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# Micropropagation of an endangered orchid *Anoectochilus formosanus*

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## Abstract

A rapid and efficient procedure is outlined for *in vitro* clonal propagation of an elite cultivar of jewel orchid (*Anoectochilus formosanus*). Multiple shoot proliferation was induced in shoot tip explants on Hyponex (H3) media supplemented with 1 mg $dm^{-3}$ benzyladenine or 1 - 2 mg $dm^{-3}$ thidiazuron (TDZ). Addition of activated charcoal (1 g $dm^{-3}$) to the TDZ containing medium promoted multiple shoot formation (11.1 shoots per explant). However, the regenerated shoots had slow growth rate and failed to elongate. This problem was overcome by transferring the shoot clumps to a hormone free H3 medium supplemented with 2 % sucrose and 0.5 g $dm^{-3}$ activated charcoal. Rooting was induced in 100 % of the regenerated shoots in the same media. The plantlets were acclimatized and established in greenhouse.

*Additional key words*: activated charcoal, benzyladenine, sugar, thidiazuron.

## Introduction

The genus *Anoectochilus* (*Orchidaceae*) consists of approximately 40 species distributed throughout Southeast Asia, New Caledonia and Hawaii. It is one of the so-called jewel orchids, which are grown primarily for its beautiful foliage as well as in folk medicine against hypertension, lung and liver disease in many countries especially in China and Taiwan. *Anoectochilus* is conventionally propagated by seed, however the germination rate is very low and now-a-days this orchid is under the threat of extinction due to over collection from natural resources (Belitsky and Bersenev 1999). A tissue culture procedure for clonal propagation of seedlings was developed in Taiwan (Chow *et al*. 1982). But this available information fails to provide a comprehensive protocol and understanding of micro-propagation of *Anoectochilus*. Moreover, for mass propagation, regeneration from shoot tip and/or nodal explants from adult plants are superior to seed culture due to year round availability of plant materials and an exponential propagation rate. We, thus report an easy, fast and reliable *in vitro* regeneration system for the propagation system of this species.

## Materials and methods

Plants of *Anoectochilus formosanus* collected from Lamdong province in Vietnam were used as a source of explants. Shoot tips (1 - 2 mm in height) were disinfected with 70 % ethanol for 10 s followed by surface sterilization with 2 % sodium hypochlorite for 10 min and then washed thoroughly in sterile distilled water. Explants were initially cultured on MS medium supplemented with 0.5 mg $dm^{-3}$ benzyladenine (BA), 0.7 % agar and 3 % sucrose. Cultures were maintained in the culture room at 25 °C and 16-h photoperiod with a photon flux density of 40 μmol $m^{-2}$ $s^{-1}$ for 8 weeks. For further experiments, shoot tips and nodal explants were obtained from actively growing *A. formosanus* plantlets.

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*Abbreviations*: AC - activated charcoal; BA - benzyladenine; H3 medium - Hyponex medium; KC medium - Knudson medium; MS medium - Murashige and Skoog medium; PFD - photon flux density; TDZ - thidiazuron.

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Three different basal media, Murashige and Skoog (1962, MS), Knudson (1946, KC) and modified Hyponex (Kano 1965; H3) were tested. For shoot multiplication and shoot growth H3 media were supplemented with different concentration and combination of BA (0.1, 0.5, 1, 2 and 3 mg $dm^{-3}$), thidiazuron (TDZ; 0.1, 0.5, 1, 2 and 3 mg $dm^{-3}$), kinetin (0.1, 0.5, 1, 2 and 3 mg $dm^{-3}$), sucrose (1, 2, 3, 5 and 7 % ), and activated charcoal (0, 0.5, 1, 3 and 5 g $dm^{-3}$) depending on the objective of the experiment. The pH of the medium was adjusted to 5.8 before sterilization. All media used in the present experiment were solid (0.7 % agar) and were autoclaved at 121 °C for 20 min. Shoot tip and/or nodal explants were cultured in 40 × 130 mm test tubes containing 30 $cm^{-3}$ medium. All the cultures were incubated at 25 °C and 16-h photoperiod provided by cool white fluorescent lamp with a photon flux density PFD) of 40 μmol $m^{-2}$ $s^{-1}$.

Developing plantlets (1 - 1.5 cm) were separated and subcultured onto solid H3 medium containing 2 % sucrose and 0.5 % activated charcoal for further growth and rooting. After twelve weeks well-rooted shoots were rinsed with sterile water to remove residual rooting media and transferred to 50 × 35 × 9 cm plastic trays containing peatmoss and kept in a growth chamber and a day/night temperature of 25 /20 °C, 16-h photoperiod with PFD of 200 μmol $m^{-2}$ $s^{-1}$ and 80 % relative humidity. After 2 weeks 45 plantlets were transferred to greenhouses under shade with low natural light (15 - 20 μmol $m^{-2}$ $s^{-1}$) and temperature of 25 ± 2 °C. The trays were covered with PVC plastic bag to keep high humidity 80 - 90 %.

