (Figure 5). This result highlights the notable plant growth-promoting effect and enhanced survival rate up to 91.58%.

Discussion

A. rotundifolia, an endemic jewel orchid renowned for its striking foliar venation, remains underexplored in terms of its ornamental and horticultural potential. Considering its rarity and threatened status, the development of an efficient *in vitro* regeneration protocol is essential for its conservation. In the present study, the internodal explants cultured on MMS medium supplemented with 1.0 mg/L *meta*-topolin have yielded promising results in terms of shoot initiation and multiplication. Modified Murashige and Skoog medium with reduced Nitrogen salts is found to be effective for raising rooted plantlets in the orchid species, namely *D. hamiltonii* (Koppada *et al.,* 2022) and *P. amabilis* (Tavares *et al.,* 2012). Cytokinins are generally known to induce cell division and enhance shoot bud formation by suppressing the apical bud and stimulating the proliferating lateral buds. Among them, *meta*-topolin has been reported to facilitate shoot regeneration by delaying senescence directly or through callus-mediated pathways (Abdalla & Dobranszki, 2024). In recent years, *m*T has been widely employed in the micropropagation of several orchid species, including *T. variegata* (Davis *et al.,* 2025); *P. insectifera* (Devi *et al.,* 2025); *Laeliocattleya* hybrid (Ramírez-Mosqueda *et al.,* 2025a); and *V. planifolia* (Ramírez-Mosqueda *et al.,* 2025b; Manokari *et al.,* 2021). Meanwhile, the application of auxins IAA in the concentration of 0.5 mg/L has promoted shoot elongation in the present study. Similar observations have been reported in *D. heyneanum* (Kaladharan *et al.,* 2024a); and *P. insigne* (Diengdoh *et al.,* 2017), indicating the contrasting physiological roles of auxins. Another auxin, NAA, is widely known to promote root induction in orchids, which is consistent with the results obtained in the study. Comparable findings have been reported in *A. odorata* (Thiyam *et al.,* 2025); and *C. mossiae* (Kaladharan *et al.,* 2024b). However, despite successful regeneration and rooting under *in vitro* conditions, subsequent acclimatisation and hardening resulted in relatively low surviving individuals, indicating the need for further optimization of post-culture conditions.

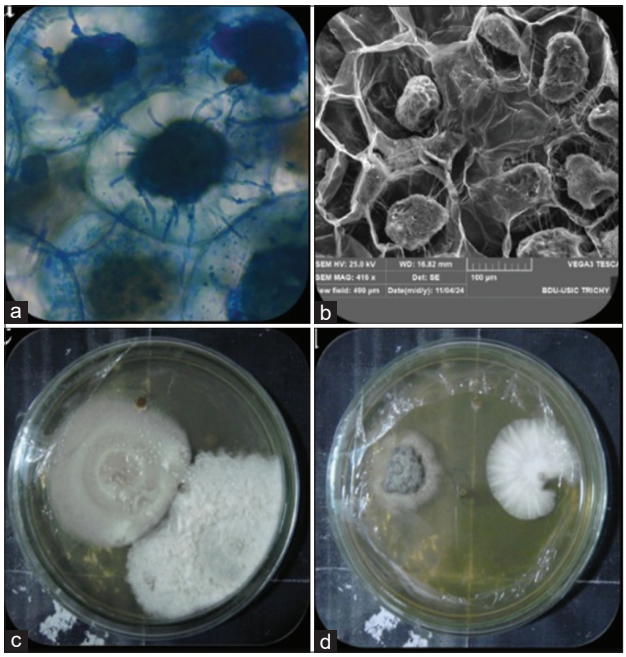

Figure 2: Identification of fungal pelotons a) Peloton under light microscope, b) Peloton under Scanning Electron Microscope, c and d) Emergence and colonization of endophytic fungi from rhizome tissue

This study also documents, for the first time, the isolation of endophytic fungi from *A. rotundifolia,* uncovering four distinct isolates with varying degrees of association with orchid hosts. Endophytes play a crucial role in the biology of terrestrial orchids; in addition to facilitating seed germination, they contribute to the biosynthesis of secondary metabolites and growth regulators (Ma *et al.,* 2018). For instance, *D. eschscholtzii*, previously isolated from the roots of *P. exul* (Ridl.) Rolfe has been reported to produce novel binaphthyl compounds such as Daldionin, along with diverse polyketide congeners (Barnes *et al.,* 2016). Similarly, members of the *Colletotrichum* genus, predominantly regarded as necrotrophic pathogens responsible for anthracnose, have been reported to exist as biotrophic endophytes in selected orchid species. These asymptomatic strains not only exhibit compatibility with their hosts but have also been successfully applied in symbiotic seed germination protocols (Parthibhan *et al.,* 2017; Ma *et al.,* 2018; Ábrahám *et al.,* 2025).

