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Enhancing Germination of *Habenaria janellehayneana* (Orchidaceae): Insight from Asymbiotic and Symbiotic Methods

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Abstract

Habenaria janellehayneana Choltco, Moloney, & Yong Gee (Orchidaceae) is a lithophytic orchid with striking pink flowers that is endemic to Phitsanulok Province, northern Thailand. Only a few populations of this species are found in Phu Hin Rong Kla National Park. To maintain rare plant species in *ex situ* collections thereby preventing extinctions, along with the aim of mass propagation for ornamental reasons, it is crucial that suitable propagation methods are developed. In this paper, we describe protocols for the asymbiotic and symbiotic germination of *H. janellehayneiana.* Of the four growing media tested, germination percentages were greatest on ½ VW (18.97%), followed by ½ MS (14.20%), MS (12.46 %), and VW (11.93 %) at 16 weeks, and protocorm development was most advanced (stage 4) within 10 weeks. Of the three plant growth regulators tested, including 6-benzylaminopurine (BAP), gibberellic acid (GA), and thidiazuron (TDZ), at 0, 1, 3, and 5 mg/L concentrations, 1 mg/L BAP significantly enhanced seed germination ($P < 0.05$) when compared to the control (8.47%). For symbiotic seed germination, two non-mycorrhizal endophytic fungi isolates of the genera *Aspergillus* and *Colletotrichum* increased seed germination by 14.03% and 11.00% respectively, when compared to the control (6.15 %). These findings demonstrate that it is possible to germinate the seeds of *H. janellehayneana* via both asymbiotic and symbiotic method, with a symbiotic approach providing the best outcomes, and this could assist in the conservation of this and other rare terrestrial orchids, as well as increase their value in the ornamental market.

Keywords: Micropropagation, Mycorrhiza, Ornamental plant, Terrestrial orchids.

INTRODUCTION

Although, orchids often constitute a significant proportion of regional floras in terms of species numbers (Christenhusz and Byng, 2016; Fay, 2018), they are characterized by low reproductive success (Neiland and Wilcock, 1998; Zhang and Gao, 2021) and are thus typically present at low abundance (Zhang and Gao, 2021). The reasons for this appear to be associated with ecological specialization at key life cycle stages, notably their requirement for myco-heterotrophic germination (Rasmussen *et al*., 2015; Yeh *et al*., 2019), as well as their subsequent transition to autotrophism for seedling establishment (Rasmussen *et al*., 2015) and dependence on specific vectors (mostly insects) for pollination (Swarts and Dixon, 2009; Ackerman *et al*., 2023). Not only does this restrict the geographic range and ecological amplitude of many species, but it also renders them highly sensitive to extraneous threats. As a result, orchids are regarded as facing a disproportionately high degree of extinction risk as compared with other taxa (Fay, 2018), with declining numbers and population fragmentation causing genetic erosion and a breakdown in key ecological processes (Gale *et al*., 2018).

In vitro propagation is frequently highlighted as a useful means of propagating rare and threatened orchids for ex-situ conservation (Stewart and Kane, 2007; Swarts and Dixon, 2009; Fay, 1992; Fay, 2018), and tissue culture technology has been widely applied to the mass propagation of various orchids of significant commercial value (Abebe *et al.*, 2009; Mohanty *et al*., 2012; Paek *et al.*, 2011; Zeng *et al.*, 2016; Zanello *et al.*, 2022). However, established *in vitro* protocols are limited to just a few high-profile genera or species and are not always transferable to less well studied taxa, particularly those, often rarer species with specific requirements for germination. Several approaches have been tested to overcome the difficulties of orchid seed germination, including both asymbiotic and symbiotic techniques, the use of mature/immature seeds, light/dark treatments, sterilization, scarification treatments, and modified culture systems (Arditti and Ghani, 2000; Rasmussen *et al.*, 2015; Setiaji *et al.*, 2021; Nongdam *et al*., 2023).

This genus *Habenaria* Willd. (Orchidaceae) contains about 928 terrestrial and lithophytic species and is characterized by the presence of a combination of derived floral traits with showy petals (Pridgeon *et al.*, 2001; Batista *et al*., 2013; Govaerts *et al.*, 2019). *Habenaria janellehayneana* Choltco. B. Moloney & Yong, a rare terrestrial species with pink flowers, was newly named in 2017 by Choltco *et al*. (2017) after concluding that it ought to be segregated from the widespread *H. rhodocheila* complex. Unlike *H. rhodocheila* Hance and *H. erichmichelii* Christenson, the stigmas of this species are basally parallel but convergent and touching (or nearly so) towards the apex. The species is native to Phitsanulok in northern Thailand and is regarded as a priority for conservation in the country (International Cooperation and Cooperation Group Wildlife and Wild Flora Protection Division, 2013; POWO, 2023). Because it has comparatively large, showy pink flowers, its population has declined due to poaching and the impacts of disturbance.

