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The role of different medium and plant hormones on multiple shoots of Jewel orchids (Anoectochilus setaceus Blume)

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Abstract. Use of *Anoectochilus setaceus* species have increased in the past few years due to the antidepressant and antiviral activities found in extracts of those plants. As a result of its potential as a pharmaceutical, a new system was developed for *in vitro* culture of this species. The goal of this investigation was to produce multiple shoot via *in vitro* techniques for *Anoectochilus setaceus*. The basal MS and Knud medium were tested and shown to be equally suitable of them for shoot culture of *A. setaceus*. Othe cultures were initiated from shoots inoculated onto MS medium supplemented individually with six different concentrations of 6-Benzylaminopurine (BAP) and Kinetin (Kn). The highest number of shoots was obtained on medium supplemented with 0.6 mg l^{-1} BAP (3.8 shoot/explant). Out of all the investigated concentrations of Kn, the best result was obtained on medium supplemented with 1.0 mg l^{-1} Kn (3.2 shoot/explant).

Keywords: Jewel orchids (*Anoectochilus setaceus*), plant hormones, the multiple shoot induction.

1. Introduction

Anoectochilus is a genus of about 50 orchids (family Orchidaceae) belonging to the subfamily *Orchidoideae* [1]. They are sometimes called "Jewel orchids" because of their attractive foliar venation. Found in Yunnan China, Assam India, Bangladesh, eatern Himalayas, Nepal, western Himalayas, Sri Lanka, Myanamar, Thailand, Vietnam, Sumatra and Java in broadleafed, evergreen, humid primary forests in soils dampened by mists and splash along steep watercourses at elevations of 800 to 1800 meters as a creeping, ascending, warm to cool growing terrestrial orchid in rich humus in damp crevasses with subcordate to ovate-acute, velvety, dark lime-green reticulated with gold leaves that are puple black on the underside that blooms on a 10" (25 cm) long, several (5) flowered inflorescence with successively opening flowers occuring in the summer [2-3].

Medicinal plants have been the subjects of man's curiosity since time immemorial [2].

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Almost every civilization has a history of medicinal plant use [3]. Approximately 80% of the people in the world's developing countries rely on traditional medicine for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts [4]. Interest in phytomedicine has exploded in the last few years, and about 500 different plant species are used as key ingredients, and many are still being collected from the wild [5].

Plants can be regenerated and mass propagated in vitro either by shoot morphogenesis or somatic embryogenesis. Many important Chinese traditional medicinal herbs have been successfully regenerated in vitro. Each has a particular group of bioactive compounds. Taxol (plaxitaxol), a complex diterpene alkaloid found in the bark of *Taxus* tree is one of the most promising anticancer agents due to its unique mode of action on microtubular cell system [6]. The root of *Panax ginseng* C.A.Mayer, has been widely used as a tonic and preciousmedicine since ancient times [7]. The primary bioactive constituents of ginseng are ginsenosides, a group of triterpenoid saponins [8]. Berberine is an isoquinoline alkaloid found in roots of *Coptis japonica* [9].

In this paper, we established in vitro culture of *A. setaceus* and some attempts have been made to increase the number of adventitious shoots in vitro culture.

2. Materials and Methods

Plant material

Jewel orchids (*Anoectochilus setaceus*) plants were collected at Tam Dao National Park, Vinh Phuc province. The samples were first soaked in 70% (v/v) ethanol for 30 seconds and then treated with 10% (w/v) sodium hypochlorite (NaOCl) for 15 min, followed by 3-4 rinses with sterile water for surface sterilization. The sterilized samples were grown on MS basal medium [10] without growth regulators to produce plantlets. The subcultures were carried in our laboratory as stock plants.

Shoot elongation and rooting

After culture for 8 weeks, the adventitious shoots regenerated from explants were transferred to hormone-free MS medium for shoot elongation. When the shoots reached 0.5-1.5 cm in height, they were transferred onto basal MS medium supplemented with 0.1-2.0 mg l^{-1} BAP (6-Benzylaminopurine) and Kn (Kinetin or 6- Furfurylaminopurine) [11] for shooting and rooting.

Culture conditions and data analysis

Uniform culture conditions were applied in all experiments. All experiments were conducted 3 replicates with 250 ml conical flasks in each and 12-15 explants were cultured in every 250 ml conical flask. The pH of the media was adjusted to 5.7 before autoclaving. The media was autoclaved for 15 min at 121°C. Cultures were incubated at 25 ± 2°C under a 16 h photoperiod with cold white fluorescent light mixed with incandescent light at 55.6 μmol m^{-2} s^{-1}. All data were analyzed using standard applied method.