The dualistic nature of *F. oxysporum,* typically recognized as a plant pathogen, is particularly noteworthy. Although implicated in root rot diseases in *Cymbidium* orchids, certain strains have demonstrated endophytic, growth-promoting behaviour in other orchid species (Benyon *et al.,* 1996; Jiang *et al.,* 2019). Similarly,

Table 3: Colony morphology and microscopic features of fungal endophytes isolated from *Aenhenrya rotundifolia*

Fungal Strain	Colour of the fungal isolate	Taxonomic Designation	Phylum	Characters of mycelium	Spore morphology
AR-1	White with brown at the bottom	*Daldinia eschscholtzii*	Ascomycota	Septate hyphae	Nodulisporium like conidiophores
AR-2	Pinkish white in colour	*Fusarium oxysporum*	Ascomycota	Filaments, septate	fusiform macroconidia
AR-3	Pure white	*Colletotrichum boninense*	Ascomycota	Septate, branched	cylindrical
AR-4	The white colour turned into green	*Aspergillus sydowii*	Ascomycota	acute branched septate	Globose or sub-globose

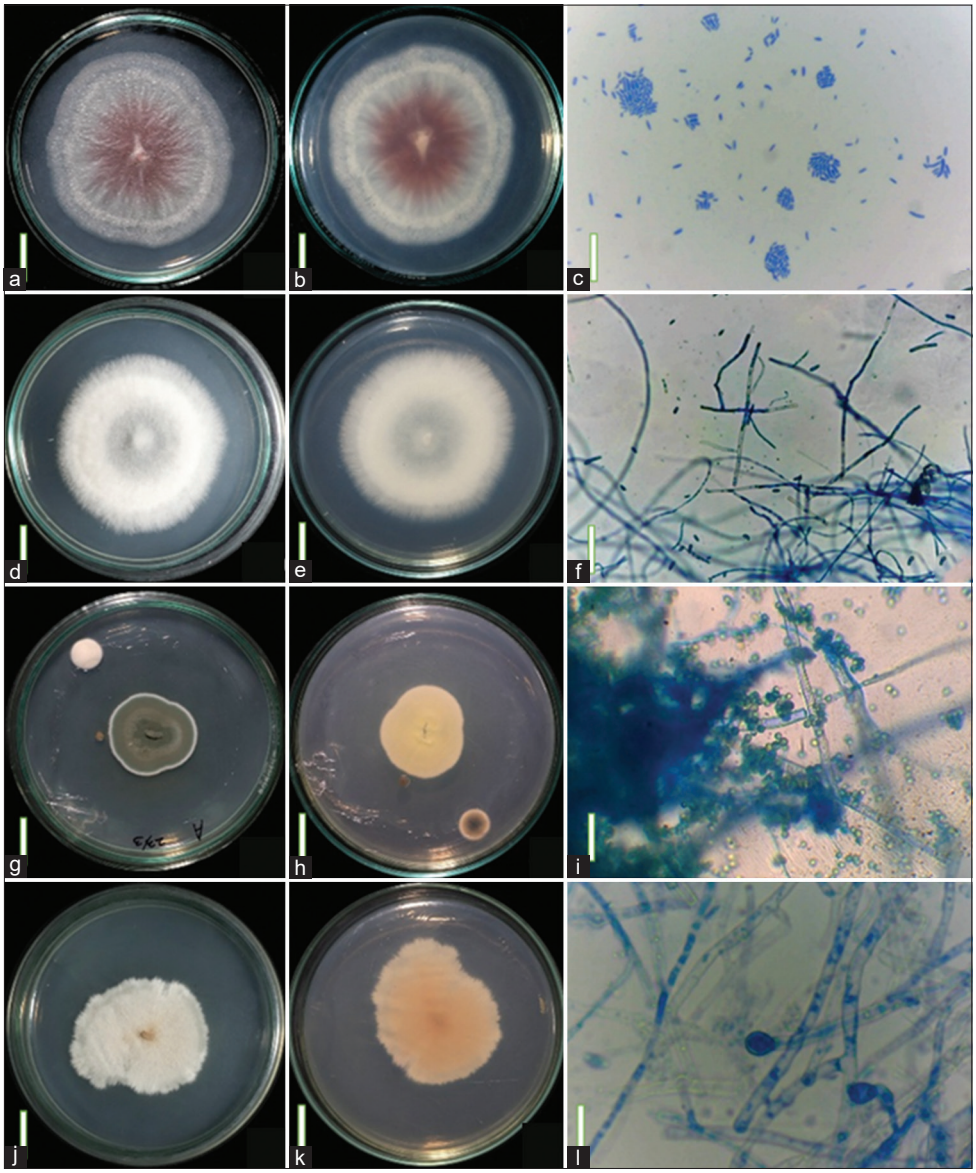

Figure 3: Cultural and spore morphology of endophytic fungi isolated from *Aenhenrya rotundifolia*. a, b) Aerial view and Basal views *Fusarium oxysporum* colony, c) spore morphology of *Fusarium oxysporum*, d, e) Aerial view and Basal views of *Colletotrichum boninense* colony, f) spore morphology of *Colletotrichum boninense*, g, h) Aerial view and Basal views *Aspergillus sydowii* colony, i) spore morphology of *Aspergillus sydowii*, j, k) Aerial view and Basal views of *Daldinia eschscholtzii* colony and l) spore morphology of *Daldinia eschscholtzii.* (Scale: a, b, d, e, g, h, j, k - 2 cm; c, f, i, l - 10 µm)

