

Seed Germination of *Haemaria discolor* var. *dawsoniana* and the Use of Mycorrhizae

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Abstract

Haemaria discolor var. *dawsoniana* is an orchid with beautiful red-green striped leaves, which can be used as an ornamental or for medical purposes. Seeds of *H. discolor* in Murashige & Skoog (MS) medium or Hyponex (#1,2 and 3) agar media germinated. In MS medium, the germination rate was optimal in 20 and 30 g/l of sucrose, but the germination rate was only 34%. However, in Hyponex #1 and H. #3 agar media, the germination rate was 60–63% within 60 days at room temperatures. Vegetative growth of the *H. discolor* seedlings in phytotrons was best with a 30/25°C day/night temperature regimes. Light microscopy and SEM observation showed that the cortex of roots could be infected by the orchid mycorrhizal fungi (OMF), and typical orchid mycorrhizal structures of "tolypophagy" with young and old pelotons were present in the same root. Of seven *Rhizoctonia* spp. isolates, only R.01 and R.02 enhanced seed germination of *H. discolor*, but the germination rate was only 5% and 37% respectively. For the inoculation of *Rhizoctonia* sp. *in vitro*, oat meal agar (OMA) medium was a simple effective medium. The growth of the *H. discolor* seedlings *in vitro* was enhanced by the inoculation of *Rhizoctonia* sp. encoded as R.01 and R.02. For practical use, seeds of *H. discolor* should be asymbiotically germinated in Hyponex # 3 agar medium with 20 g of sucrose, and the protocorms or small seedlings should be inoculated near the roots with R.01 or R.02 isolate after subculturing into OMA medium.

Keywords: *Haemaria*, *Rhizoctonia* sp., seed germination, seedling production, Hyponex agar, oat meal agar (OMA), orchid mycorrhizal fungi (OMF)

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1. Introduction

Haemaria discolor var. *dawsoniana* was introduced to Taiwan from Burma more than 5 years ago. It is an orchid with red-green striped leaves and blooms from late December to March. The whole plant of *H. discolor* is also used as an herbal medicine for curing lung diseases, such as pulmonary tuberculosis (Chou, 1997; Lo, 1992; Teuscher, 1978). As a result of a limited supply, relatively high prices are demanded on the flower market of Taiwan for this orchid (Chou and Chang, 1999a and b).

In the National Taiwan University (NTU) green houses, *H. discolor* has bloomed every year for the past 5 years, but never bore any seeds. However, trained workers can achieve 100% pollination rate under controlled condition.

The stems usually grow less than 20 cm (3–8 nodes) per year, and stem cutting, the usual means of propagation need 2 nodes for success. Therefore, for mass production of this orchid, both seed production and germination are required.

In nature, seed germination of orchid plants usually requires the presence of orchid mycorrhizal fungi (OMF) (Hadley, 1982; Hadley and Smith, 1983). However since micropropagation techniques became well developed, seed germination of orchids has been generally achieved by asymbiotic tissue culture methods. Subsequent inoculation of OMF with micropropagated *Anoectochilus formosanus* Hayata can greatly enhance the growth of the plantlets *in vivo* and *in vitro* (Sheu, 1994; Tsai, 1997). In the present study an attempt was made to compare the effects of OMF and tissue culture techniques with respect to seedling production in *H. discolor*.

2. Materials and Methods

Fifteen replicates were tested for each treatment. For all the symbiotic inoculations, R.01–R.07 isolates cultured in water agar for 5–10 days were cut into $1 \times 3 \text{ mm}^2$ blocks for *in vivo* or *in vitro* inoculation. All the data were computer analyzed using Microsoft Excel and Costat.

Isolating, culturing and purifying Rhizoctonia spp. from Haemaria discolor and terrestrial orchid roots from Taiwan

Isolating orchid mycorrhizal fungi: Orchid roots rinsed in tap water were sterilized with 1% sodium hypochlorite solution, and subjected to ultrasonic vibration for 15 min. The roots were rinsed with sterilized water 3 times and then cut into small pieces (1–2 mm in thickness) in a laminar flow cabinet. The root pieces were cultured in darkness on 1/6 usual concentration of Difco PDA (Tsai, 1997; Chou, 1997).

