Propagation of *Haemaria discolor* via *in vitro* seed germination

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Abstract

In vitro propagation protocol for *Haemaria discolor* (Ker) Lindl. var. *dawsoniana* by artificial cross-pollination and asymbiotic germination of seeds has been developed. Fruit set (100 %) was obtained when the pollinia and ovules of various aged flowers were used for pollination. *In vitro* germination of seeds obtained from capsules of various ages was achieved on half-strength Murashige and Skoog's (MS) medium supplemented with 3 % sucrose and 0.85 % agar. The germinated seedlings were cultured on half-strength MS medium with 0.2 % activated charcoal, 8 % banana homogenate, 0.1 mg dm^{-3} 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (TDZ) and 1 mg dm^{-3} α-naphthaleneacetic acid (NAA). Ninety-six percent of plantlets survived after hardening in greenhouse.

Additional key words: *in vitro* propagation, Jewel orchid, pollination.

Introduction

Haemaria discolor (Mandarin: Xue-ye-lan or Cai-ye-lan) belongs to a group of terrestrial orchids commonly known as "Jewel Orchids" due to their attractive foliage. It is native to the regions from southern China and Burma down through the Malay Archipelago, Sumatra, Java, and other islands of Indonesia (Hawkes 1970, Teo 1978). It is a slow growing perennial plant and seedlings mature and reproduce through seeds after 2 - 3 years of growth and does not need special conditions for the growth like under a bell jar, which are usually essential for its more delicate allies (Hawkes 1970). The leaf-extracts of *H. discolor* were found to contain asparagine, glutamine, histidine, serine and threonine (Arditti 1992).

For conservation and commercial cultivation of this species, an efficient *in vitro* propagation system is desirable. Micropropagation of *Haemaria discolor* has been achieved using nodal explants (Teo 1978) and by seed germination (Chou and Chang 1999b). To the best of our knowledge *in vitro* propagation by protocorm tissues for this jewel orchid has not been reported so far. The present studies were carried out with the objectives on one hand to resolve the optimum period for cross-pollination and fruit set and on the other hand to standardize the concentration of plant growth regulators in culture medium for asymbiotic development of seedlings and acclimatization of plantlets in greenhouse conditions.

Materials and methods

Plants: Thirty plants (approx. 12 - 15 cm in height) of *H. discolor* grown in the greenhouse at Agricultural Research Institute (ARI) in central Taiwan for two years were used in the present study. The plants were replanted in plastic trays containing peat moss:*Vermiculite*:*Perlite* (1:1:1 by volume) in September 2000 and were maintained in growth chamber (*Model 624 HD, Hotech Instruments Corp.*, Taipei, Taiwan) under cool white fluorescent tubes (*Philips*, Eisenhoven, The Netherlands) at irradiance of 47 μmol m^{-2} s^{-1}, with a 14-h photoperiod, 90 % relative humidity, and 25/20 ºC day/night temperature. The plants were initially covered with a polythene sheet and irrigated once a week with tap water.

Received 13 January 2004, *accepted* 3 March 2005.

Abbreviation: TDZ - 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea; NAA - α-naphthaleneacetic acid; MS - Murashige and Skoog's medium; DAF - days after flowering.

Acknowledgement: This research was supported by grant (91AS-3.1.1-CI-C3) from the Council of Agriculture, Executive Yuan of Taiwan and grants (NSC-89-2317-B055-002 and NSC-91-2317-B324-001) from the National Science Council of Taiwan. This paper is Agricultural Research Institute Contribution No. 2158.

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Pollination: A pair of bipartite pollinia was picked and deposited on the stigma of a flower of different plant. To determine the optimum period for cross-pollination, flowers were classified into five groups: Group A, 0, 1, 2 days after flowering (DAF); Group B, 6 - 8 DAF; Group C, 12 - 14 DAF; Group D, 18 - 20 DAF; and Group E, 24 - 26 DAF. The pollinated plants were maintained in the growth chamber for 30 d. For each group fifteen flowers were pollinated.

