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Conservation of *Anoectochilus formosanus* Hayata by artificial cross-pollination and in vitro culture of seeds

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Abstract. *Anoectochilus formosanus* is an important ethnomedicinal plant of Taiwan. We have optimized a method for mass propagation of *A. formosanus* by artificial cross-pollination and asymbiotic germination of seeds. The success of pollination and fruit set was found to be dependent on the developmental stage of male and female gametophytes. Fruit set of hand-pollinated flowers was 86.7%. The seeds from 7-week-old capsules were germinated by culturing on half-strength Murashige and Skoog's (MS) medium supplemented with 0.2% activated charcoal and 8% banana homogenate for four months. Germinated seedlings were cultured in half-strength liquid MS medium containing 2 mg/l N^6-benzyladenine (BA) in 125-ml Erlenmeyer flasks for two months. Before *ex vitro* establishment of seedlings, seedlings with well-developed rhizomes and shoots were cultured on half-strength MS medium with 0.2% activated charcoal, 8% banana homogenate, 2 mg/l BA and 0.5 mg/l α-naphthaleneacetic acid (NAA) for further growth. Around 90% seed-derived plants survived two months after transfer to peat moss:vermiculite potting mixture and incubation in the growth chamber.

Keywords: *Anoectochilus formosanus*; Conservation; Endangered plant; In vitro propagation; Jewel orchid; Medicinal herb; Orchidaceae; Pollination; Pollinia; Seed germination.

Introduction

Anoectochilus formosanus (Mandarin: Jin-xián-liàn; Japanese: Kim-sòan-liân or Kinsenren) ($2n = 2x = 24$, Hsu, 1971) is an important medicinal herb of the family Orchidaceae. *Anoectochilus formosanus* belongs to a group of terrestrial orchids commonly known as "Jewel Orchids" (Cavestro, 1994) due to their attractive foliage (Teuscher, 1978). The genus *Anoectochilus* has 30 to 40 species found in Sri Lanka, Malaysia, India, Japan, southern China, Australia and the South Pacific Islands (Teuscher, 1978). *Anoectochilus formosanus* is one of four species of this genus found in Taiwan (Liu and Su, 1978). It is an endemic species, 20-30 cm tall, with velvety-textured, dark-green leaves that have intricate reticulations of silver veins above and pale purple on the lower surface. *Anoectochilus formosanus* is distributed between 800 m to 1,500 m in the central mountain range and Lanyu Island in Taiwan, on Ryukyu Island in Japan and in China's Fujian Province (Anon, 1999). The plants grow in deep shade on moist forest floors with a rich layer of humus, often in the vicinity of streams.

In Taiwanese folk remedies, the whole plant of *A. formosanus*, fresh or dried, is boiled in water and taken internally in the treatment of chest and abdominal pains (Hu, 1971), diabetes, nephritis (Chiu and Chang, 1995), fever, hypertension, impotence, liver and spleen disorders, and pleurodynia (Kan, 1986). The fresh herb is applied externally for snake-bite (Kan, 1986). It has been observed that *A. formosanus* contains substances affecting arachidonic acid metabolism, involved the function of the cardiovascular system (Mak et al., 1990; Huang et al., 1991). The aqueous extract of *A. formosanus* was found to possess anti-viral (Chan et al., 1994), anti-inflammatory, and liver-protective properties (Lin et al., 1993). The extract of dried *A. formosanus* was found to contain 4-hydroxycinnamic acid, β-sitosterol β-D-glucopyranoside (Takatsuki et al., 1992) and three butanoic acid glucosides (Du et al., 1998). Recently, a compound 3-(*R*)-3-β-D-glucopyranosyloxybutanolide, named Kinsenoside isolated from *A. formosanus* and *A. koshunensis*, was found to possess an anti-hyperliposis effect (Du et al., 2001). A patent has been registered for anti-diabetic and anti-atherosclerotic properties of compounds isolated from aqueous extract of *A. formosanus* (Takeshita et al., 1995).

Anoectochilus formosanus is a slow growing perennial herb with seedlings that mature and reproduce through seeds after 2-3 years of growth. It flowers only once a year in the winter between October and December. Indiscriminate collection of these plants, often before they have a chance to bloom, has reduced the species towards rarity. Reduced population size of *A. formosanus* may lead to reduced gene flow, inbreeding depression, and reduced fitness. Due to high cost of the herb (the current market price of the fresh and dry herb collected from its natural habitat is around US$320 and $3,200 per kilogram respectively) and increasing demand, other related species such as *A. koshunensis*, *Goodyera* spp., and *Zebrina pendula* are often found as adulterants in Taiwan's drug market (Lin and Namba, 1981a; 1981b).