Screening and purification of inoculum: The growth of mycelium was observed, and a single hypha was removed to a new plate of PDA medium in order to obtain a pure culture. The cultures were identified by light microscopy as *Rhizoctonia* spp. because of the constriction of the mycelium in the branching areas, the dolipore septa, the angles of the branching mycelium, the nucleus number, the monilioid cells of the aged mycelium and the size and colour of the mycelium (Sneh et al., 1991).

Pathogenicity test for Rhizoctonia spp. on mungbean sprouts (Chen, 1995)

Mungbean seeds were sown in growth medium (peatmoss:vermiculite=1:1 by volume), to which 5% "V-8 juice" was added. Mungbean seedlings were inoculated with small blocks (1 × 3 mm²) of only one *Rhizoctonia* isolate. The growth of the seedlings was observed for at least 7 days. Only those *Rhizoctonia* isolates which grew on healthy sprouts were selected as inocula.

Hand pollination and orchid capsule collection (Chow, 1997)

After the flower buds of *H. discolor* had been open for 3–4 days, and the pollen was light yellow in colour, hand pollination was carried out. Seed capsules were collected for germination *in vitro* 40–45 days after pollination.

Light microscopy and SEM observation for the orchid mycorrhiza

The morphology and root structures of non-mycorrhizal control plants grown in MS medium and mycorrhizal plants grown in OMA medium inoculated with R.01 isolate for 348 days were compared by light microscopy and by SEM observations.

Roots of orchid were rinsed with water and were sectioned with a vibratome, stained with 0.05% aniline blue for 15–20 min at room temperature and examined under a light microscope. Roots were collected, dissected and fixed by 3% glutaraldehyde for 1–2 days, then were dehydrated with acetone series, and critical point dried (CPD) in liquid carbon dioxide. Finally, the root segments were coated with gold for 90 sec using Biorad ion coater. The non-mycorrhizal and mycorrhizal root segments were examined using ABT 60 of SEM. Photos were taken on Fuji 120 black and white film.

Asymbiotic germination by tissue culture techniques

Seed capsules were collected 40–45 days after hand pollination, and surface disinfected with a cotton ball swab moistened with 70% ethyl alcohol, then

were sterilized with 1% of sodium hypochlorite solution for 10–15 min. Then the capsules were rinsed three times with sterilized water. Seeds were sprinkled onto the surface of agar media in glass test tubes (50 cm³ in volume). The incubation room was at 25±3°C, with 2 Klux of inflorescence light and 85–92% of relative humidity. Two agar media were used as follows:

MS medium with various sucrose concentration test: On MS medium with various concentrations of sucrose (Sigma product): 0, 10, 20, 30, 40 and 50 g/l, with tryptone 3 g, and agar (Sigma) 7.8 g, the pH of the medium was adjusted to 5.2.

Hyponex agar medium test: Hyponex agar media were prepared by using one of the Hyponex products [i.e. Hyponex #1(H. #1; NPK=7-6-9), H.#2 (NPK=20-20-20), H.#3 (NPK=10-30-20), H.#4 (NPK=25-5-20), and H.#5 (NPK=30-10-10)] 3 g, with tryptone 3 g, sucrose (Sigma) 20 g and agar (Sigma) 7.8 g in 1 liter of water, then the pH was adjusted to 5.2.

Only those seeds with appearance of papillae were counted as germinated seeds throughout the asymbiotic and symbiotic germination tests.

Symbiotic germination of Haemaria discolor in vitro

Seeds from surface sterilized seed capsules as mentioned above were sprinkled onto the surface of oat meal medium (OMA; oat meal 2.5 g + Difco agar 11.5g + distilled water and make up to 1000 ml, then adjust pH to 5.25) in 50 cm³ glass test tubes. The incubation conditions were the same as those for asymbiotic germination. A total of seven *Rhizoctonia* spp. orchid mycorrhizal fungi (OMF) isolates coded as R.01–R.07 were isolated from wild grown orchids. Only one isolate was used for each treatment.