3. Results and discussion

3.1. Effect of basal media on growth of Anoectochilus setaceus cultured in vitro

The basal MS and Knud medium were tested and the results were shown to be equally

suitable of them for shoot tip culture of *A. setaceus* (Table 1). The culture of shoots in these media showed good growth after 8 weeks culture. Shoot length was about 3.5-5.0 cm with 5 to 10 leaves per shoot. Shoot tips of *A. setaceus* cultured on MS or Knud without PGRs developed 2-5 additional shoots within 8 weeks of culture.

Table 1. The effects of different basal media on multiple shoot induction of *A. setaceus*

Medium	Avg length of shoots (cm) (Mean ± SE)	Number of shoot/explant	Number of leaves/planlet
MS	4.4 ± 0.9	3.5 ± 1.5	7.5 ± 2.5
Knud	4.6 ± 0.5	3.2 ± 1.7	6.3 ± 3.3

Basically, a nutrient medium consists of all of the essential major and minor plant nutrient elements, vitamins, plant growth regulators, and a carbohydrate as carbon source with other organic substances as optional additives [12]. The medium described by [10] (MS medium) is most commonly used. The growth response of *A. setaceus* also showed the best result on the MS medium.

The mineral requirement of orchids is generally not high, and salt poor medium is usually recommended. Beside the MS medium, the modified [13] also often used for micropropagation of orchids such as *Cattleya*, and *Cymbidium* [14]. Knud medium frequently used for the growth of orchids performed very poorly in this experiment when compared to MS medium. Probably salt poor in Knud medium is also further suitable for growth of *A. setaceus*. For further experiments on shoot growth and proliferation, we used MS media because it is commonly, commercially and available.

3.2. The effects of different concentrations of BAP on multiple shoot induction of Jewel orchids (Anoectochilus setaceus Blume) cultured in vitro

The application of molecular approaches with medicinal plants would also benefit from the development of cell, tissue and organ culture systems for *in vitro* growth and regeneration of medicinal plants. In addition, such tissue culture systems could also prove useful for large-scale biotechnological production of medicinal plant phytochemicals [15]. Furthermore, uniform plant growth with consistent plant material can be achieved, plants can be grown in sterile, standardized conditions and are free from biotic and abiotic contamination.

This study describes the basic procedures for the establishment on multiple shoot induction of *A. setaceus*. Samples (0.5-1.5 cm in stem length) were separately transferred to MS medium supplemented with 0.1-2.0 mgl^{-1} of BAP and Kn (Fig. A). The formation of new shoots was observed in all media studied except in the control group (hormon-free medium), indicating that *A. setaceus* is highly responsive to plant growth regulators. Regeneration frequency, mean number and length of shoots per explant were recorded after all hormone experiments. In the first stage of our experiment, the number of shoots changed, depending on the different concentrations of BAP. When the number of shoot was compared, there were statistically significant differences among the concentrations of BAP

tested (Table 2). The highest and the lowest number of shoots were obtained on the medium supplemented with 0.6 mg l^{-1} (Fig. B) and 0.1 mg l^{-1} of BAP (3.8-1.7 shoot/explant, respectively).

Table 2. The effects of concentrations of BAP on multiple shoot induction of *Anoectochilus setaceus*

Concentrations of BAP (mg l^{-1})	Avg No of shoots/explant (Mean ± SE)	Avg length of shoots (cm) (Mean ± SE)
Control	1.0 ± 0.2	2.0 ± 0.3
0.1	1.7 ± 0.2	3.5 ± 0.3
0.3	2.9 ± 0.3	4.9 ± 0.5
0.6	3.8 ± 0.2	6.6 ± 0.4
1.0	3.4 ± 0.3	6.6 ± 0.4
1.5	3.0 ± 0.2	6.3 ± 0.3
2.0	2.8 ± 0.3	6.4 ± 0.2

3.3. The effects of different concentrations of Kinetin on multiple shoot induction of Jewel orchids (Anoectochilus setaceus Blume) cultured in vitro

All the investigated concentrations of Kn showed shoot production. However, the best result was obtained on the medium supplemented with 1.0 mg l^{-1} Kn (Fig. C). From the results presented in Table 3, it appears that the number of shoot rises by increasing the concentration of Kn. However, smaller and shorter shoots were formed as the concentration of Kn increased in the culture medium. Excessive shoot length and root formation were observed on the medium containing low concentrations of Kn (from 0.6 to 1.5 mg l^{-1}) (Fig. D).