Habenaria species are notoriously difficult to propagate *in vitro* due to inherent barriers to seed germination and seedling establishment, with capsule maturity, medium nutrient content, culture method, growth factors and mycorrhizal fungi all being important factors (Stewart and Zettler, 2002; Keel *et al*., 2011). Several researchers have attempted symbiotic culture of *Habenaria* seeds, which has been shown to promote seed germination (Stewart and Kane, 2006a; Sangmanee *et al*., 2012). Further, Stewart and Kane (2006b) reported the method of asymbiotic seed germination of *H. macroceratitis*, but no leaf formation was observed. Sangmanee *et al*. (2012) examined the growth of *H. erichmichelii* in the presence of mycorrhizae and found that average plant height was increased when the culture medium was inoculated with fungal strains of *Humicola* sp. and *Oidiodendron* sp., but not with *Fusarium* sp., *Nodulisporium* sp. and *Trichoderma* sp. Symbiotic seed germination of *H. janellehayneiana,* on the other hand, has never been reported. The aim of the present study was therefore to find the best conditions for both symbiotic and asymbiotic germination of this important species. Various media, plant growth regulators, and different fungal isolates, were examined.

MATERIALS AND METHODS

Plant material and seed storage

Habenaria janellehayneana is a terrestrial orchid that mostly grows on moist rocks besides streams and waterfalls in Phitsanulok Province, Thailand (Fig. 1). Mature undehisced plant capsules (7–8 weeks old) of *H. janellehayneana* (n=3) were collected with a permit from Phu Hin Rong Kla National Park in 2018. We used paper bags with silica gel for capsule storage until dehiscence, then stored the resulting brown seeds at 4°C in a sterile Eppendorf tube. Seed vigor was tested within 7 days after staining in a 1% triphenyl tetrazolium chloride (TTC) test at 30 ±2 °C for seven days (Lauzer *et al*., 1994), with embryos becoming orange or reddish in color considered viable.

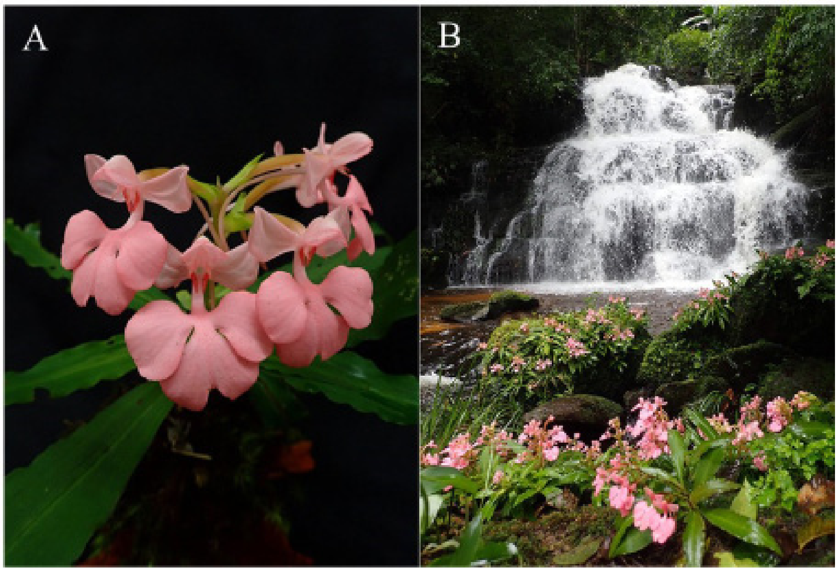

Fig. 1. *Habenaria janellehayneana* at Phu Hin Rong Kla National Park, Thailand. A: Flower morphology; B: Habitat.

Fungal isolation and identification

Roots and rhizomes of plant specimens at vegetative and reproductive stages were collected in a sterile plastic bag, transported to the laboratory within 24–48 h, and refrigerated at 4°C before use. In the laboratory, the roots and rhizomes were then cleaned with tap water, trimmed into 1 cm sections and sterilized in a five min immersion in 0.5% NaOCl. Under a stereomicroscope, the segments were dissected transversely, and pelotons were taken from the cortical cells with a dissecting needle. The pelotons were washed with sterile distilled water five times, placed on a potato dextrose agar (PDA) plate adding both streptomycin and tetracycline at 100 mg/mL concentration, and incubated for 48–72 h at 30 ± 2 °C in the dark. Each fungal mycelia colony was sub-cultured on a fresh PDA media for purification.