Asymbiotic growth responses to temperature in vivo and the inoculation of orchid mycorrhizal fungi (OMF) for seedlings in vitro

Temperature test *in vivo*: Asymbiotic micropropagated seedlings with 2 leaves (about 1.0 cm height) were transplanted into 4 inch pots which contained peatmoss:vermiculite:perlite=1:1:1 v/v/v. The growth of seedlings was compared at various day/night temperature; 15/13, 20/15, 25/20 and 30/25°C in phytotrons.

Inoculation of OMF and various growth media test *in vitro*: Asymbiotic seedlings of 0.5 cm height were subcultured into 50 ml of test tubes containing 15 ml of agar medium. They were cultured in a 25±3°C cultural room with 2 Klux of light illumination in the following 3 agar media (Warcup, 1973). Per liter of medium contains as follows: (1) OMA (2.5 g/l of oat meal and 11.5 g Difco agar), (2) War Ma (WM, including NaNO₃ 0.3 g, KH₃PO₄ 0.2 g, MgSO₄·7H₂O 0.1 g,

KCl 0.1 g, cellulose powder 10 g, malt extract 0.1 g and Difco agar 11.5 g), and (3) War Ye (WY, including NaNO_3 0.3 g, KH_2PO_4 0.2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g, KCl 0.1 g, cellulose powder 10 g, yeast extract 0.1 g and Difco agar 11.5 g).

Symbiotic seedlings were inoculated with R.01 isolate by $1 \times 3 \text{ mm}^2$ of water agar block with small amount of mycelia. After 3 months, the growth response of plants on the various media were compared.

Growth comparison of non-mycorrhizal and mycorrhizal seedlings after inoculation with OMF for 4 months on OMA medium *in vitro*: *H. discolor* seedlings 0.5 cm tall were inoculated with R.01 or R.02 mycelium *in vitro*. Growth responses were compared after inoculation for 4 months.

3. Results and Discussion

Isolating Rhizoctonia spp. as inocula for H. discolor

H. discolor is an unique orchid as it has beautiful foliages with a dark red-green upper surface and red-colored lower surface (Fig. 1). The numerous small white and yellow flowers are very attractive (Fig. 2).

Seven *Rhizoctonia* spp. were isolated and cultured from terrestrial orchids of Taiwan. These were identified as *Rhizoctonia* spp. (Harley and Smith, 1983; Sneh et al., 1991; Tsai, 1997; Chou, 1997).

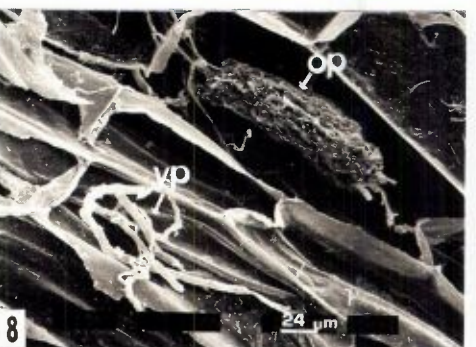
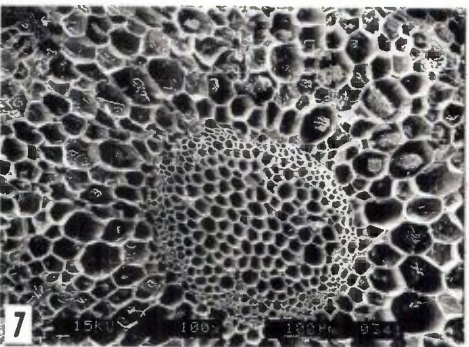
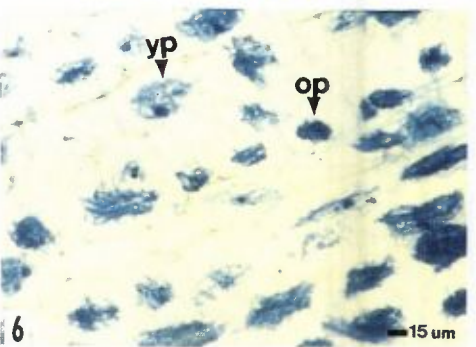
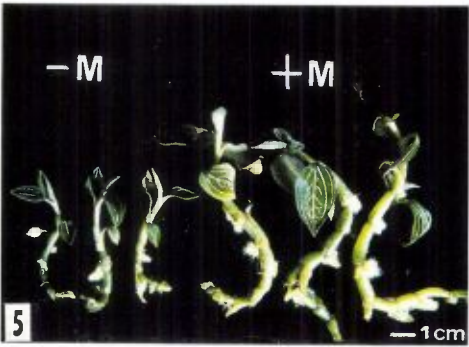
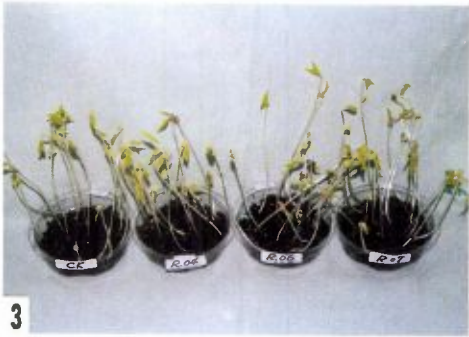
A total of seven lines of *Rhizoctonia* spp. were separately inoculated onto mungbean sprouts for 7 days. None of the 7 *Rhizoctonia* spp. caused any pathogenic symptoms on the mungbean sprouts (Fig. 3). The absence of pathogenic symptoms on mungbean sprouts indicated their suitability as inocula for orchid plants.