The pollen germination studies were carried out as described for *Anoectochilus formosanus* (Shiau *et al*. 2002). The number of pollen tubes emerging from massulae was counted and the percentage of pollen germination was calculated to determine viability of pollen. Pollen tubes from at least 20 massulae were scored in each group and the experiments were repeated thrice.

Seed germination: Capsules formed after 15, 20, 25, and 30 d of hand pollination were surface-disinfected in 70 % ethanol for 30 s, followed by treatment with 0.5 % sodium hypochlorite (*Clorox, The Clorox Co*., Oakland, CA, USA) with two drops of *Tween 20*® (*Hayashi Pure Chemical Industries Ltd*., Osaka, Japan) under ultrasonic vibration (*Branson Ultrasonic Cleaner, Branson Cleaning Equipment Co.*, Shelton, CT) for 15 min and rinsed three times with sterile distilled water. The seeds from the surface sterilized capsules were cultured by spreading as thinly as possible over the surface of the culture medium in 22 × 120 mm glass test tubes containing half-strength Murashige and Skoog (1962; MS) basal medium. Three capsules (thirty tubes) were cultured to evaluate the optimum seed germination. The cultures were incubated for four months at 25 ± 1 °C under cool white fluorescent tubes at irradiance of 38 μmol m^{-2} s^{-1} with a 14-h photoperiod. The percentage of seed germination was scored after 45 d of culture.

Growth of seedlings: The seedlings obtained from the capsules collected after 30-d of pollination were used in the further studies. Four-month-old seedlings with well developed shoot and rhizomes, were re-cultured in Erlenmeyer flasks, each containing 100 cm^3 of half-strength MS medium containing 0.2 % activated charcoal, 8 % m/v banana homogenate, with 0.01, 0.1, or 1.0 mg dm^{-3} TDZ alone or in combination with 0.2, 0.5, or 1.0 mg dm^{-3} NAA. All media were supplemented with 3 % sucrose and 0.85 % *Difco Bacto* agar (*Difco Laboratories*, Detroit, MI, USA). The pH of medium was adjusted to 5.7 ± 0.1 before autoclaving at 121 °C, 105 kPa for 15 min. The culture vessels were capped with two layers of aluminum foil before autoclaving and sealed with three layers of *Parafilm M*® (*American National Can*$_{TM}$, Menasha, WI, USA) after inoculation. Twenty seedlings were cultured per flask and five flasks per combination of plant growth regulators. The cultures were incubated under the conditions described above for five months.

Acclimatization of plantlets: The plantlets (240) with well-developed rhizomes and shoots were washed thoroughly under tap water for 2 - 3 min to remove agar. These were planted in plastic trays (40 plants per tray) containing a autoclaved mixture of peat moss:*Vermiculite*:*Perlite* (1:1:1 by volume). The plants were initially covered with a polythene sheet and kept in growth chamber for one month to maintain high humidity (above 90 %) and then transferred to greenhouse (above 70 % humidity) for further hardening. They irrigated once in a week with tap water. The percentage survival of plants was calculated after two months.

Statistical analysis: Fisher's protected least significant difference (LSD) test (Fisher 1935), standard deviation or 95 % confidence limit of binomial variation was used for statistical analysis.

Results and discussion

Synchronization of flowering and hand-pollination: Plants cultured in growth chambers flowered after six months (in March 2001) of replanting. In controlled environmental conditions of the growth chambers uniform flowering pattern was observed (Fig. 1*A*,*B*). Similar to other species of Jewel orchids the flowers of *H. discolor*

Table 1. Effect of pollen and ovule maturity on cross-pollination, fruit set and embryo germination in *Haemaria discolor*. Means ± SD. Means followed by the same letter within a column are not significantly different from each other at the 5 % level as determined by LSD test. Values in parentheses are 95 % confidence limits of binomial distribution.