Experiments were set up in completely randomized design and repeated three times. Each treatment had three replications. Observations on the number of shoots, shoot height, fresh mass and dry mass, number of leaves were recorded after 3 months of culture. Data were subjected to Duncan's multiple range test using *SAS* program (*Version 6.12*, SAS Institute, Cary, USA).

## Results and discussion

Of the three basal media (MS, KC and H3) tested, MS and H3 were found more suitable for shoot tip culture of *A. formosanus* than KC medium, frequently used for growth of orchids. Shoot tips of *A. formosanus* cultured on MS or H3 medium without phytohormones supplement developed 2 - 3 shoots within 3 months of culture (Table 1). For further experiments on shoot proliferation study using different phytohormone supplement we used H3 medium because it is cheaper than MS and commercially available.

In all the media tested shoot bud differentiation occurred within 30 d of culture and was free from any intervening callus or protocorm like body formation. An optimal concentration of TDZ (1 - 2 mg $dm^{-3}$) or BA (1 mg $dm^{-3}$) developed an average of 5.1 - 5.2 shoots per explant (Table 2) but increasing concentration of cytokinins often led to lower proliferation rate and stunted growth. TDZ is now being widely used for micropropagation of several plants including many orchids because of its tremendous ability to induce organogenesis (Ernst 1994, Chen and Piluek 1995, Nayak *et al*. 1997, Zhao *et al*. 2003/4) but higher concentration of TDZ is sometimes associated with morphological abnormalities as has been reported in several species (Huetteman and Preece 1993).

A series of experiments were conducted to enhance the shoot proliferation rate and we found that addition of activated charcoal (AC) to the H3 medium supplemented with 2 mg $dm^{-3}$ TDZ was essential for multiple shoot formation in the explants (Table 3). Production of multiple shoots was dependent on the amount of charcoal in the medium (Fig. 1*A*). Concentration dependent increase in proliferation rate was observed. Among the different concentrations of AC tested, maximum proliferation rate was recorded at 1 g $dm^{-3}$ AC. Higher concentration reduced the proliferation rate. Addition of BA to AC containing media had little effect on proliferation rate (data not shown). On the contrary, AC did not promote shoot proliferation when nodal explants were used (Table 3, Fig. 1*B*). Similar observation was also found by Majada *et al*. (2000). One possible explanation of the effects of charcoal on shoot proliferation is that it improves aeration (Ernst 1974). A second possibility is that the charcoal adsorbs ethylene

Table 1. Growth responses of *A. formosanus* shoots cultured under different basal media after three months of culture. Means with different letters within columns are significantly different according to Duncan's multiple range tests at 5 % level.

| Media | Shoot length [cm] | Number of leaves [$shoot^{-1}$] | Number of shoots [$explant^{-1}$] | Fresh mass [mg $plant^{-1}$] | Dry mass [mg $plant^{-1}$] |
|---|---|---|---|---|---|
| MS | 3.6 a | 7.7 a | 3.2 a | 455 a | 42 a |
| KC | 2.4 b | 5.9 b | 1.0 b | 178 b | 19 b |
| H3 | 3.7 a | 7.6 a | 2.0 b | 457 a | 43 a |

Table 2. Effect of BA, kinetin and TDZ on *in vitro* shoot proliferation of *A. formosanus* after three months of culture. Means with different letters within columns are significantly different according to Duncan's multiple range tests at 5 % level. NS -, * -, ** -, *** - non-significant or significant at $P$ = 0.05, 0.01, and 0.001, respectively.