Seed capsules collection

Only light yellow to yellow pollens resulted in capsule formation after hand pollination. Capsules were collected when still in a light bronze colour, i.e. 40–45 days after pollination to ensure a full development of seeds with high germination rate (Fig. 4). Seeds in those capsules which had already split exhibited a poor germination rate.

Light microscopy and SEM observation of the orchid mycorrhiza

Better growth was observed for mycorrhizal seedlings (+M) on OMA medium than for non-mycorrhizal plants grown in MS medium (–M) (Fig. 5). Light microscopy showed that numerous young and old pelotons were present in the cortex of the roots (Fig. 6). SEM observation on mycorrhizal roots showed



typical orchid mycorrhizal structure, "tolytophagy", with young and old pelotons present in the cortex of same root (Figs. 7 and 8). It is the common mycorrhizal structures for the majority of orchids (Hadley, 1982).

Asymbiotic germination by tissue culture techniques

MS medium with various sucrose concentration test: The asymbiotic germination rate of *H. discolor* was 33 and 34% in MS medium with 20 or 30 g of sucrose/l, respectively, but zero in the absence of sucrose (Fig. 9).

Hyponex medium test: Seed germination rates were 60 and 63% in Hyponex #1 and H. # 3 agar media respectively, but only 37% in H. # 2 medium. Hyponex #4 and #5 agar media which had a high nitrogen contents, both resulted in no germination (Fig. 10).

Symbiotic germination in vitro

Previous experience showed that MS medium induced very rapid growth of OMF, that could lead to death of seedlings *in vitro*, so appropriate "inoculation media" are needed. On OMA medium, seeds of asymbiotic *H. discolor* did not germinate, while seeds which were inoculated with OMF R.01 or R.02 did germinate. The other five *Rhizoctonia* isolates did not result in any germination (Fig. 11). Seeds inoculated with R.02 OMF exhibited a 37% germination rate, and the same isolate also stimulates the growth of another orchid-*Anoectochilus formosanus* Hayata which has medical applications (Sheu, 1994; Chou, 1997; Tsai, 1997).

Figs. 1-8. See opposite page.

Figure 1. Morphology of the *Haemaria discolor* var. *dawsoniana* plants with flower buds.

Figure 2. Close up of *H. discolor* flowers.

Figure 3. The pathogenicity of *Rhizoctonia* spp. was tested by inoculation on mungbean sprouts for 7-10 days. Only those *Rhizoctonia* spp. which caused no harmful symptom should be used as inoculum.

Figure 4. Capsules of *H. discolor* were formed by hand pollination and should be collected 40-45 days after pollination for germination.

Figure 5. Growth of mycorrhizal *H. discolor* (+M, R.01) seedlings in OMA medium was much higher than those of non-mycorrhiza ones (-M) grown in MS medium.