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Development of a rapid in vitro propagation system for *A. formosanus* would help in germplasm conservation for commercial cultivation and also alleviate the pressure on the natural populations. In Taiwan, *A. formosanus* is cultivated on a limited scale by a few farmers. Cultivation is difficult as it is delicate and highly susceptible to stem and rhizome rot caused by *Fusarium oxysporum* (Hsieh et al., 1994). This problem could be overcome either by artificial cross-pollination, using different healthy/elite individuals of *A. formosanus*, which may result in the production of robust seedlings which are less likely to get infected by the disease, or in vitro propagation of elite plants. After cross-pollination, around 8,000 to 12,000 plants could be produced using a single capsule by in vitro sowing of seeds. In vitro mass propagation of *A. formosanus* has been achieved using shoot tip and/or nodal explants (Chow et al., 1982; Ho et al., 1987; Liu et al., 1987; Tai, 1987; Yen et al., 1996) and seeds from the capsules collected from wild plants (Ho et al., 1987; Lee et al., 1992; Yen et al., 1996). However, no data is available on percentage germination of in vitro cultured seeds. In a recent report, micropropagation of two endangered species of *Anoectochilus*, *A. sikkimensis* and *A. regalis*, has been achieved using nodal explants (Gangaprasad et al., 2000). However, the authors reported difficulty in obtaining seed capsules and failure in embryo culture for both the species. Furthermore, propagation by protocorm tissues, a commonly used method for other orchid species, has, to our knowledge, not been reported with jewel orchids.

Anoectochilus formosanus readily cross pollinates with *A. koshunensis*, a species which is not as important in folk medicine, and it is difficult to identify the two species and their hybrid until they flower because the plants are so similar in appearance (Lin, 1988). Recently, Cheng et al. (1998) standardized a method for correct identification of *A. formosanus*, *A. koshunensis*, and their hybrid based on RAPD markers.

The aims of the present investigation were: (1) to obtain synchronous flowering in plants of *A. formosanus* collected from their natural habitat; (2) to resolve the optimum period for cross-pollination and fruit set; and (3) to standardize conditions for asymbiotic germination of seeds and *ex vitro* establishment of plantlets.

Materials and Methods

Plant Material and Synchronization of Flowering in Plants

Fifty plants (approx. 10-15 cm in height) of *Anoectochilus formosanus* Hayata were collected from the Shuisheta Shan mountain area (121°00' N, 23°50' W, altitude about 1,100 m) of Nantou County in central Taiwan in the month of October and replanted in 49.5 × 29.5 × 6.5 cm plastic trays in a potting mixture of peat moss:vermiculite (1:1 by volume). The plants were covered with a polythene sheet and kept in a growth chamber (Hotech Instruments Corp., Model 624 HD, Taipei, Taiwan) under cool white fluorescent light at 47 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Philips, Holland), with a 16 h photoperiod per day, 90% relative humidity, and a 25/20°C day/night temperature for two months. The plants were irrigated once in a week with tap water. After two months, the photoperiod was reduced to 14 h, and the day/night temperature was lowered to 21/16°C for the succeeding four months. Once flowering was initiated, day/night temperatures were increased to 24/19°C, keeping the photoperiod to 14 h. To predict the number of flowers on a spikelet on a particular plant, the diameter of the stem just below the largest leaf and the length and width of the largest leaf were measured in centimeters using a digital caliper (Digimatic Caliper, Mitutoyo, Japan). The product of these three values was recorded as a "plant growth index." The graph was plotted as the number of flowers vs. the plant growth index using the data recorded from seventy flowering plants (Figure 1).

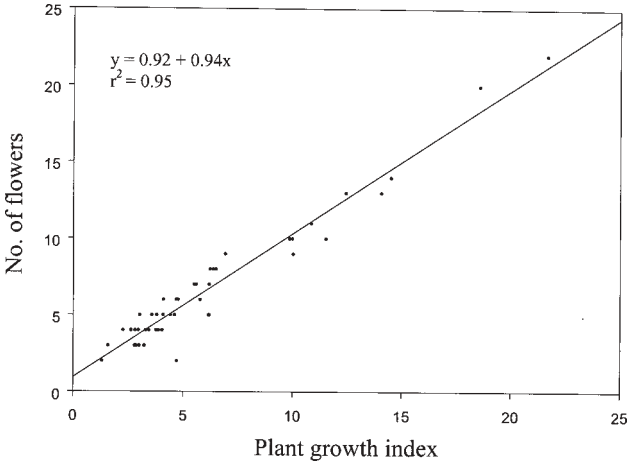

Figure 1. Relation between plant growth index and number of flowers borne on a spikelet for *Anoectochilus formosanus*.