The characterization and identification of fungi followed the methods by Zhu *et al*. (2008) for Rhizoctonia species. Genomic DNA of 14-day-old fresh fungal cultures was extracted using a universal and automated nucleic acid extraction system including MagLEAD 12gC machine (Hitachi Co., Ltd.) and a prefilled reagent cartridge for nucleic acid extraction MagDEA® Dx SV kit (Precision System Science Co., Ltd.). Five loci were amplified and sequenced, including beta-tubulin (tub), chitin synthase 1 (chs-1), actin (act), glyceraldehyde-3-phosphate dehydrogenase (gadph), and the internal transcribed spacer regions (ITS). Genes were amplified and sequenced using the primer pairs ITS-1F + ITS4 (Gardes and Bruns, 2013; White *et al*., 1990), GDF1 + GDR1 (Guerber et al., 2003), CHS-354R + CHS-79F (Carbone and Kohn, 1999), ACT-512 F + ACT-783R (Carbone and Kohn, 1999), and Bt2a + Bt2b (Glass and Donaldson, 1995), respectively. The PCR mixture with a total volume of 25 µL contained 5 ng of genomic DNA, 1.25 unit of Taq DNA polymerase (GeneDireX, Inc.), and 0.2 µM of each primer. PCR amplifications were performed in T100 thermal cycler (Bio-Rad Laboratories Ltd., Thailand). The following thermocycling conditions were used: İnitial denaturation at 95 °C for 3 min, followed by 35 cycles of 40 s at 94 °C, 45 s at 54 °C (for ITS and tub2 gene) or 52 °C (for gadph, chs-1, and act genes), and 1 min at 72 °C, followed by a final step of extension at 72 °C for 7 min. Purified PCR amplicons were used to perform direct PCR sequencing of both DNA strands with Applied Biosystems™ 3500 Genetic Analyzer (Thermo Fisher Scientific (Thailand) Co. Ltd.). Using BioEdit (v.7.2.5; Hall, 1999), Forward and reverse primers were assembled to obtain consensus sequences that were subsequent deposited in GenBank. The resulting sequence data were edited and subsequently evaluated using BLAST-n (Altschul *et al*., 1997) to determine affiliation to other sequenced relatives.

For phylogenetic analysis, multiple DNA sequences of act, chs-1, chs-1, gadph, ITS, and tub2 were concatenated for isolate SUT-HJ-I04, and ITS and tub2 were concatenated for isolate SUT-HJ-I35. The DNA sequences were aligned using ClustalW multiple alignment (Thompson *et al*., 1994) and manually adjusted where necessary using BioEdit (v.7.2.5). For phylogenetic analysis, DNA sequences from the Colletotrichum boninense species complex and C. gloeosporioides were used as outgroups for isolate SUT-HJ-I04, while sequences from the Aspergillus species complex section Terrei and A. neoflavipes in section Flavipedes were used as an outgroup for isolate SUT-HJ-I35. Maximum Likelihood (ML) phylogenetic tree with bootstrap (1000 replicates) were constructed with RaxML v.8 (Stamatakis, 2014) and plotted with FigTree (v.1.4.4; http://tree.bio.ed.ac.uk/software/figtree/).

The following four different basal media modified with 2% sucrose, 15% coconut water and 0.8% agar were used to test their influence on seed germination and protocorm formation: (1) Vacin and Went (VW; Vacin and Went, 1949), (2) ½ VW, (3) Murashige and Skoog (MS; Murashige and Skoog, 1962), and (4) ½ MS. Separately, we also enriched the ½ VW medium with the following three plant growth regulators at 0, 1, 3, and 5 mg/L concentrations to assess their impact on germination and early growth: 6-benzylaminopurine (BAP), gibberellic acid (GA), and thidiazuron (TDZ).

Seeds were sterilized in 10% Clorox for 10 min, rinsed with distilled water, sterilized in 3% hydrogen peroxide for 10 min, and washed in sterilized water three times for five min before sowing on each medium. About 100 surface sterilized seeds were sprinkled in a Petri dish containing 20 mL of solidified media, sealed with parafilm, and kept at 25 ± 2 °C in darkness for four weeks and then transferred to a 16 h light/8 h dark cycle for 12 weeks. All treatments consisted of four independent replicates. A 1–5-point growth scale was used to evaluate germination and development, as described by Stewart and Zettler (2002): No germination (stage 0), embryo swollen with production of rhizoid (stage 1), enlarged embryo with testa ruptured (stage 2), protomeristem appearance (stage 3), first leaf emergence (stage 4), and first leaf elongation (stage 5). For the evaluation of seed germination, 100-150 seeds per plate and four replications were marked. The seed germination percentage formula shown below was then used to determine seed germination at each stage. The state of each seed was determined by stereomicroscope examination.