| Cytokinins | Concentration [mg dm$^{-3}$] | Shoot length [cm] | Number of shoots [explant$^{-1}$] | Fresh mass [mg plant$^{-1}$] | Dry mass [mg plant$^{-1}$] |
|---|---|---|---|---|---|
| Control | 0 | 3.4 a | 1.7 g | 473 e | 42 f |
| BA | 0.1 | 3.5 a | 2.1 g | 516 e | 47 e |
| | 0.5 | 3.2 b | 3.9 de | 698 cd | 57 a |
| | 1.0 | 3.0 bc | 5.1 a | 661 cd | 56 abc |
| | 2.0 | 3.0 bc | 4.9 abc | 727 bc | 59 a |
| | 3.0 | 2.9 cd | 4.9 abc | 706 cd | 56 abcd |
| KIN | 0.1 | 2.9 cde | 2.0 f | 515 e | 46 e |
| | 0.5 | 2.9 cd | 4.8 abc | 816 a | 57 a |
| | 1.0 | 3.0 bc | 4.9 abc | 787 ab | 56 ab |
| | 2.0 | 2.9 cd | 4.3 dc | 692 cd | 52 cd |
| | 3.0 | 2.7 e | 4.4 bcd | 643 d | 52 d |
| TDZ | 0.1 | 2.9 cd | 3.3 ef | 518 e | 46 e |
| | 0.5 | 3.0 bc | 4.6 abc | 821 a | 57 a |
| | 1.0 | 3.0 bc | 5.2 a | 808 a | 56 a |
| | 2.0 | 2.9 cd | 5.2 a | 682 cd | 52 cd |
| | 3.0 | 2.7 de | 5.0 ab | 675 cd | 52 cd |
| Cytokinins | | ***$^{z}$ | ** | * | ** |
| Concentration | | *** | *** | *** | *** |
| Interaction | | NS | *** | *** | NS |


Fig. 1. *Anoectochilus formosanus* shoot tip explants (*A*) and of nodal explants (*B*) grown for three months on H3 medium supplemented with 2 mg dm$^{-3}$ TDZ combined with activated charcoal in different concentrations. *Bar* = 1 cm.

(Ernst 1975), which can inhibit growth and proliferation. But it remains unclear, why the same medium had an opposite effect resulting in poor proliferation in nodal explants. This may be due to the physiological status of the material at different explant types used in the present experiment. Shoots developed on a TDZ containing medium had slow growth as well as small leaves. The inhibition of shoot growth by TDZ may be consistent with its high cytokinin activity (Mayer and Staden 1988, Preece and Imel 1991, Huetteman and Preece 1993). To overcome this problem, transfer shoot cultures to a medium lacking TDZ has been suggested by many workers.

Table 3. Effect of different concentrations of activated charcoal on *in vitro* proliferation of shoot tip (S) and nodal (N) explants of *A. formosanus* cultured on H3 media supplemented with 2 mg $dm^{-3}$ TDZ after 12 weeks in culture. Means with different letters within columns are significantly different according to Duncan's multiple range tests at 5 % level; *** - significant at $P = 0.001$.

| Charcoal [mg $dm^{-3}$] | Explants | Shoot length [cm] | Number of shoots [$explant^{-1}$] | Fresh mass [mg $plant^{-1}$] | Dry mass [mg $plant^{-1}$] |
|---|---|---|---|---|---|
| 0 | S | 2.9 $e^z$ | 4.8 c | 447 f | 37 d |
| | N | 3.0 e | 3.6 d | 819 cd | 45 cd |
| 0.5 | S | 3.1 e | 5.7 b | 917 bc | 78 b |
| | N | 4.8 a | 1.0 e | 1205 a | 92 a |
| 1.0 | S | 3.8 d | 11.2 a | 1339 a | 98 a |
| | N | 4.4 bc | 1.0 e | 927 bc | 75 b |
| 3.0 | S | 4.1 cd | 1.8 e | 716 de | 58 c |
| | N | 4.5 ab | 1.0 e | 950 bc | 77 b |
| 5.0 | S | 4.7 ab | 1.1 e | 652 e | 50 cd |
| | N | 3.9 d | 1.0 e | 995 b | 87 ab |
| Charcoal | | *** | *** | *** | *** |
| Explants | | *** | *** | *** | *** |
| Interaction | | *** | *** | *** | *** |

Table 4. Effect of sucrose concentration on growth of *Anoectochilus formosanus* in H3 medium for threes months. Means with different letters within columns are significantly different according to Duncan's multiple range tests at 5 % level.

| Sucrose [%] | Shoot length [cm] | Number of shoots [$explant^{-1}$] | Number of nodes [$shoot^{-1}$] | Fresh mass [mg $plant^{-1}$] | Dry mass [mg $plant^{-1}$] |
|---|---|---|---|---|---|
| 1 | 3.3 b | 1.0 d | 5.8 b | 375 c | 28 c |
| 2 | 4.0 a | 1.4 c | 6.2 a | 736 a | 56 a |
| 3 | 4.1 a | 1.6 bc | 6.3 a | 721 a | 57 a |
| 5 | 2.9 c | 2.0 ab | 5.0 c | 489 b | 52 ab |
| 7 | 2.2 d | 2.1 a | 5.0 c | 358 c | 47 b |