Table 4: NCBI accession details of fungal endophytes isolated from the rhizome of *Aenhenrya rotundifolia*

Fungal isolates	Name of the fungus	GenBank accession number	Sequence length	Query cover in percentage	Percentage identity	Closely related blast match
AR-1	*Daldinia eschscholtzii*	OL350847.1	491	100	99.8	*Daldinia eschscholtzii*
AR1	*Fusarium oxysporum*	OQ691947.1	560	89	98.65	*Fusarium oxysporum*
AR2	*Colletotrichum boninense*	OQ691949.1	812	100	99.5	*Colletotrichum boninense*
AR3	*Aspergillus sydowii*	OQ691946.1	546	100	100	*Aspergillus sydowii*

A. sydowii, isolated from the root pelotons of the endemic orchid *H. brachyphylla.* This species produces enzymes such as cellulase and ligninase and enhances phosphate solubilization, contributing to overall plant nutrition (Avhad *et al.,* 2023).

The subsequent co-cultivation of regenerated plantlets with *F. oxysporum* under *ex situ* conditions significantly enhanced plantlet survival. The incorporation of a suitable fungal component in the potting mixture can therefore promote improved plant growth and development (Kaladharan *et al.,* 2024a). Microbial endophytes, both fungal and bacterial species, have been employed as bioinoculants to improve the growth of propagated orchid species significantly, which is evident in earlier reports on *Dendrobium* hybrids (Sukamto *et al.,* 2025); *V. ticolor* (Rineksane *et al.,* 2026); *M. Kumbia*, *Cattleya* Irene Finney and *C. tubulare* (Manuel & Jeannette, 2020).