$$\text{Seed germination (\%)} = \frac{\text{number of seeds germinated in each stage}}{\text{number of total mature seeds}} \times 100$$

Symbiotic seed germination

We evaluated the efficacy of 35 fungal isolates (8 *Rhizoctonia*-like and 27 endophyte isolates) in facilitating *H. janellehayneana* symbiotic seed germination using the modified method of Stewart and Kane (2006a). The seed surface disinfection was the same as described above. About 100 viable seeds were sown on a nylon mesh and placed onto 110/ oatmeal agar (OMA) which had has its pH adjusted to 5.5. A 5 mm-diameter plug was then excised from the edge of 7-day old, actively growing mycelium of each fungal inoculum (Yam and Arditti, 2009), and this was inoculated onto the oatmeal agar medium with uninoculated plates serving as a control. Four replicates of each treatment were wrapped in parafilm and kept at 25 ± 2 °C in darkness for four weeks, followed by 12 weeks at 16 h light/8 h dark. The germination and developmental stages were graded in the same manner as described above.

Statistical analysis

A completely randomized design (CRD) was used to set up all of the studies. To normalize variability, the data were transformed to the square root of the arcsine before analysis. The statistical software package SPSS V16.0 (SPSS Inc., Chicago, USA) was used for ANOVA, and the means were compared using Duncan's Multiple Range Test (P=0.05).

RESULTS AND DISCUSSION

Asymbiotic seed germination

The TTC test of *H. janellehayneana* seeds revealed a mean stainability of 14.89 ± 1.77%, which was very low and could be caused by low pollination rates in nature. Within four weeks after sowing, seeds were swollen and were scored as stage 1 (embryo swollen; Fig. 2) in all tested media. At 16 weeks, the ½ VW media showed the highest germination (18.97%), followed by ½ MS (14.20%), MS (12.46%), and VW (11.93%).

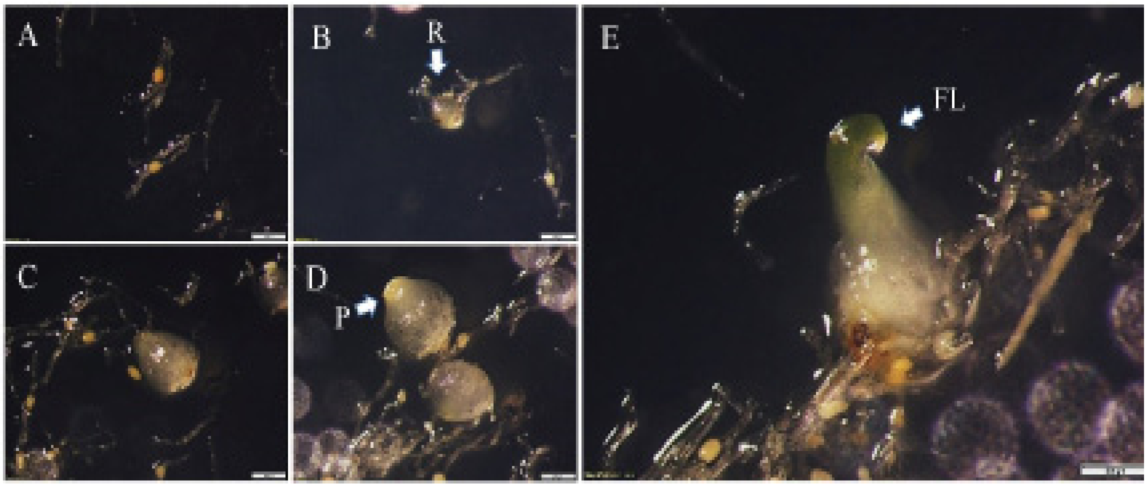

Fig. 2. Protocorm developmental stages of *H. janellehayneana* on ½ VW agar. A: Stage 0 (no germination); B: Stage 1 (embryo swollen with rhizoids present); C: Stage 2 (embryo enlargement with ruptured testa); D: Stage 3 (protomeristem appearance); E: Stage 4 (first leaf emergence); FL: First emerged leaf; P: Protomeristem; R: Rhizoids. bar = 500 µm.

All media supported advanced protocorm development up to stage 4 (leaf emergence) within 10 weeks, with no significant difference among them for stages 1–4; however, ½ VW had the highest frequencies for all stages (Table 1).

Table 1. Effect of basal media on seed germination and development of *H. janellehayneana* for 16 weeks.