Pollination

A pair of bipartite pollinia was picked up using tweezers and deposited on a stigma of a flower on another plant. It was not necessary to emasculate flowers of *A. formosanus* because its floral anatomy precludes self-pollination. To resolve the optimum period for cross-pollination, flowers were classified into five groups: -1 (the day before the opening of a flower), 0 (the day flower opens), 1 (one day after flowering) (DAF) (Group A), 2-4 days after flowering (DAF) (Group B), 5-7 DAF (Group C), 8-10 DAF (Group D) and 11-13 DAF (Group E) (Table 1). Fifteen flowers were pollinated for each group. Flowers were individually marked with tags. After pollinating plants, the capsules were allowed to ripen by maintaining plants in the growth chamber at 25 ± 1°C under cool white fluorescent light at 47 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 16 h photoperiod per day for 7 weeks.

Pollen Germination Studies

Anoectochilus formosanus belongs to subfamily Spiranthoideae. Two major pollinaria types inhabit this subfamily, granulate and massulate or sectile pollinia

Table 1. Effect of pollen and ovule maturity on pollination and fruit set in *Anoectochilus formosanus*.

Pollinia age	No. pollen tubes	% Fruit set and Ovule age (DAF)				
(DAF)[1]	per massula[2]	A (-1,0,1)	B (2-4)	C (5-7)	D (8-10)	E (11-13)
A (-1,0,1)	36.2[b]	40.0 (16-68)[3]	53.3 (27-79)	33.3 (12-62)	26.7 (8-55)	0.0 (0-22)
B (2-4)	56.4[a]	46.7 (21-73)	86.7 (60-98)	73.3 (45-92)	33.3 (12-62)	6.7 (0-32)
C (5-7)	40.0[b]	26.7 (8-55)	80.0 (52-96)	66.7 (38-88)	20.0 (4-48)	6.7 (0-32)
D (8-10)	14.8[c]	0.0 (0-22)	40.0 (16-68)	33.3 (12-62)	26.7 (8-55)	0.0 (0-22)
E (11-13)	8.9[d]	0.0 (0-22)	0.0 (0-22)	0.0 (0-22)	0.0 (0-22)	0.0 (0-22)

[1]Days after flowering (DAF).
[2]Means within a column followed by the same letter are not significantly different from each other at the 5% level as determined by LSD test.
[3]Values in parentheses are 95% confidence limits of binomial distribution.

(Balogh, 1982). Since *A. formosanus* has sectile pollinia with packets of tetrads, the number of pollen tubes emerging from massulae was counted to determine pollen viability (Table 1). The number of pollen tubes formed per massula was used to represent effect of pollen maturity on pollen germination (Table 1). Pollinia were collected from all groups. Massulae of pollinia were dusted on a microscopic glass slide with a drop of pollen germination medium containing 10% sucrose + 0.01% boric acid in distilled water, pH 5.8. The medium was autoclaved at 121°C, 105 kPa for 15 min. The slide was rested on glass rods over a moistened filter paper in a 14.5 cm glass Petri dish and incubated at 25°C under high humidity conditions in darkness for 36 h. The massulae with germinated pollen grains were stained with a drop of 1% acetocarmine (Serva, Heidelberg, New York) for 10 min and examined/photographed under a microscope (Nikon UFX-II, Japan) at a magnification of 150X. Pollen grains in *A. formosanus* are packaged as columns of tetrads, precluding the counting of pollen tubes emerging from individual pollen grains. Hence, the viability of pollen was determined by counting the number of pollen tubes emerging per massula (Table 1). For each group of flowers, pollen tubes from at least 20 massulae were counted. Experiments were performed thrice, on three separate occasions.