Pollinia age [DAF]	Pollen germination [%]	Number pollen tubes [massula^{-1}]	Fruit set [%] ovule maturity [DAF] 0,1,2	6-8	12-14	18-20	24-26
A (0,1,2)	94.9[a]	74.5 ± 31.3	100 (69-100)	100 (69-100)	100 (69-100)	100 (69-100)	50 (19-81)
B (6-8)	93.3[a]	64.2 ± 27.9	100 (69-100)	100 (69-100)	100 (69-100)	100 (69-100)	60 (26-88)
C (12-14)	87.6[b]	33.2 ± 15.5	100 (69-100)	100 (69-100)	100 (69-100)	100 (69-100)	40 (12-74)
D (18-20)	53.7[c]	13.3 ± 11.2	100 (69-100)	100 (69-100)	90 (55-100)	80 (44-97)	40 (12-74)
E (24-26)	21.6[d]	7.7 ± 9.4	100 (69-100)	100 (69-100)	90 (55-100)	70 (35-93)	50 (19-81)

arise on a spikelet, has a pollen cap and a characteristic lip (Fig. 1*C*). The flower has pollinarium having pair of bipartite pollinia, which is attached to a well-developed sticky base, the viscidium (Fig. 1*D*) (Freudenstein and Rasmussen 1996). The differentiation and development of ovules in orchid flowers are pollination dependent (Zang and O'Neill 1993). The fruit set is defined as retention of a firm light green ovary. In the present study, there has been no significant effect of pollen age on the fruit set. In contrast to the *A. formosanus* (Shiau *et al*. 2002), maximum percentage of fruit set (100 %) was obtained when the pollinia and ovules of various aged flower were used for pollination (Table 1). The number of pollen tubes per massula was higher in the 0- to 2-d-old flowers. The massulae (Fig. 1*E*) from group A flowers showed the maximum germination of pollen tubes (Fig. 1*F*, Table 1).

