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

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**Abstract**

*Anoectochilus setaceus* Blume is a terrestrial orchid which is having ornamental and medicinal value. It is declared threatened orchid in Vietnam due to overexploitation and also due to 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 benzylamino purine (BAP) or kinetin (Kn; 0.1 – 2 mg/l). MS medium supplemented with 0.6 mg/l Kn induced 5.4 shoots per explants. Among the various levels of sucrose tested (0 - 7%) for shoot regeneration, 2% sucrose was found suitable for shoot regeneration and growth. Rooting of shoots was achieved on the shoot regeneration medium itself. 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, heart, lung and liver diseases (Mak et al. 1990, Chiu and Chang 1995, Shih et al. 2002). Various bioactive compounds like flavonoid glycosides (He et al. 2006) and kinsenosides have been isolated from *Anoectochilus* which have shown hepatoprotective, hypoglycemic and anti-inflammatory effects (Wu et al. 2007, Zhang et al. 2007 and Hsiao et al. 2011). *A. setaceus* is one such species which was once widely distributed in rain forests Vietnam and now it is considered as rare and threatened plant in Vietnam and listed in the Red data book because of overexploitation and habitat destruction (Anonymous, 2007). Propagation of this plant is by seeds; however, 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 for 10 min and then washed thoroughly in sterile water. Explants were initially cultured on Murashige and Skoog (1962, MS) medium supplemented with 0.5 mg/l benzylamino purine (BAP), 0.7% agar and 3% sucrose. Cultures were maintained in the culture room at 25 oC 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* platelets.

**Shoot multiplication**

Three different media, half strength MS, full strength MS and Knudson (1946, KC) were tested. For shoot multiplication and shoot growth, MS medium supplemented with different concentrations of BAP (0.1, 0.3, 0.6, 1.0, 1.5 and 2.0 mg/l) kinetin (0.1, 0.3, 0.6, 1.0, 1.5 and 2.0 mg/l), 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 121 oC for 20 min. Explants were cultured in 250 ml bottles containing 80 ml medium. All cultures were incubated 25 oC 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 regenerated plants**

Developing shoots (1 - 2 cm in length) were separated and sub-cultured onto solid MS medium 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 and kept in growth chamber and day/night temperature of 25/20 oC, 16 h photoperiod with photosynthetic photon flux of 200 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 ± 2 oC.

All experiments were set up in completely randomized design and repeated two times. Each experiment had three replications. Observations on number of shoots, 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, on 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 shoot were regenerated on full strength medium and the average shoot length was 4.4 cm. A number of tissue culture media were tested for orchid tissue culture (Arditti and Ernst 1993) and relatively simpler media like Knudons C (Knudson 1946) and Vacin-Went (Vacin and Went 1949), which include manganese as sole microelement along with macroelements is 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 its low cost. Nutrient requirement for orchid tissue cultures are reported to be species specific (Mohanty et al. 2012), it was, therefore, we have tested in the present study, a fairly simple nutrient medium like Knudson C and full and half strength MS medium for in vitro propagation of *A. setaceus* and we got good shoot regeneration on full strength MS medium from shoot tip explants.

Shoot tip culture is used as 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 was 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 used tested on shoot regeneration and results showed that 2% sucrose was best in induction shoots (Table 3). 5-8 shoots were regenerated on medium supplemented with 2% sucrose and this concentration is also responsible for shoot elongation (average shoot length was 6.6 cm; Fig. 1b), however, higher concentrations of sucrose was not beneficial on regeneration and growth of the shoots. These results are in agreement with 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 medium supplemented with different concentration of sucrose (0, 1, 2, 3, 5, and 7%) were sub-cultured on to the same media where they have come from. The shoots involved in root regeneration 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). The protocol developed here is simple and more efficient when compared to in vitro propagation methods reported by others for *Anoectochiuls* species. Two-five shoots were regenerated in *A. formosanus* on Hyponex medium supplemented with benzyladenine and thidiazuron and subculturing of shoots to fresh Hyponex medium containing activated charcoal and 2 mg/l thidiauron was essential for shoot proliferation and elongation, one more subculturing was essential for subsequent root induction (Ket et al., 2004). Whereas, transferring of regenerated shoots was essential to Knudson medium supplemented with naphthalene acetic acid (1 mg/l) for induction of rooting in *A. setaceus* (Thach and Mien 2012). In the present studies optimal of 5.4 shoots were regenerated from shoot tip explants on MS medium supplemented with 0.6 mg/l Kn and rooting of shoots was achieved on shoot regeneration medium itself.

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 under different media after eight weeks of culturea.

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Media Number of shoots/explants Length of the shoots (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 Duncan’s multiple range test at 5% level.

Table 2. Effect of BAP and Kinetin supplemented to full strength of 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

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

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