Seed Germination

Capsules from hand-pollinated plants were collected 7 weeks after pollination, approximately one week before dehiscence. They were surface-disinfected in 70% ethanol for 30 s, followed by 0.5% sodium hypochlorite (Clorox, The Clorox Co., Oakland, CA) with two drops of Tween 20® per 100 ml (Hayashi Pure Chemical Industries Ltd., Osaka, Japan) under ultrasonic vibration (Branson Ultrasonic Cleaner, Branson Cleaning Equipment Co., Shelton, CT) for 15 min and rinsed five times with sterile distilled water. After sterilization, capsules were cut open with a surgical blade in the laminar air flow. Seeds were removed and sown by spreading them as thinly as possible over the surface of the culture medium in 22 × 120 mm glass test tubes (approximately 10 test tubes per capsule), each containing 10 ml of medium. The medium consisted of half-strength Murashige and Skoog's (MS) basal medium (Murashige and Skoog, 1962) (half-strength MS inorganic salts and vitamins + 100 mg/l myo-inositol + 3% sucrose, referred to hereafter as half-strength MS medium), 0.9% Difco Bacto agar (Difco Laboratories, Detroit, MI), pH 5.7 (A); half-strength MS basal medium + 0.2% activated charcoal (Sigma), pH 6.0 (B); and half-strength MS basal medium + 0.2% activated charcoal + 8% (w/v) banana homogenate (semi-ripe banana, cultivar Pei-Chiao) (C) (Table 2). A blender was used to evenly disperse the banana homogenate in medium C. Three capsules (thirty tubes) were evaluated per treatment. All media contained 0.9% Difco Bacto agar. The pH of all media was adjusted with 1N NaOH or HCl 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) after culture, to avoid media dehydration during the long culture period. The cultures were incubated at 25 ± 1°C under cool white fluorescent light at 38 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 16 h photoperiod per day for four months. The percentage seed germination was recorded

Table 2. Response of *Anoectochilus formosanus* seeds after in vitro culture on different media.

Medium*	% Seed germination after two months of culture	% Green, healthy seedlings after four months of culture	Fresh weight (g) per hundred seedlings after four months of culture
A	77.6 ± 3.5	81.0 ± 2.9	0.55 ± 0.02
B	78.1 ± 3.3	65.4 ± 3.8	0.56 ± 0.02
C	77.9 ± 2.1	81.4 ± 0.9	1.94 ± 0.02

[1]A: Half-strength MS (Control); B: Half-strength MS + 0.2% activated charcoal; C: Half-strength MS + 0.2% activated charcoal + 8% banana homogenate.
±: Standard deviation.

after two months of culture and the percentage of green seedlings and the weight of 100 seedlings were recorded after four months of culture (Table 2).

Growth of Seedlings

Four-month-old seedlings cultured on medium C (Section 2.4) were transferred to half-strength liquid MS medium supplemented with 2 mg/l BA in a 125-ml Erlenmeyer flask, each containing 10 ml of medium, for rapid growth. The pH of the medium was adjusted to 5.2 ± 0.1 prior to autoclaving. The flasks were placed on an orbital shaker (Model SK-302A, Sun Kuan Instruments Co., Taichung, Taiwan) with rotary motion of 100 rev/min, and incubated for two months at 25 ± 1°C under cool white fluorescent light at 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 16 h photoperiod per day. Thirty flasks were cultured with ten seedlings per flask.

Seedlings with well-developed shoots and rhizomes were transferred to half-strength MS medium supplemented with 0.9% Difco Bacto agar, 0.2% activated charcoal, 8% w/v banana homogenate, 2 mg/l BA + 0.5 mg/l NAA in 500-ml Erlenmeyer flasks, each containing 100 ml of medium and incubated for four months under the conditions described for the germination of seeds. Twelve flasks were cultured with twenty-five seedlings per flask.

Ex Vitro Establishment of Plantlets

One hundred and eighty seed-derived plants with well-developed rhizomes and shoots were washed thoroughly under tap water for 2-3 min to remove traces of adhering agar. Plants were then planted in 16 × 10.5 × 4 cm plastic containers (30 plants per container) containing coconut fibre as a substrate. The containers were incubated in the growth chamber for 2 weeks under light (44 $\mu mol \cdot m^{-2} \cdot s^{-1}$)