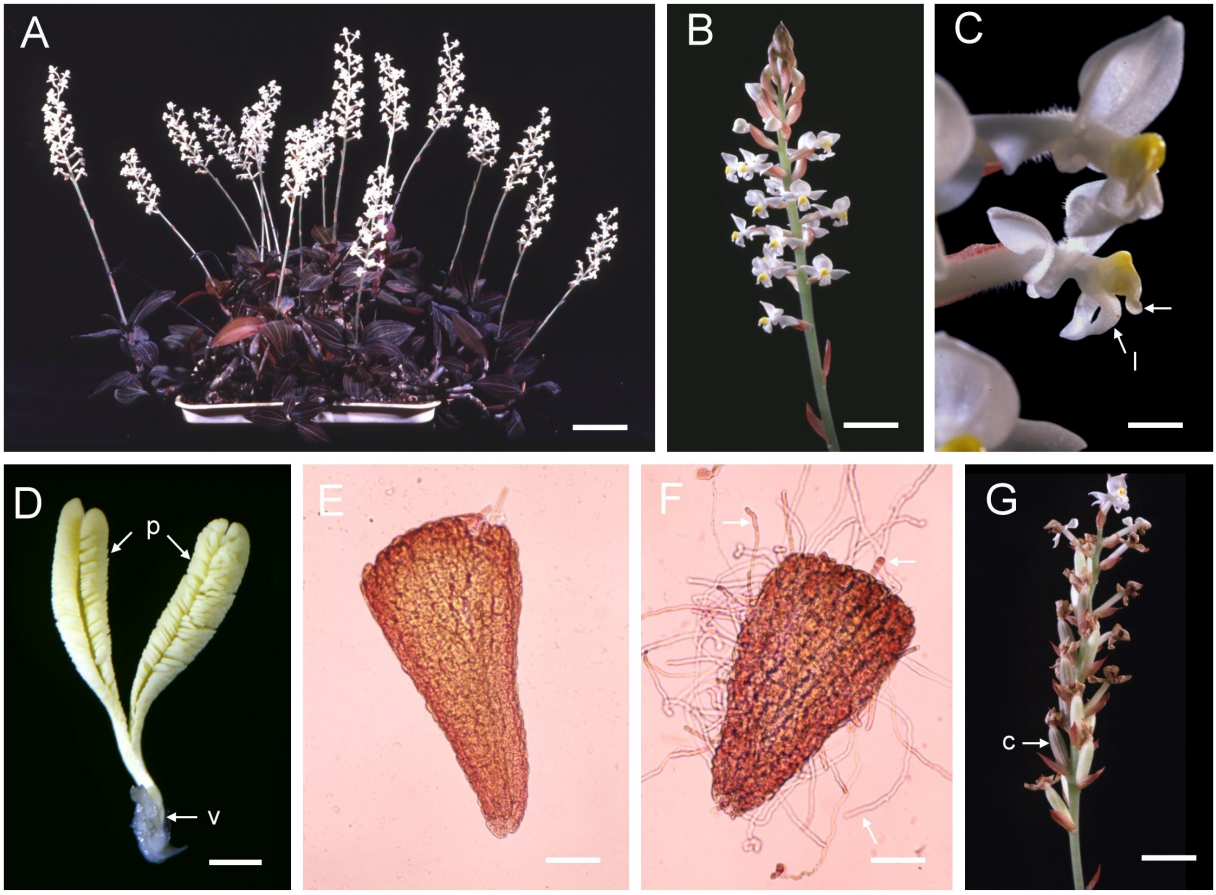

Fig. 1. Flowering in *Haemaria discolor*. *A* - Synchronous flowering in plants after replanting in peat moss:*Vermiculite*:*Perlite* for four months (*bar* 10.59 cm). *B* - The inflorescence of *H. discolor* (*bar* 3.25 cm). *C* - A spikelet with cream-yellow flowers having a characteristic lip (l) and the pollen cap (*arrow*) holding the pollinarium (*bar* 3.91 mm). *D* - A pollinarium showing a pair of bipartite pollinia (p) attached to a viscidium (v) (*bar* 550 µm). *E* - A massula consisting of packets of pollens (*bar* 50 µm). *F* - A massula showing emergence of pollen tubes (*arrows*) after incubation in pollen germination medium for 18 h (*bar* 58 µm). *G* - Mature capsules (c) formed 30 d after hand-pollination (*bar* 3.12 cm).

Seed germination *in vitro*: Germination of orchid seeds was considered difficult, as specific nutritional and environmental conditions are needed (Arditti *et al*. 1990, Rasmussen 1995). Immature capsules are most suitable for *in vitro* germination as it is easy to surface disinfect them than mature ones (Yam and Weatherhead 1988) and also embryos become viable and develop normally prior to the capsule ripening (Arditii 1967 and references cited therein, Yam and Weatherhead 1988, Mitchell 1989). Usually the orchid seeds lack endosperm and cotyledons and lipid droplets are the only storage materials localized within the embryo proper itself (Arditti and Ernst 1984). These lipids are utilized during germination of orchid seeds (Manning and van Staden 1987) and cytokinin is needed for the lipid mobilization. However, seeds of *H. discolor* could be germinated on medium devoid of cytokinin. This may be because of the presence of sufficient level of endogenous cytokinin required for initial stages of germination. Significant variation (9.0 - 44.5 %) was observed among capsules collected at four different periods after

pollination, tested for seed germination on half strength MS medium (Table 1). Thirty-day old capsules (Fig. 1*G*) showed highest seed germination after 45 d of incubation. For germination studies Chou and Chang (1999a), used capsules after 30 - 45 d of hand pollination. However, we found that in the *H. discolor* plants grown under controlled environmental conditions, the capsule matures in 25 - 30 d after pollination. In many European terrestrial orchids growing in natural conditions capsule matures in about 6 - 7 weeks from the day of pollination (Mitchell 1989).

Growth of seedlings: Although the seeds germinated on the plant growth regulator free medium (Fig. 2*A*,*B*) but

Table 2. Influence of seed maturity (d after pollination) on germination of *Haemaria discolor* after culture for 45 d. Means followed by the same letter within a column are not significantly different from each other at the 5 % level as determined by LSD test.