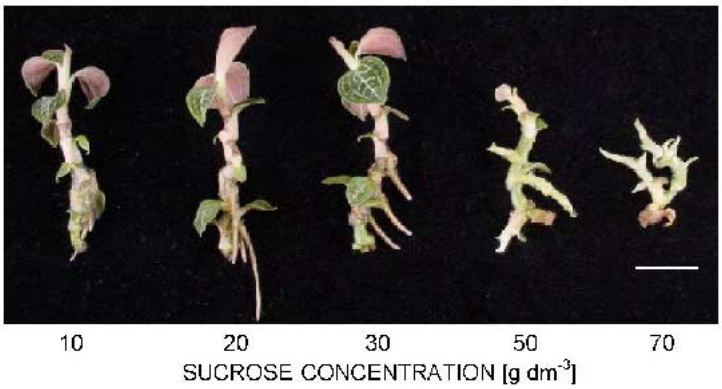


Fig. 2. Growth of *Anoectochilus formosanus* on H3 medium with different sucrose concentrations for three months. *Bar* = 1 cm.

Different sucrose concentrations and AC were used (without phytohormone) with the aim to stimulate *in vitro* shoot and root development. The best results were obtained when 2 % sucrose was used (Table 4, Fig. 2). The effect of reduced sucrose concentration on plantlet growth in *A. formosanus* similar with other plant such as in *Dianthus caryophylus* and *Lycopersicon esculentum* (Schnapp and Preece 1986) and *Campsis chinensis* (Paek and Kwang 1993) where growth rate of explants decreased with more negative water potential relating with sucrose concentration in the medium. These observations also supported by others (Klimaszeska *et al*. 1985, Schnapp and Preece 1986) where the growth and development generally increases with increasing sugar concentration until an optimum and then decreased at very high concentration.

Table 5. Effect of charcoal concentration on growth of *Anoectochilus formosanus* on H3 medium with 2 % sucrose for three months. Means with different letters within columns are significantly different according to Duncan's multiple range tests at 5 % level.

| Charcoal [mg $dm^{-3}$] | Plant height [cm] | Number of shoots [$explant^{-1}$] | Length of root [cm] | Fresh mass [mg $plant^{-1}$] | Dry mass [mg $plant^{-1}$] |
|---|---|---|---|---|---|
| 0 | 3.0 c | 2.3 a | 2.5 c | 478 c | 43 b |
| 0.5 | 4.0 a | 1.2 b | 3.6 ab | 708 a | 59 a |
| 1 | 3.6 b | 1.2 b | 3.5 b | 642 ab | 58 a |
| 3 | 3.7 b | 1.3 b | 4.1 a | 516 bc | 45 b |
| 5 | 3.7 b | 1.0 b | 3.9 ab | 502 bc | 46 ab |

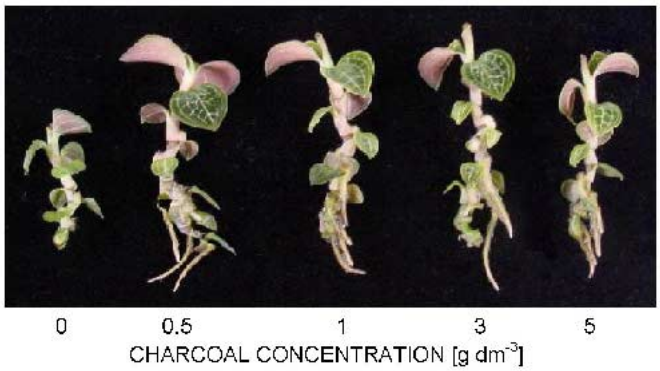


Fig. 3. Growth of *Anoectochilus formosanus* on H3 medium with different concentrations of activated charcoal for three months. *Bar* = 1 cm.

Fig. 4. Acclimatized and flowering *Anoectochilus formosanus* plants after 18 months in pots in greenhouse. *Bar* = 3 cm.

Addition of AC to the culture medium resulted in more vigorous growth, rooting and production of more expanded leaves than on other media. AC at 0.5 g $dm^{-3}$ induced maximum shoot growth with 100 % rooting from

the base of the regenerated shoot (Table 5, Fig. 3). Similar reports have been found in several species (Ernst 1974, Hyndman *et al*. 1982, Paek and Yeung 1991). The positive effect of AC can be interpreted by considering at least two aspects, reduction of light at the base of the shoots, thus providing an environment conductive to the accumulation of auxin or cofactors (Druart and Wulf 1993) and/or absorption of inhibitory substances such as polyphenols from the media (Fridborg and Eriksson 1975).

Regenerated shoots with well-developed roots were transferred to peatmoss and kept in a humidity chamber with 80 % relative humidity for hardening. The survival percentage was 100 % after 4 weeks in the greenhouse (Fig. 4). No phenotypic variation was observed among the regenerants.

Rapid and efficient multiplication rate, rooting and successful transfer of plantlets to the greenhouse makes this protocol suitable for large scale multiplication as well as *ex situ* conservation of this important jewel orchid.

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