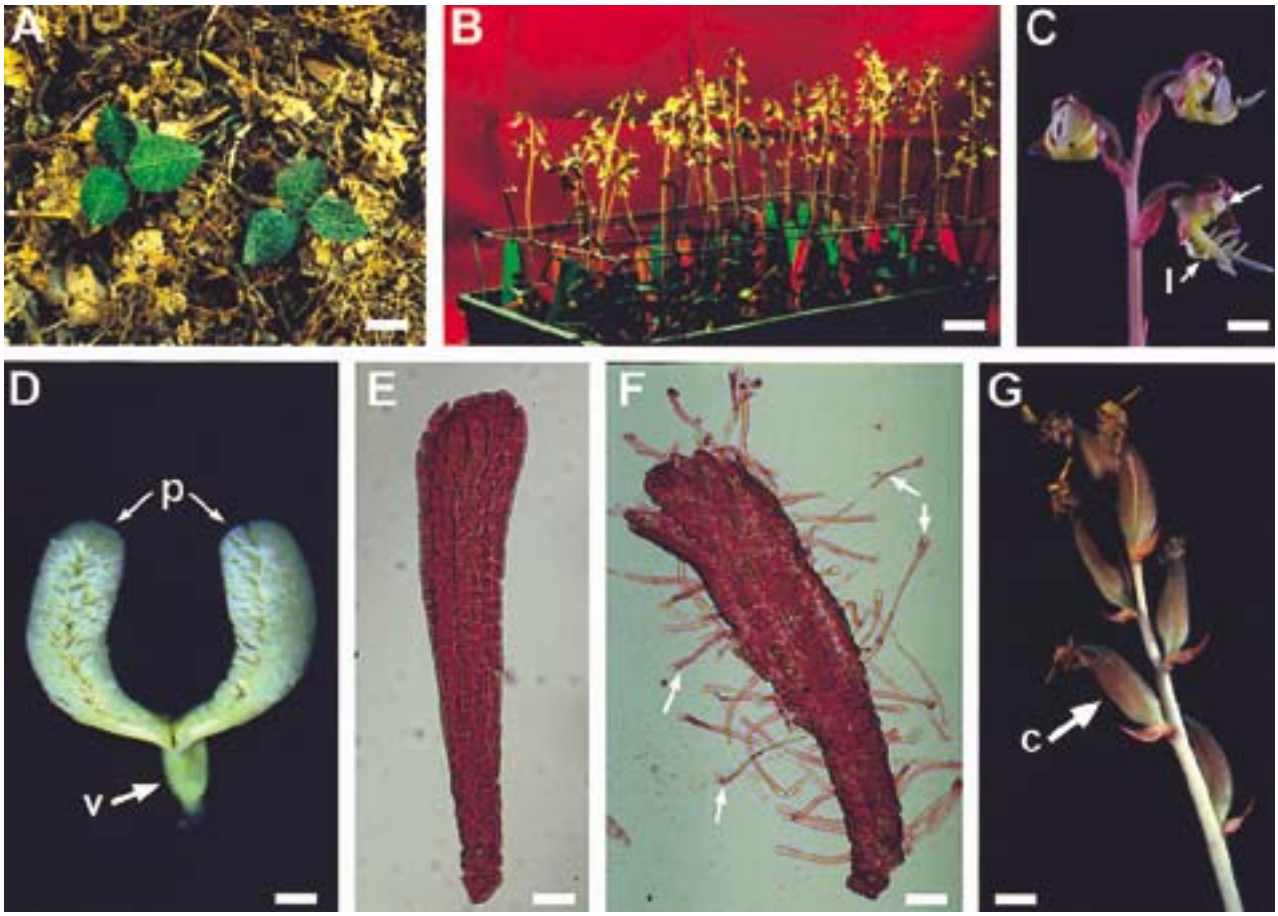

Figure 2. A-G, Flowering and capsule formation in *Anoectochilus formosanus*. (A) Plants of *Anoectochilus formosanus* growing under natural conditions in the Shuisheta Shan mountain area, Nantou County, Taiwan. Bar 23 mm; (B) Synchronous flowering in plants collected from the natural habitat after replanting in peat moss:vermiculite (1:1 by volume) and incubation in the growth chamber for eight months. Bar 59.53 mm; (C) A spikelet with cream-yellow flowers having a characteristic lip (l) with a distinct spur exerted beyond the lateral sepals and the pollen cap (arrow) holding the pollinarium. Bar 9.43 mm; (D) A pollinarium showing a pair of bipartite pollinia (p) attached to a viscidium (v). Bar 705 µm; (E) A massula consisting of packets of pollen tetrads. Bar 44 µm; (F) A massula showing emergence of pollen tubes (arrows) after incubation in pollen germination medium for 36 h. Bar 47 µm; (G) Mature capsules (c) formed 7 weeks after hand-pollination. Bar 5.1 mm.

for a 16 h photoperiod per day and day/night temperatures of 25/20°C. Plants were then replanted in five 49.5 × 29.5 × 6.5 cm plastic trays (60 seed-derived plants per tray) containing a mixture of autoclaved potting mixture peat moss: vermiculite (1:1 by volume). The trays were kept in the growth chamber under a light intensity (44 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for a 16 h photoperiod per day and day/night temperatures of 25/20°C. The plants were initially covered with a polythene sheet for one month to maintain high humidity (above 90%) and irrigated once in a week with tap water. The percentage survival of plants was calculated after two months.