Seed maturity [d]	Seed colour	Seed germination [%]
15	White	9.3 [c]
20	Hazel	30.1 [b]
25	Hazel	43.2 [a]
30	Tawny	44.5 [a]

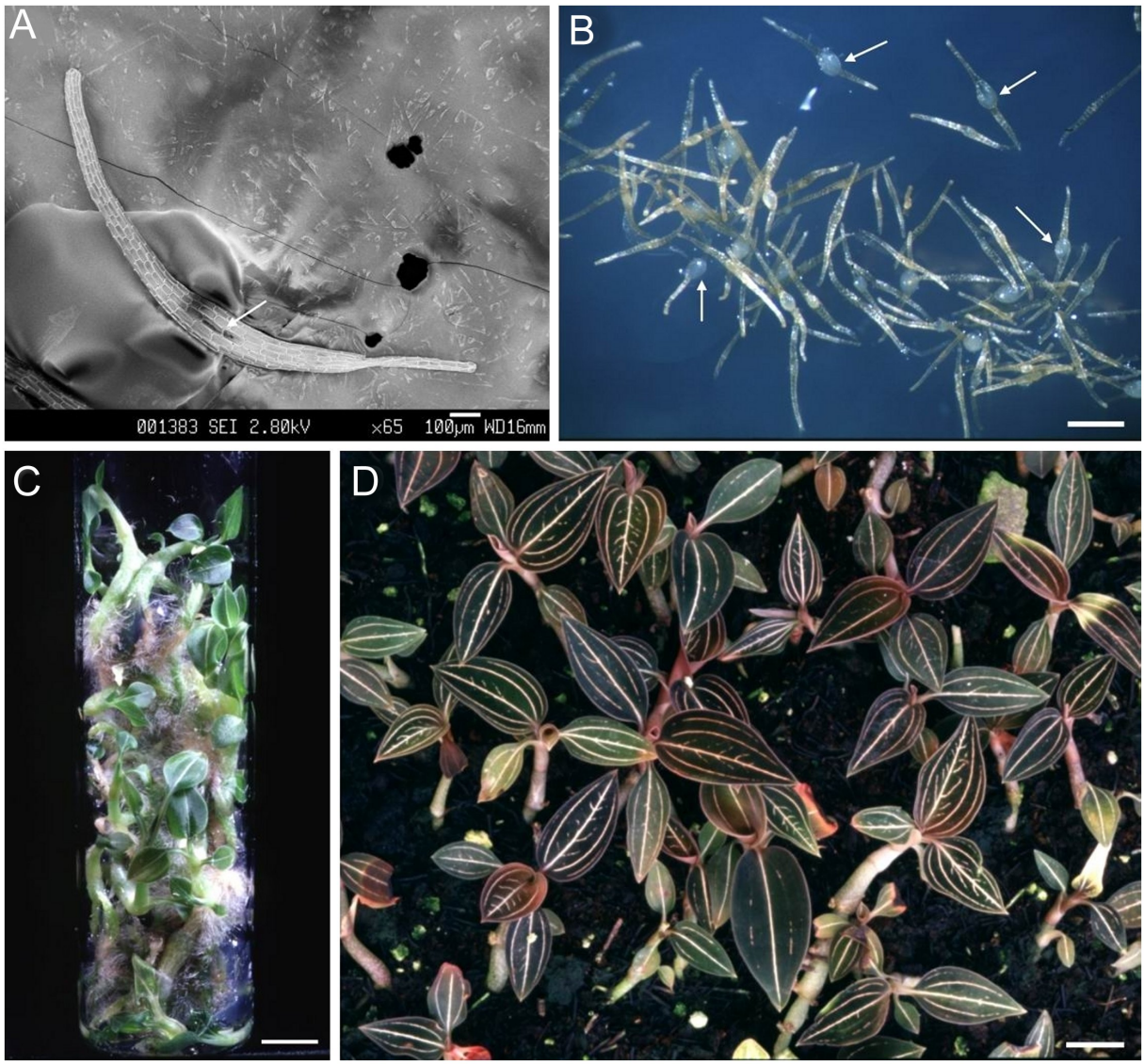

Fig. 2. Asymbiotic seed germination and acclimatization of plant of *Haemaria discolor*. *A* - SEM of the seed (*bar* 100 µm). *B* - Germination of seeds, 30 d after sowing on half-strength MS medium devoid of plant growth regulators or any additives (*bar* 0.8 mm). *C* - Seedling after 4 months of culture (*bar* 6.51 mm). *D* - Hardened healthy plants in greenhouse 5 months after transfer to peat moss:*Vermiculite*:*Perlite* (bar 2.04 cm).