Media	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Total
			(%)			
VW	6.70 ± 1.75	2.66 ± 1.50	1.64 ± 1.05	0.91 ± 1.06	0	11.93 ± 2.66^{b}
½VW	12.00 ± 3.00	2.75 ± 2.62	1.92 ± 1.41	2.28 ± 1.12	0	18.97 ± 2.47^{a}
MS	9.40 ± 4.32	1.39 ± 1.87	0.69 ± 0.46	0.97 ± 1.37	0	12.46 ± 2.49^{b}
½MS	9.01 ± 3.19	2.67 ± 2.48	1.33 ± 0.94	1.18 ± 0.49	0	14.20 ± 4.66^{ab}

*In each column, means with similar letter(s) are not significantly different (P < 0.05) using the Duncan's Multiple Range Test. Each mean value is determined by stereomicroscopic examination.

These results are similar to those previously reported by Thummavongsa *et al*. (2022), in which both half- and full-strength MS and VW media supported germination of *H. rhodocheila*, but ½ VW gave better performance overall. This might, perhaps, be due to its phosphate-rich regime, although different species in the same genus might be expected to have different media preferences. Stewarts and Kane (2006) showed that, among six tested media, percent seed germination of *H. macroceratitis* was greatest on both KC and LM (about 89%). On the other hand, *H. edgeworthii* Hook.f. ex. Collett exhibited the highest seed germination rates on a MS with 1.0 µM α-naphthalene acetic acid (NAA) (Giri *et al*., 2012).

Different types and concentrations of plant growth regulators had different effects on seed germination and growth of *H. janellehayneana* (Table 2). The addition of BAP, GA, and TDZ resulted in enhanced seed germination percentages, ranging from 8.33% to 13.16%, as compared with the control (8.47%). Media with 1 mg/L BA added gave the highest germination percentage (13.16%), which significantly differed from the control and 1 mg/L TDZ treatments. Seeds grown on media with 1, 3, 5 mg/L BAP and 3 mg/L GA proceeded to stage 4 (protocorm), as did those on the control, whereas seeds on media with 1, 3 mg/L GA and 3 mg/L TDZ stopped at stage 3. On the other hand, seeds on media with 5 mg/L TDZ added stopped at stage 1, indicating that a high TDZ concentration has an inhibitory effect on protocorm development (Table 2).

Table 2. Effect of plant growth regulators on seed germination and development of *H. janellehayneana* cultured on modified ½VW media for 16 weeks.

Treatment	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Total
			(%)			
control	6.98 ± 1.95	0.55 ± 1.11^{ab}	0.75 ± 0.58^{abc}	0.18 ± 0.37^{ab}	0	8.47 ± 1.87^{b}
1 mg/L BAP	10.01 ± 2.78	0.98 ± 0.89^{ab}	1.79 ± 0.98^{a}	0.37 ± 0.43^{ab}	0	13.16 ± 3.51^{a}
3 mg/L BAP	7.69 ± 3.15	0.76 ± 0.66^{ab}	0.89 ± 1.04^{abc}	0.71 ± 0.82^{ab}	0	10.06 ± 3.59^{ab}
5 mg/L BAP	9.64 ± 3.12	0.61 ± 0.42^{ab}	0.49 ± 0.62^{bc}	0.24 ± 0.49^{ab}	0	10.99 ± 3.39^{ab}
1 mg/L GA	7.80 ± 1.52	0.71 ± 0.12^{ab}	0.56 ± 0.38^{bc}	0^{b}	0	9.08 ± 1.04^{ab}
3 mg/L GA	8.29 ± 2.82	1.24 ± 0.88^{a}	0.18 ± 0.36^{bc}	0.24 ± 0.49^{ab}	0	9.95 ± 2.37^{ab}
5 mg/L GA	8.99 ± 3.27	0.60 ± 0.72^{ab}	0^{c}	0^{b}	0	9.59 ± 3.82^{ab}
1 mg/L TDZ	8.15 ± 1.69	0.18 ± 0.36^{ab}	0^{c}	0^{b}	0	8.33 ± 1.71^{b}
3 mg/L TDZ	8.69 ± 3.57	0.36 ± 0.42^{ab}	0.55 ± 1.11^{ab}	0^{b}	0	9.62 ± 3.41^{ab}
5 mg/L TDZ	8.97 ± 1.39	0^{b}	0^{c}	0^{b}	0	8.97 ± 1.39^{ab}

*In each column, means with similar letter(s) are not significantly different (P < 0.05) using the Duncan's Multiple Range Test. Each mean value is determined by stereomicroscopic examination.

