***IN VITRO* PROPAGATION OF THREATENED TERRESRTIAL ORCHID *ANOECTOCHILUS SETACEUS* BLUME**

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**Abstract**

*Anoectochilus setaceus* Blume is a terrestrial orchid which has ornamental and medicinal value. It is declared as a threatened orchid in Vietnam due to overexploitation and habitat loss. In the present study we have developed a simple and efficient *in vitro* propagation protocol for *A. setaceus*. Multiple shoots were regenerated from shoot tip explants on Murashige and Skoog (MS) medium supplemented with 0.1 – 2 mg/l benzylamino purine (BAP) or kinetin (Kn). MS medium supplemented with 0.6 mg/l Kn induced 5.4 shoots per explants. Among the various levels of sucrose tested (0 - 7%), 2% sucrose was found suitable for shoot regeneration and growth. Rooting of shoots was also achieved on the shoot regeneration medium. The plants were acclimatized in pots using peatmoss as medium and established in greenhouse.

**Key words**: Jewel orchid, multiple shoots, micropropagation, plant regeneration, ornamental plant

**INTRODUCTION**

*Anoectochilus* is an important tropical terrestrial orchid distributed throughout Southeast Asia and it is popularly called as “Jewel orchid” because of its beautiful foliage. Many species of *Anoectochilus* are used in Chinese folk medicine for many years in the treatment of hypertension, diabetes, and heart, lung and liver diseases (Mak et al. 1990, Chiu and Chang 1995, Shih et al. 2002). Various bioactive compounds have been isolated from *Anoectochilus* such as flavonoid glycosides (He et al. 2006) and kinsenosides, which have shown hepatoprotective, hypoglycemic and antiinflammaotry effects (Wu et al. 2007, Zhang et al. 2007 and Hsiao et al. 2011). *A. setaceus* is one of such species which was once widely distributed in rain forests of Vietnam but is now considered as rare and threatened plant in Vietnam and listed in the Red Data Book because of overexploitation and habitat destruction (Anonymous, 2007). This species can be propagated by seeds, but germination rate is very low because of mychorrhizal requirement. Mircorpropagtion is the suitable means for such rare and threatened plants and therefore, in the present study we attempted *in vitro* propagation of *A. setaceus* using shoot tip explants and developed a simple, rapid and efficient protocol.

**MATERIALS AND METHODS**

**Plant material and culture initiation**

Plants of *Anoectochilus setaceus* Blume were collected from Tam Dao National Park, Vinh Prhuc Province, Vietnam. Shoot tips (1–2 mm in length) were disinfected with 70% ethanol for 10 s followed by surface sterilization with 2% sodium hypochlorite solution for 10 min and then washed thoroughly with sterile water. Explants were initially cultured on Murashige and Skoog (1962) medium (MS) supplemented with 0.5 mg/l benzylamino purine (BAP), 0.7% agar and 3% sucrose. Cultures were maintained in the culture room at 25oC for 16-h photoperiod with a photon flux density of 40 µmol m-2 s-1 for 8 weeks. For further experiments, shoot tips were obtained from actively growing *A. setaceus* plantlets.

**Shoot multiplication**

Three different media, half strength MS, full strength MS and Knudson C medium (1946, KC) were tested. For shoot multiplication and shoot growth, MS medium supplemented with different concentrations of BAP or kinetin (0.1, 0.3, 0.6, 1.0, 1.5 and 2.0 mg/l) and sucrose (0, 1, 2, 3, 5, and 7%) were tested 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 solidified with 0.7% agar and were autoclaved at 121oC for 20 min. Explants were cultured in 250 ml bottles containing 80 ml medium. All cultures were incubated at 25oC for 16-h phtoperiod provided by cool white fluorescent lamps with a photon flux density of 40 µmol m-2 s-1.

**Plantlet regeneration and acclimatization**

Developing shoots (1-2 cm in length) were separated and sub-cultured onto agar-solidified MS for further growth and rooting. After eight weeks, rooted shoots were rinsed with sterile water to remove residual medium and transferred to 50 x 35 x 9 cm plastic trays and 5 cm wide plastic pots containing peatmoss. They were kept in growth chamber with day/night temperature of 25/20oC, 16 h photoperiod with photosynthetic photon flux of 200 µmol m-2 s-1, and 80% relative humidity. After 4 weeks, plantlets were transferred to greenhouse under shade with low natural light (15-20 µmol m-2 S-1) and temperature of 25 ± 2oC.

All experiments were set up in completely randomized design and repeated two times. Each experiment had three replications. Number of shoots and shoot length were recorded. Data were subjected to Duncan’s multiple range test.

**RESULTS AND DISCUSSION**

Half strength MS, full strength MS and KC media were tested for shoot regeneration from the shoot tip explants, and the results revealed that full strength MS medium is superior for shoot regeneration (Table 1). After eight weeks of culture, 3 - 5 shoots with average length of 4.4 cm were regenerated on full strength MS. A number of tissue culture media were tested for orchid tissue culture (Arditti and Ernst 1993) and relatively simple media like Kndons C and Vacin-Went (Vacin and Went 1949), which include manganese as sole microelement along with macroelements, are enough for seed germination. However, balanced medium with macro- and microelements is essential for the micropropagation of orchids from shoot tip, nodal or leaf explants of orchids. Ket et al. (2004) used Hyponex medium for plant regeneration of *A. formosanus* due to its low cost.