Figure 6. Light microscopy of the mycorrhizal *H. discolor*. Note that the parenchyma cells in the cortical tissues formed young (yp) and old pelotons (op) in the same root.

Figure 7. SEM microscopy of the mycorrhizal *H. discolor*. Note it was the "tolytophagy" type of infection.

Figure 8. SEM observation of the mycorrhizal *H. discolor*. Note the young peloton (yp) and the old peloton (op) were present in the adjacent cells in the same root.

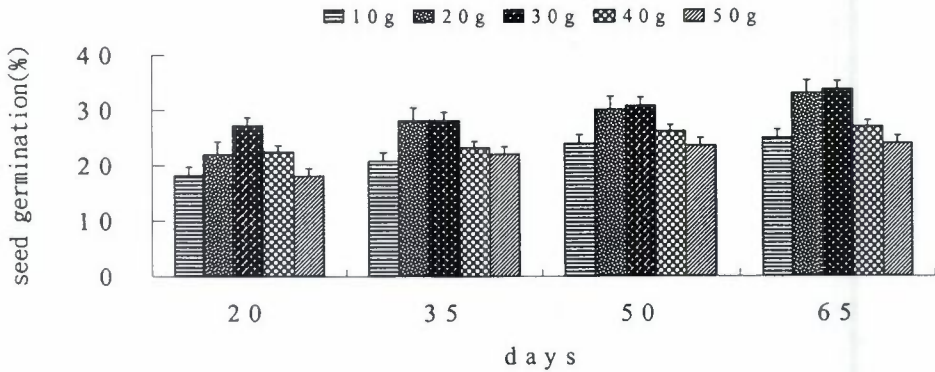


Figure 9. Asymbiotic germination of *Haemaria discolor* var. *dawsoniana* in MS medium with various sucrose concentration *in vitro*.

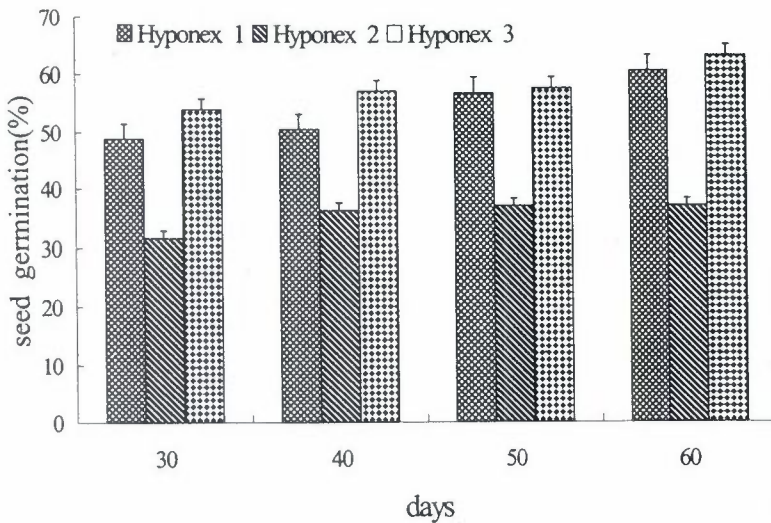


Figure 10. Asymbiotic seed germination of *Haemaria discolor* var. *dawsoniana* as influenced by Hyponex agar media *in vitro*. (Note: among Hyponex #1-5, only Hyponex 1-3 were effective for seed germination).

The R.01 inoculation lead to a maximum of 10% germination rate, Chou (1997) reported that this OMF could greatly enhance the later seedling growth in *H. discolor* both *in vivo* and *in vitro*. Results here indicate that both R.01 and R.02 are effective inocula for *H. discolor*. Only the growth enhancement by R.01 isolate was significant for plant height and node number than that induced by