Our findings agreed with those of several previous researchers who have described the asymbiotic seed germination of other terrestrial orchid species and found low germination and slow development. Stewart and Kane (2006b) reported that seeds of H. macroceratitis placed on ML and MM media supplemented with BAP only attained stage 4 protocorms within 16 weeks. Similarly, Piyatrakul (2014) observed that only 5.48% of H. rhodocheila seeds germinated on a modified VW medium (CMU1 with 0.1 mg/L NAA and 1 mg/L BAP added) after 20 weeks, and no stage 5 protocorms were observed. However, Thammavongsa *et al*. (2022) reported a seed germination range of 15.78–27.92% of the same orchid species on ½VW medium with the presence of stage 5 protocorms.

Symbiotic seed germination

We obtained thirty-five fungal isolates from the roots and rhizomes of H. janellehayneana at the vegetative and reproductive stages. The hyphae were noticed after seven days of culture. The morphological characteristics of these isolates on PDA were white, light purple to yellow in color, and some were identified as Rhizoctonia-like fungi according to Sneh *et al*. (1991). The results of co-culture of H. janellehayneana seeds with all 35 fungi isolates for 16 weeks are shown in table 3.

Table 3. Effect of fungal isolates on germination and development of *H. janellehayneana* seeds for 16 weeks.

Treatment	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Total
			(%)			
OMA	5.25 ± 0.86[b]	0.67 ± 0.45	0.22 ± 0.44	0	0	6.15 ± 0.92[c]
OMA + HJ-I04	10.10 ± 1.66[a]	0.89 ± 0.72	0	0	0	11.00 ± 1.78[b]
OMA + HJ-I35	10.87 ± 2.58[a]	2.24 ± 1.94	0.91 ± 1.06	0	0	14.03 ± 1.03[a]

*In each column, means with similar letter(s) are not significantly different ($P < 0.05$) using the Duncan's Multiple Range Test. Each mean value is determined by stereomicroscopic examination.

Only seeds inoculated with one of two fungal isolates, namely SUT-HJ-I04 and SUT-HJ-I35, began to swell and germinate. Seeds treated with the SUT-HJ-I35 isolate exhibited the highest germination rate (14.03%), which was significantly higher compared to that for the other isolates, and they reached stage 3 protocorms, whereas the seeds treated with SUT-HJ-I04 stopped developing at stage 2, suggesting high mycorrhizal specificity or potentially a requirement for mycobiont switch (Umata *et al*., 2022). The results of BLAST searches using the ITS sequence data from these two fungal isolates are shown in table 4. The BLAST search identified SUT-HJ-I04 and SUT-HJ-I35 as Colletotrichum boninense and Aspergillus terreus, with 99.85 % and 100.00 % identity, respectively (Table 4).

Table 4. BLAST searches using the ITS sequence data of fungal isolates from *H. janellehayneiana*.

Isolate	Accession no.	Identity (%)	BLAST search result (Accession no./ taxonomic affiliation)
SUT-HJ-I4	OR074487	99.84	*Colletotrichum boninense* (MF076585.1)/Glomerellales
SUT-HJ-I35	OR074489	100.00	*Aspergillus terreus* DTO 403-C9 (MT316343.1)/Eurotiales

Further phylogenetic analysis based on multiple gene sequences indicated that SUT-HJ-I35 was grouped with *A. terreus* indeed (Fig. 3) but isolate SUT-HJ-I04 should be identified as *C. karstii* (Fig. 4). Our data suggest that these non-mycorrhizal fungi are more important for seed germination than previously thought.