Statistical Analysis

Linear regression analysis was used to examine the relationship between the plant growth index and flower number in plants. 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

Anoectochilus formosanus has a short, once-a-year flowering period (Liu and Su, 1978). In nature, it is believed to be cross-pollinated by insects. To enable artificial cross-pollination, it is necessary to obtain flowering in different individuals at the same time. Artificial cross-pollination could also be accomplished by using pollinia stored at a low temperature (7.2°C) (Meeyot and Kamemoto, 1969) or ultra-low temperature (-79°C) (Ito, 1965). In *A. formosanus*, flowers are borne only on mature plants. After initiation of flowering in different individuals of *A. formosanus*, inflorescences developed slowly,

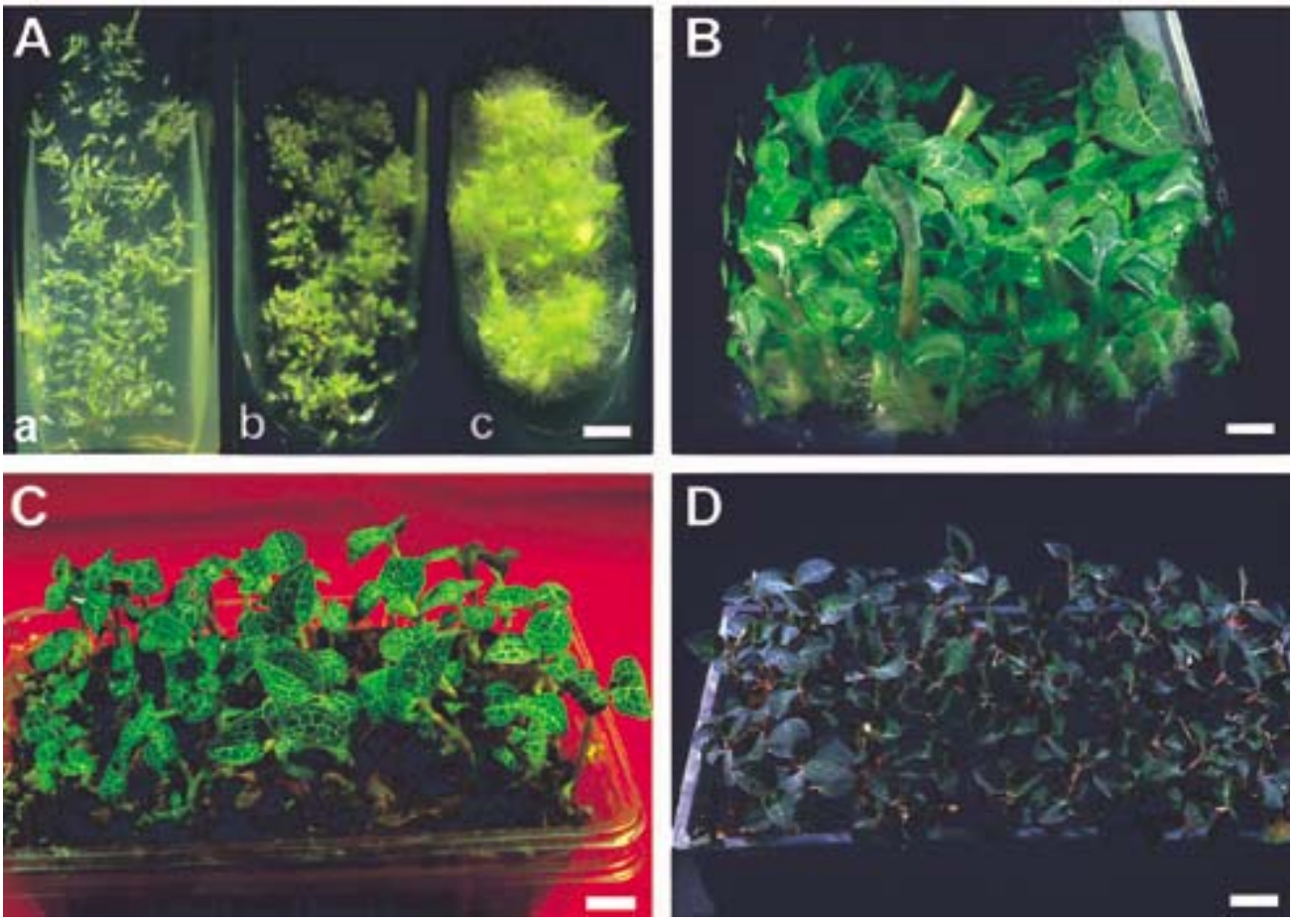

Figure 3. A-D, Asymbiotic seed culture and *ex vitro* establishment of seedlings of *Anoectochilus formosanus*. (A) Germination of seeds, four months after sowing on agar-gelled half-strength MS medium -without any additives (a); with 0.2% (w/v) activated charcoal (b); and with 0.2% activated charcoal + 8% (w/v) banana homogenate (c). Bar 5.84 mm; (B) Well-developed seedlings after fourmonths of culture on half-strength MS medium supplemented with 2 mg/l BA + 0.5 mg/l NAA. Four-month-old seedlings were cultured in half-strength liquid MS medium supplemented with 3% sucrose + 2 mg/l BA for two months in 125-ml Erlenmeyer flasks and incubated on a rotary shaker, prior to culture on aforementioned agar-gelled medium. Bar 8.84 mm; (C) Ten-month-old seed-derived plants after 2 weeks of culture on coconut fibre substrate in 16 × 10.5 × 4 cm plastic containers and incubation in the growth chamber. Bar 1.25 cm; (D) Healthy seed-derived plants four months after transfer to 49.5 × 29.5 × 6.5 cm plastic trays containing peat moss:vermiculite (1:1 by volume) and incubation in the growth chamber. Bar 3.64 cm.

and flowering took 7-8 weeks. The number of flowers produced per inflorescence depended on plant size and vigor (plant growth index), and we found a strong correlation between the plant growth index and the number of flowers borne on a spikelet (Figure 1).

Synchronization of Flowering and Hand-Pollination

Plants collected from their natural habitat (Figure 2A) showed uniform flowering (Figure 2B) eight months after replanting and transfer in the growth chamber. The flowers were borne on a spikelet and had a pollen cap (arrow) and a characteristic lip (l) (Figure 2C). Figure 2D shows pollinarium of *A. formosanus* composed of a pair of bipartite pollinia (p) attached to a sticky base, the viscidium (v), similar to the pollinarium of *Ludisia discolor* (Freudenstein and Rasmussen, 1996). The maximum fruit set (86.7%) was obtained when pollinia and ovules from 2-4 day old flowers (group B) were used for pollination (Table 1). The massulae (Figure 2E) from group B flowers also showed the maximum germination of pollen tubes (Figure 