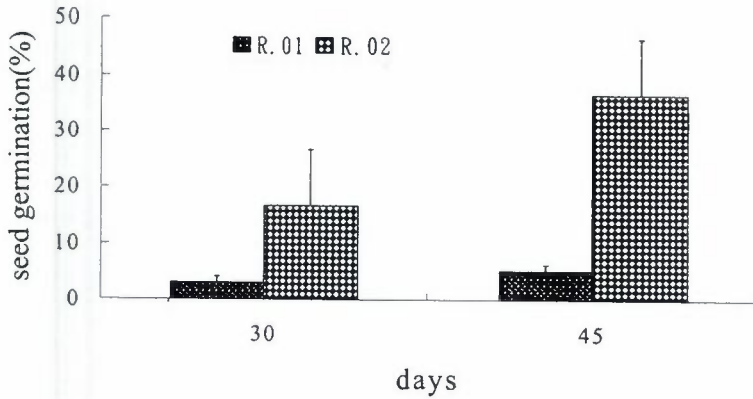


Figure 11. Symbiotic germination of *Haemaria discolor* var. *dawsoniana* in vitro as influenced by *Rhizoctonia* spp. Note that among 7 kinds of *Rhizoctonia* spp., only R.01 and R.02 inocula could enhance seed germination, while other five isolates (R.03–R.05) all showed no effect.

R.02 isolate (Table 3). So R.01 is preferred after seed germination. But R.02 is good for both seed germination and seedling growth.

Although the symbiotic germination rate (37%, Fig. 11) of *H. discolor* was lower than the asymbiotic germination (60–63%, Fig. 9) on optimal media, the symbiosis seemed to enhance development of the protocorms and the growth of seedlings large enough to be transplanted (Fig. 5). The symbiotic germination technique showed potential for the propagation of some rare species, e.g. *Diuris punctata* var. *albo-violacea* and the conservation of other species (Clements and Ellyard, 1979). Thus, the two *Rhizoctonia* spp. isolates, R.01 and R.02 have potential for commercial use.

Asymbiotic growth responses to temperature in vivo and the inoculation of orchid mycorrhizal fungi (OMF) for seedlings in vitro

Temperature test: Vegetative growth of the non-symbiotic *H. discolor* was highest at 30/25°C day/night, and the fresh weight of the plant was much greater than those at 15/13°C and 25/20°C (Table 1). This refutes an earlier report that the optimum growth temperature was approximately 24°C (Jones, 1979). Thus our *H. discolor* plants adapt to the weather here and become heat stable in Taiwan.

Inoculation of OMF and various growth medium tests: Orchid plantlets or seedlings grown in MS media inoculated with *Rhizoctonia* spp. isolates of OMF *in vitro* were fungus covered within 10 days, and finally died. Thus, for successful symbiotic cultures, ordinary tissue cultured media should not be

chosen. On OMA medium, it was found that the growth of symbiotic *H. discolor* plants was greatly enhanced following inoculation by R.01 OMF. But the other two media, namely WM and WY were not effective for the growth of seedlings (Table 2).

Table 1. Asymbiotic seedling growth of *Haemaria discolor* var. *dawsoniana* plants *in vivo* as affected by day/night temperature for 3 months in phytotrons

Temp. (°C)	Plant height (cm)	Leaf no.	Leaf length (mm)	Leaf width (mm)	Root no.	Node no.	Fresh weight (mg)
15/13	2.0c	3a	4.0c	2.7b	1a	3b	56.2c
20/15	2.5c	3a	5.7c	3.7b	1a	3b	60.1c
25/20	3.2b	3a	7.0b	5.7b	2a	4b	95.1b
30/25	5.1a	3a	16.5a	9.8a	2a	6a	306.5a
35/30	3.7b	3a	8.3b	4.0b	1a	3b	120.1b

15 replicates grown in "King Garden #3" growth medium (peatmoss:vermiculite:perlite=1:1:1 v/v/v) were tested for each treatment, in which 1.0 cm height of asymbiotic micropropagated seedlings were used. Means in each column followed by the different letters were significantly different ($p=0.05$) as determined by Duncan's multiple range test.