Table 3. Influences of various concentrations of TDZ and NAA [mg dm^{-3}] on the seedling growth of *Haemaria discolor* growth parameters were determined after five months of culture. Means ± SD. Means followed by the same letter within a column are not significantly different from each other at the 5 % level as determined by LSD test.

TDZ	NAA	Height [cm]	Shoot diameter [cm]	Fresh mass [g $plant^{-1}$]
0.0	0.0	4.4 ± 0.4	0.33^{bc}	0.28^{ef}
0.01	0.0	4.0 ± 0.1	0.31^{c}	0.27^{f}
0.1	0.0	5.0 ± 0.1	0.36^{b}	0.35^{d}
1.0	0.0	4.4 ± 0.4	0.33^{bc}	0.25^{g}
0.01	0.2	5.8 ± 0.6	0.33^{bc}	0.38^{c}
0.1	0.2	5.0 ± 0.9	0.36^{b}	0.38^{c}
1.0	0.2	4.3 ± 0.5	0.33^{bc}	0.29^{e}
0.01	0.5	5.4 ± 0.8	0.37^{ab}	0.38^{c}
0.1	0.5	5.6 ± 0.1	0.36^{b}	0.42^{b}
1.0	0.5	4.6 ± 0.8	0.33^{bc}	0.25^{g}
0.01	1.0	4.9 ± 0.1	0.35^{bc}	0.35^{d}
0.1	1.0	5.3 ± 0.7	0.41^{a}	0.44^{a}
1.0	1.0	4.4 ± 0.5	0.33^{bc}	0.29^{e}

their growth was very slow. To enhance the growth, the seedlings were transferred to media supplemented with TDZ alone or in combination with NAA (Table 3). Activated charcoal and banana homogenate along with the phytohormones have been used for asymbiotic germination of orchid seeds (Ernst 1974, Yam and Weatherhead 1990 and references cited therein). Activated charcoal has been reported to promote multiple shoot formation in nodal segments of *A. formosanus* (Ket *et al*. 2004). In concurrence with the report on *Paphiopedilum ciliolare* (Pierik *et al*. 1988), activated charcoal and banana homogenate were not necessary during initial stage of seed germination but had positive influence on further development of the seedlings. The inclusion of TDZ and NAA in medium was found beneficial for seedling growth. Seedlings (Fig. 2*C*) transferred to half-strength MS medium supplemented with 0.85 % *Difco Bacto* agar, 0.2 % activated charcoal, 8 % m/v banana homogenate, 0.1 mg dm^{-3} TDZ and 1 mg dm^{-3} NAA, developed further into healthy plantlets and showed higher seedling height (5.3 ± 0.7 cm), seedling mass (0.44 g) and shoot diameter (0.41 cm) compared to other media combination (Table 3).

Acclimatization of plantlets: Five-month-old plants cultured on various media were randomly selected and hardened in greenhouse. The high humidity prevented the wilting of healthy plants transferred to a peat moss:*Vermiculite*:*Perlite* potting mixture. The *in vitro* raised plantlets became established in potting mix in plastic trays (Fig. 2*D*) with an acclimatization rate of 96 % after 4 months of incubation in the greenhouse under high humidity. The *in vitro* raised plants have been maintained since December 2001. These show morphological similarity with those grown naturally. This technique of *in vitro* germination and development of healthy plantlets could be helpful for re-plantation in their natural habitat or cultivation at large scale for the ornamental or pharmaceutical product of interest.

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