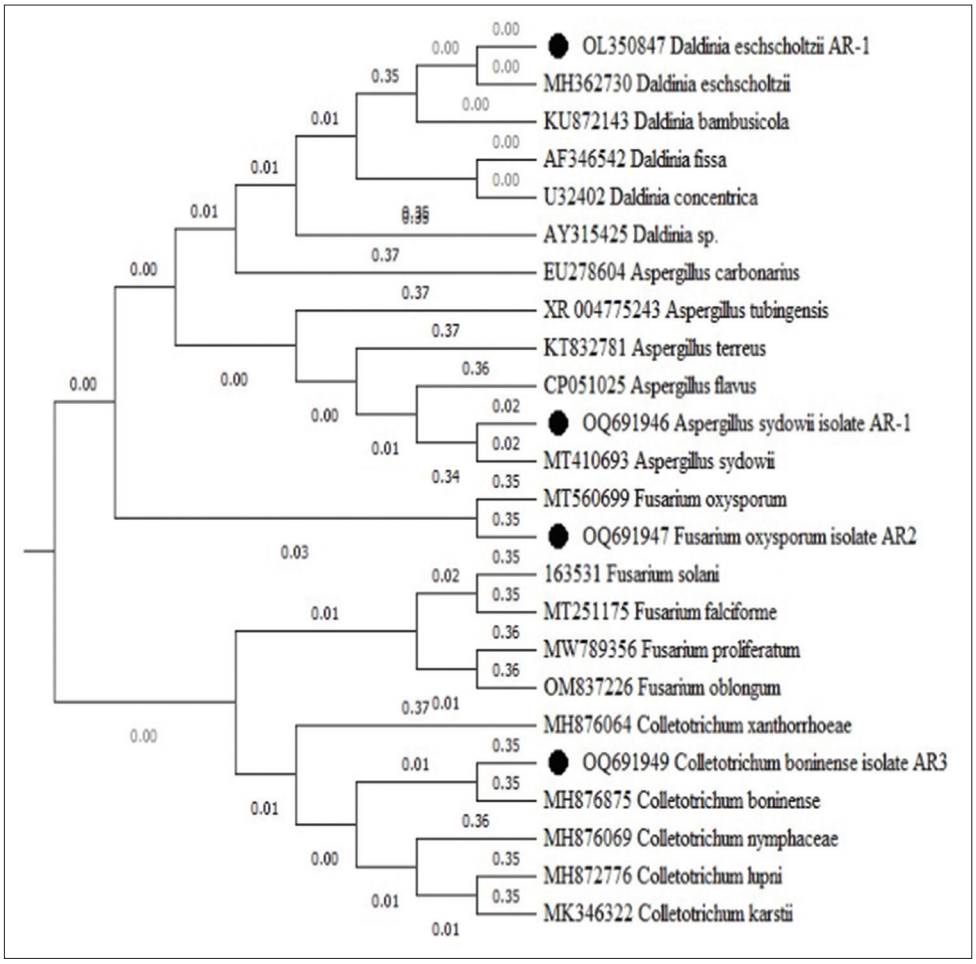

Figure 4: Phylogenetic tree of fungal endophytes isolated from the rhizome of *Aenhenrya rotundifolia* based on ITS rDNA sequences

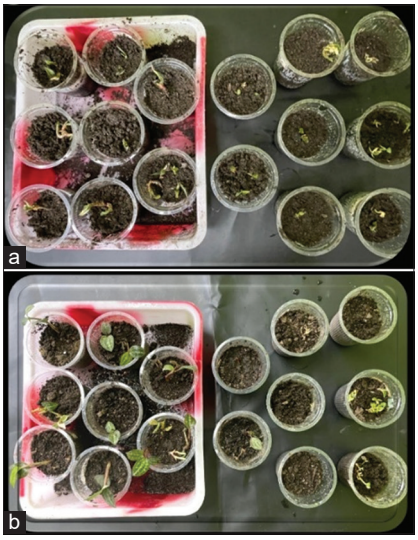

Figure 5: Co-cultivation experiment with *Fusarium oxysporum* to assess its effect on plant growth. a) Plantlets on Day 0 and b) Plantlets on Day 20. N.B. Here, the co-cultivated plants are represented inside the tray while the remaining is marked as untreated controls

Conclusion

A successful *in vitro* regeneration protocol was developed for the orchid *A. rotundifolia* using MMS medium. Internodal explants served as an ideal means for shoot initiation on *meta*-topolin augmented media, while efficient shoot elongation and rooting were achieved on IAA and NAA, respectively. Four fungal symbionts, namely *D. eschscholtzii*, *F. oxysporum*, *C. boninense*, and *A. sydowii* were also isolated and identified from the rhizome tissue of *A. rotundifolia*. Co-cultivation with *F. oxysporum* in the hardening substrate significantly enhanced the survival rate of the plantlets. The standardised methodology can therefore be effectively utilised as an *ex situ* conservational strategy to preserve this endangered species and may facilitate its future reintroduction into natural habitats.

Author contributions

Palaniyappan Subramanian: performed the experiments, analyzed and interpreted the data, performed the molecular identification, data collection and wrote the original manuscript. Shivakrishnan Kaladharan: visualised and edited the final manuscript. Ahamed Sherif Naseruddin: formal analysis and validation. Senthil Kumar Thirupathi: resources, data curation, and supervision. All authors read and approved the final manuscript.

Acknowledgements

The authors gratefully acknowledge the financial support provided by Department of Science and Technology-Science and Engineering Research Board (DST-SERB), Government of India (Ref. No. CRG/2019/000367, dated 18.02.2020). The authors also extend sincere thanks to the Tamil Nadu Forest Department, Government of Tamil Nadu, for granting the necessary permissions and support for conducting the survey.

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