Shoot tip culture is used as the most reliable technique for tissue culture of sympodial orchids like *Dendrobium, Cymbidium, Arundina, Phaius and Anoectochilus* (Chugh et al. 2009; Paek and Murthy, 2002). In the present study, on all the media shoot bud differentiation occurred within 4 weeks of culture from shoot tip cultures of *A. setaceus* and shoot bud differentiation was devoid of either callus or protocorm formation. BAP and Kn in the range of 0.1 – 2.0 mg/l was tested for shoot regeneration in *A. setaceus* and an optimal regeneration was achieved on medium supplemented with 0.6 mg/l (Table 2; Fig. 1a). An average of 5.4 shoots per explant were developed on this medium and similarly BAP has been widely used for micropropagation of several orchid species (Sheelavanthmath et al. 2000, 2005, Murthy and Pyati 2001, Pyati et al. 2002). It was seen that thidiazuron is more effective than other cytokinins in inducing shoot bud differentiation from various explants (Ernst 1994, Nayak et al. 1997). However, the drawback of using thidiazuron in regeneration studies includes difficulty in elongation and rooting of the shoots. This may be due to the high cytokinin activity and persistence of thidiazuron in the tissues compared to BAP or other cytokinins (Huetteman and Prece, 1993). In the present study, shoots regenerated on BAP containing medium were very much elongated (6.6 cm in length, Table 2) and problems of shoot elongation was not observed.

Most orchid tissue culture media require the addition of sugar as a source of carbon. Sucrose is an entirely satisfactory and inexpensive carbohydrate (Arditti and Ernst 1993), however the level of sucrose used in the orchid tissue culture media may differ from species to species. Different sucrose levels (0, 1, 2, 3, 5 and 7%) were tested for shoot regeneration and the results showed that 2% sucrose was the best in induction of shoots (Table 3). Five to eight shoots were regenerated on medium supplemented with 2% sucrose and this concentration is also suitable for shoot elongation (average shoot length was 6.6 cm; Fig. 1b). However, higher concentration of sucrose was not beneficial for regeneration and growth of the shoots. These results are in agreement with the previously reported results that growth and development increased with increase in sugar concentration until optimum and then decreased at very high concentration (Ket et al. 2004, Kilmazeska et al., 1995, Schnapp and Preece 1986).

The shoots which developed on MS supplemented with different concentration of sucrose (0, 1, 2, 3, 5, and 7%) were sub-cultured on to the same media where they had derived from. The shoots produced roots at the base of shoots within four weeks of culture (Fig. 2a). The regenerated plantlets were transferred to peatmoss and kept in humidity chamber with 80% relative humidity for hardening. The survival percentage was 100% after four weeks in growth chamber (Fig. 2b). The plants were then transferred to plastic pots containing peatmooss and kept in green house (Fig. 2c).

In conclusion, a propagation method was developed in this investigation for the threatened orchid *Anoectochilus setaceus*. Multiple shoots were induced from shoot tip explants on MS medium supplemented with BAP or Kn and plantlets were regenerated successfully. This simple and efficient procedure could be used for large scale propagation and *ex situ* conservation of this orchid species.

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**Legend to Figures**

Fig. 1. Multiple shoots developed from shoot tip of *Anoectochilus setaceus* on MS medium supplemented with 0.6 mg/l BAP after 4 weeks of culture (1a) and after 8 weeks of culture (Fig. 1b).

Fig. 2. Growth of *Anoectochilus setaceus* on MS medium with different sucrose concentrations after 8 weeks of culture (2a). Acclimatized plantlets after 4 weeks (2b) and after 8 weeks of transplantation.

Table 1. Growth responses of *Anoectochilus setaceus* shoots cultured on different media after eight weeks of culturea.

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Media Number of shoots/explant Shoot length (cm)

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Half strength MS 3.0 b 4.2 b

Full strength MS 4.1a 4.4 a

KC 3.0 b 4.2 b

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aMeans with different letters within the columns are significantly different according to Duncan’s multiple range test at 5% level.

Table 2. Effect of BAP and kinetin supplemented to full strength MS medium on *in vitro* shoot proliferation of *Anoectochilus setaceus* shoots after eight weeks of culturea.

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Cytokinin Concentration Number of shoots/explant Length of shoots

(mg/l) (cm)

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Control 0 1.0 f 2.0 e

BAP 0.1 1.7 d 3.5 d

0.3 3.3 b 4.9 c

0.6 5.4 a 6.6 a

1.0 3.6 b 6.6 a

1.5 3.3 b 6.3 b

2.0 2.8 c 6.4 a

Kn 0.1 1.7 d 2.5 e

0.3 2.1 d 3.5 d

0.6 2.5 c 4.3 c

1.0 3.3 b 6.1 b

1.5 3.2 b 6.3 b

2.0 3.3 b 5.5 c

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aMeans with different letters within the columns are significantly different according to Duncan’s multiple range test at 5% level.

Table 3. Effect of sucrose concentration on growth of *Anoectochilus setaceus* shoots after eight weeks of culturea.

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Sucrose Number of shoots/explant Length of shoots

(%) (cm)

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0 1.2 e 2.8 c

1 1.9 e 3.0 c

2 5.4 a 6.6 a

3 4.1 b 4.0 b

5 3.8 c 3.8 b

7 2.5 d 3.9 b

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

aMeans with different letters within the columns are significantly different according to Duncan’s multiple range test at 5% level.