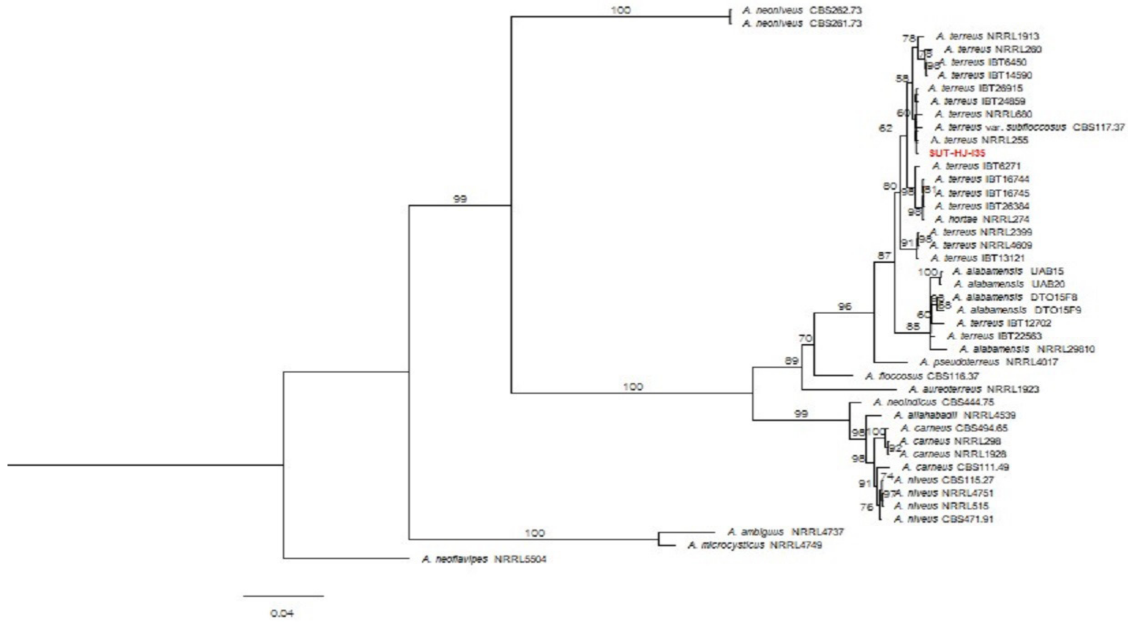

Fig. 3. Maximum Likelihood (ML) tree obtained based on phylogenetic analysis of ITS and tub2 sequence data of the isolate SUT-HJ-I35 and *Aspergillus* section Terrei. Numbers above branches are bootstrap values. Only values above 50% are indicated. The species *A. neoflavipes* NRRL5504 in section Flavipes was selected as an outgroup.

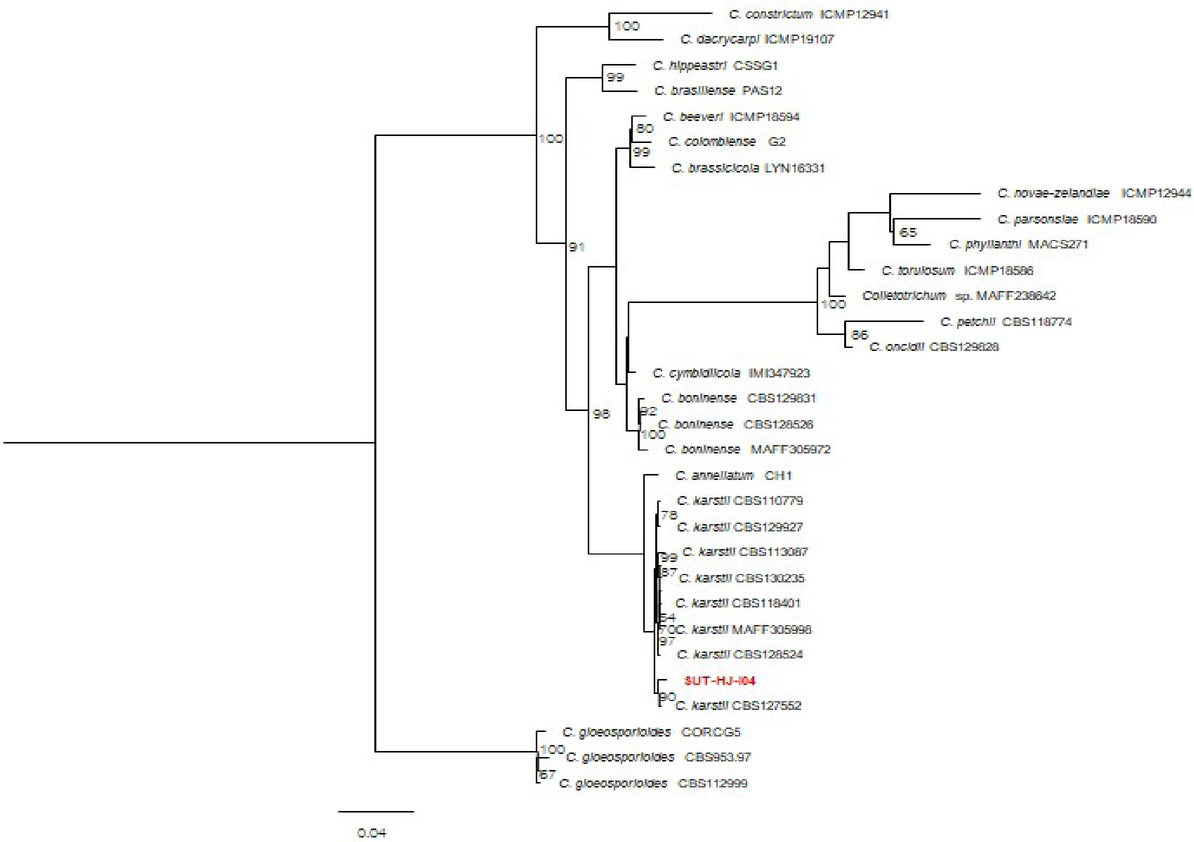

Fig. 4. Maximum Likelihood (ML) tree obtained based on phylogenetic analysis of concatenated sequences of the act, chs-1, gadph, ITS and tub2 genes of the isolate SUT-HJ-I04 and *Colletotrichum boninense* species complex. Numbers above branches are bootstrap values. Only values above 50% are indicated. The species *Colletotrichum gloeosporioides* species complex was selected as an outgroup.