2F) (Table 1). Some flowers in this group, despite hand-pollination, failed to set fruit, a result similar to values recorded for the South American orchid *Oncidium variegatum* Sw. The failure was attributed to either possible interplant incompatibilities, experimental mishandling, or selective abortion by the plant to avoid excessive fruit set and prevent reduction in plant vigor (Ackerman and Montero Oliver, 1985). When pollinia from group E i.e. 11-13 DAF was used, it failed to produce any capsules.

Asymbiotic Seed Germination

Seven-week-old capsules (Figure 2G) were selected for seed germination studies. The capsule matures about 8 weeks after pollination when the plants are grown under controlled environmental conditions. An average of 6 to 7 weeks from pollination was also found to be a suitable time for the capsule collection of many European terrestrial orchids (Mitchell, 1989). In preliminary studies, seeds from brown, seven-week-old capsules showed maximum percentage germination when sown in vitro (data not shown). Orchid embryos have been reported to become viable and develop normally prior to the capsule ripening (Arditii, 1967 and references cited therein; Yam and Weatherhead, 1988; Mitchell, 1989). Moreover, it is more convenient to surface disinfect immature capsules than mature ones (Yam and Weatherhead, 1988). Seeds of *A. formosanus* sown on different media germinated within two months of culture. The seeds sown on medium supplemented with banana homogenate and activated charcoal (medium C) not only showed a high germination percentage but also significantly higher seedling weight as compared to other media (medium A and B) (Table 2). Activated charcoal is a common additive in orchid seed germination media (Ernst, 1974). The beneficial effect of banana homogenate on the asymbiotic germination of orchid seeds has been reported earlier (Ernst, 1974; Yam and Weatherhead, 1990 and references cited therein). By contrast, in studies with *Paphiopedilum ciliolare*, Pierik et al. (1988) reported that the effect of activated charcoal and banana homogenate was inhibitory during seed germination and beneficial only for further development of seedlings. The seeds of *A. formosanus* sown on medium with banana homogenate and activated charcoal (c) germinated, turned green and grew rapidly as compared to medium with activated charcoal alone (b) or without additives (a) (Figure 3A). Four-month-old seedlings obtained on medium C grew well when transferred to half-strength MS liquid medium with 2 mg/l BA and cultured on a rotary shaker two months. The beneficial effect of liquid medium on the rapid growth of nodal explants of *A. formosanus* has been reported (Ho et al., 1987). Seedlings developed further into healthy plantlets after culture on fresh agar-gelled half-strength MS medium supplemented with 0.2% activated charcoal, 8% banana homogenate, and 2 mg/l BA + 0.5 mg/l NAA in 500-ml Erlenmeyer flasks and incubation for a further four months (Figure 3B).