Table 2. Symbiotic seedling growth of *Haemaria discolor* var. *dawsoniana* *in vitro* as grown in 3 "inoculation media" for 3 months after the inoculation of orchid mycorrhizal fungi (R.01)

Inoculation and medium	Plant height (cm)	Leaf length (mm)	Leaf width (mm)	Root no.	Root length (mm)	Node no.	Fresh weight (mg)
OMA ^a	3.8a	8.1a	5.1a	3.4a	5.2a	5.4a	370a
WM ^b	2.2b	3.4b	2.7b	1.0bc	2.5b	4.9a	60b
WY ^c	0.6c	0.5c	0.4c	0.1c	0.1c	2.1b	30b

^aOMA medium = oat meal 2.5 g + Agar 11.5 g (Difco) + distilled water, then make up to 1000 ml, and adjusted to pH 5.25; ^bWar Ma (WM) medium = NaNO₃ 0.3 g + KH₂PO₄ 0.2 g + MgSO₄·7H₂O 0.1 g + KCl 0.1 g + malt extract 0.1 g + Difco Agar 11.5 g + distilled water, then make up to 1000 ml, and adjusted to pH 5.25; ^cWar Ye (WY) medium = NaNO₃ 0.3 g + KH₂PO₄ 0.2 g + MgSO₄·7H₂O 0.1 g + KCl 0.1 g + yeast extract 0.1 g + Difco Agar 11.5 g + distilled water, then make up to 1000 ml, and adjusted to pH 5.25. 15 replicates were tested for each treatment, in which 0.5 cm tall of seedlings were used. Means in each column followed by the different letters were significantly different ($p=0.05$) as determined by Duncan's multiple range test.

Table 3. Seedling growth of *Haemaria discolor* var. *dawsoniana* in OMA medium as influenced by the inoculation of *Rhizoctonia* spp. isolates (R.01 or R.02) for 4 months *in vitro*

Inoculum	Plant height (cm)	Leaf no.	Leaf length (mm)	Leaf width (mm)	Root no.	Node no.	Fresh weight (mg)
NM control ^a	1.8c	1.7a	0.3b	0.2b	1.5c	3b	38.7b
R.01 ^b	4.2a	3.0a	0.8a	0.5a	4.0a	6a	396.5a
R.02 ^c	3.4b	2.7a	0.7a	0.5a	3.0b	5a	373.3a

15 replicates for each treatment, in which 0.5 cm height of seedlings were inoculated, then were cultured in a 25±3°C room with 2 Klux of inflorescence light. ^aNM control: non-mycorrhizal control; ^bR.01: inoculated with R.01 OMF isolate; ^cR.02: inoculated with R.02 OMF isolate. Means in each column followed by the different letters were significantly different ($p=0.05$) as determined by Duncan's multiple range test.

Growth comparison of non-mycorrhizal and mycorrhizal seedlings after inoculation with OMF for 4 months on OMA medium *in vitro*: All the growth parameters showed significant difference between mycorrhizal and non-mycorrhizal control. In the inoculation treatments, the plant height, root number and node number were higher for R.01 than R.02. Both *Rhizoctonia* isolates resulted in a 9–10 fold increase in fresh weight of growth compared with the non-mycorrhizal control on OMA medium (Table 3). The growth enhancement caused by the inoculation of OMF was higher than for asymbiotic plant on MS medium (Fig. 5) or Hyponex #3 agar medium (unpublished data). Thus R.01 and R.02 are recommended for enhancing the growth of *H. discolor* *in vitro* on OMA media for germinated seedlings. The results also indicate that symbiotic seed germination on OMA medium with the simultaneous inoculation of R.02 followed by subculturing on OMA medium is an alternative strategy for seedling production in *H. discolor*.

4. Conclusions

The growth of *H. discolor* in Taiwan indicates that high temperature 30°C was optimum for this orchid. Hyponex #1 or #3 agar medium with 20 g of sucrose/l could result in 60% asymbiotic germination. The symbiotic germination rate with the inoculation of *Rhizoctonia* isolate R.02 for *H. discolor* seeds in OMA medium was at most 37% (R.02). *In vitro* inoculation of R.01 or R.02 OMF isolate significantly stimulated the growth and development

of protocorms and the development of seedlings, and resulted in an earlier supply of plants large enough to be transplanted. It is important to note that for *in vitro* inoculation of OMF, the medium should be OMA, otherwise very rapid growth of OMF will result in death of the plantlets.

Acknowledgment

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