The finding that *Colletotrichum* and *Aspergillus* have a role in seed germination of *H. janellehayneana* is consistent with prior work. Non-mycorrhizal fungi species, including *Colletotrichum, Aspergillus, Alternaria, Penicilli, Trichoderma* and *Fusarium* species, have been isolated from many orchid species (Cig *et al*., 2018; Alomía *et al*., 2022). Endophytic *Colletotrichum* fungi from *Bletilla ochracea* (Tao *et al*., 2013), *Dendrobium* spp. (Chen *et al*., 2010; Ma *et al*., 2018; Meng *et al*., 2019; Sarsaiya *et al*., 2020) and *Pogoniopsis schenckii* (Sisti *et al*., 2019) have been reported. Despite its high pathogenicity on seedlings, Shah *et al*. (2019) reported that *Colletotrichum* enhanced the growth of adult individuals of *Dendrobium* species. *Aspergillus* fungi, on the other hand, are not yet known to promote seed germination in orchids (Ma *et al*., 2015; Cig *et al*., 2018). Moreover, *A. fumigatus* was reported as an opportunistic orchid pathogen in *Laelia* orchids (Almanza-Álvarez *et al*., 2017).

More research is needed to assess the potential physiological and ecological benefits of non-mycorrhizal fungi commonly found in orchid roots. Some fungi produce active substances that may benefit orchids by increasing their tolerance to abiotic stress, allowing them to adapt to a variety of environmental circumstances or fighting to pathogens and insects (Ma *et al*., 2015). Some fungi may even breakdown local soils and offer nutrients for orchid growth and development (Li *et al*., 2021). Using the appropriate fungal strain may improve germination success.

Since all orchids rely on mycorrhizal partners to germinate naturally, symbiotic germination is now a widely employed technique and helpful strategy for terrestrial orchid conservation efforts. The symbiotic technique has been successfully applied to germinate three *Habenaria* species from Florida, USA, including *H. repens*, *H. quinquiseta*, and *H. macroceratitis*, with germination percentages ranging from 5.8–55.1%. The highest germination rates for all species were achieved using a *Ceratorhiza* isolate (Stewart and Zettler, 2002). Only *H. repens* seedlings developed stage 5, while none of *H. quinquiseta* or *H. macroceratitis* seeds developed beyond stage 2. Similar to their results, our study showed that none of the seedlings of *H. janellehayneana* inoculated with *Colletotrichum* or *Aspergillus* developed beyond the stage 2 or stage 3, respectively. Further investigations should explore how such seedlings can continue development thereafter. Studies on other orchid species have documented a need for multiple fungal species to achieve full development. For example, Chutima *et al*. (2011) showed that endophytic fungi isolated from *Pecteilis susannae* (L.) Rafin. enhanced seed germination up to 86.20% when the seeds were also grown with *Epulorhiza* sp. Similarly, a combination of *Ceratobasidium* sp., *Flavodon* sp., and *Tulasnella* sp. isolates induced significantly higher germination rates in *Paphiopedilum villosum* (Lindl.) Stein. as compared with uninoculated control treatments (Khamchatra *et al*., 2016). In addition, using *Ceratobasidium* strains achieved a high germination frequencies of up to 80% whereas *Tulasnella* strains supported a germination percentages close to 60% (Alomía *et al*., 2017).

CONCLUSION

For asymbiotic seed germination of *H. janellehayneana*, the highest germination percentages were obtained on ½ VW or with the addition of 1 mg/L BAP, and seeds on this medium developed to stage 4 protocorms within 10 weeks. In the case of symbiotic seed germination, however, two non-mycorrhizal endophyte fungi isolates obtained from the roots of wild-grown adult *H. janellehayneana* plants promoted seed germination via a symbiotic effect in co-culture. This research suggests that these fungal isolated may be effective for symbiotic early seed germination of this orchid species, but they are less effective for further growth. More specific mycorrhizal fungi might be needed for seed germination enhancement and onward development of this (and other) terrestrial orchid species. Nevertheless, orchid growers may achieve more consistent results in the propagation of this terrestrial orchid using asymbiotic germination.

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