Ex vitro Establishment of Plantlets

Before transfer of healthy, ten-month-old seed-derived plants to peat moss:vermiculite potting mixture, they were potted into coconut fibre substrate in closed plastic containers and acclimatized for 2 weeks (Figure 3C) to prevent wilting. High survival (90%) was recorded for seedlings transplanted to peat moss:vermiculite potting mix in plastic trays (Figure 3D) and incubated in the growth chamber under high humidity conditions for four months.

Conclusions

The optimal procedure for rapid plant production in *A. formosanus* using seeds is summarized as: (i) synchronous flowering in plants of *A. fomosanus* collected from their natural habitat could be induced by growing them under controlled environmental conditions; (ii) a period between 2 to 4 days after flowering was found to be optimal for artificial cross-pollination; (iii) seeds from 7-week-old capsules when cultured on half-strength MS basal medium with 0.2% activated charcoal and 8% banana homogenate showed a high germination rate (77.9%) and well-developed rhizomes and shoots; (iv) germinated seedlings grew well when cultured in half-strength liquid MS medium containing 2 mg/l BA for two months; (v) before transplanting to peat moss:vermiculite and incubation in the growth chamber, the seedlings with well-developed rhizomes and shoots were cultured on half-strength MS medium with 0.2% activated charcoal, 8% banana homogenate, 2 mg/l BA and 0.5 mg/l NAA for further growth; (vi) ninety percent of plantlets survived two months after transfer to peat moss:vermiculite and incubation in the growth chamber.

With the methods presented here it is possible to produce healthy, disease-free plants of *A. formosanus*, which could then be released to their native protected habitats, such as national parks, away from *A. koshunensis*, with which it might hybridize. It is one of the best ways of

conserving natural populations of *A. formosanus*, therebyreducing the risk of its extinction. Similar approaches could be used in conservation of other species of *Anoectochilus*, threatened by gradual loss of habitat and/or indiscriminate collection for commercial purposes.

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台灣金線連人工授粉適期探討及應用無菌播種繁殖技術進行種原保存

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台灣金線連是本國極為珍貴的藥用植物，藉由探討人工授粉適期及應用無菌播種技術，可以大量繁殖種苗並達到種原保存之目的。試驗結果顯示，台灣金線連花朵之花粉與雌蕊成熟度是影響授粉率及果莢正常發育的重要因子，在花朵適當成熟度時進行人工授粉可獲得 86.7% 最佳結莢率。取七週齡果莢內之種胚，播種於含有 1/2 Murashige and Skoog's (MS) 基本鹽類添加 0.2% 活性碳粉及 8% 香蕉汁之固體培養基上，在種胚萌芽並培養四個月後，取生長健壯的幼苗，繼代培養於 125 ml 玻璃三角瓶內含 1/2 MS 基本鹽類添加 2 mg/l N^6-benzyladenine (BA) 之液體培養基振盪培養二個月。實生苗經液體培養後，根莖生長勢旺盛，再將其移植於含有 1/2 MS 基本鹽類添加 0.2% 活性碳粉、8% 香蕉汁、 2 mg/l BA 和 0.5 mg/l α-naphthaleneacetic acid (NAA) 之固體培養基，使其充分成長發育。育成的台灣金線連實生苗株，出瓶後移植於泥炭土與蛭石混合之介質，並置於生長箱中馴化培育二個月，其移植存活率約達 90%。

關鍵詞：台灣金線連；種原保存；瀕危植物；試管內繁殖；寶石蘭；藥草；蘭科；花粉；種子萌芽。