Table 3. The effects of concentrations of Kinetins on multiple shoot induction of *Anoectochilus setaceus* Blume

Concentrations of Kn (mg l^{-1})	Avg No of shoots/explant (Mean ±SE)	Avg length of shoots (cm) (Mean ±SE)
Control	1.2 ± 0.3	2.2 ± 0.3
0.1	1.7 ± 0.3	2.5 ± 0.4
0.3	2.1 ± 0.4	3.5 ± 0.5
0.6	2.5 ± 0.3	4.3 ± 0.4
1.0	3.2 ± 0.3	6.1 ± 0.4
1.5	3.2 ± 0.2	6.3 ± 0.3
2.0	3.3 ± 0.4	5.5 ± 0.1

The results indicated that the highest shoot number formed on the medium supplemented with 2.0 mg l^{-1} of Kn (3.3 shoots/explant). However, the morphological characteristics of shoots in this medium were not similar to plants growing in a natural environment and led to

vitrification in shoots. Therefore, a culture medium with 1.0 mg l^{-1} of Kn was sufficient to produce multiple shoot and an average of 3.2 shoots formed from each explant in this medium within eight weeks (Table 3). In the morphological observations, Kn concentrations surpassed other media (BAP concentrations) in terms of mean shoot length, leaf width and root formation.

In comparison of all treatments of two cytokinins with control group (Table 2-3), it was determined that the medium should be supplemented with exogenous hormones (PGRs) for new shoot formation.

In conclusion, shoots were successfully propagated after two subcultures in the presence of BAP or Kn. Among the cytokinins (BAP, Kn concentrations), BAP was reported to be more efficient than Kn in promoting shoot formation. Our findings are compatible with those of [16], who reported that in *H. perforatum*, BAP was found to be the most efficient in shoot formation when excised parts of seedlings were used. Also, for *H. perforatum* L., BAP was found to be the most efficient in promoting shoot regeneration when leaves [17] were used as the explant.

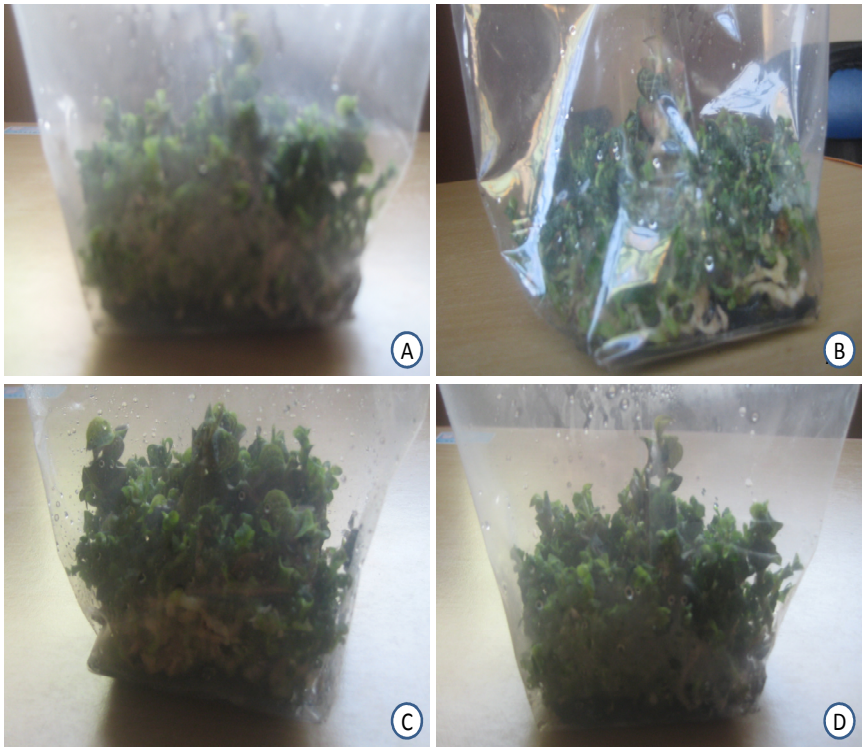

Fig 1. In vitro propagation via shoot morphogenesis: Induction of multiple shoots from shoot tips with supplemented BAP and Kin showed as A-B and C-D, respectively.

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Vai trò của môi trường và chất điều hòa sinh trưởng thực vật để nhân nhanh chồi Lan kim tuyến (*Anoectochilus setaceus* L.)

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Lan kim tuyến (*Anoectochilus setaceus* Blume) là một loại thảo dược có giá trị kinh tế cao và khả năng chữa trị các bệnh ung thư, chống tăng huyết áp, lưu thông khí huyết, kháng khuẩn, v.v. Trong những năm qua ở Việt Nam, loài Lan kim tuyến do bị thu hái với số lượng lớn để bán thuốc và xuất khẩu nên chúng đang có nguy cơ đe dọa mạnh, rất có thể sẽ bị tuyệt chủng ngoài tự nhiên nếu không có biện pháp bảo tồn hữu hiệu. Hiện nay, Lan kim tuyến được xếp trong nhóm IA của Nghị định 32/2006/CP, và nhóm thực vật đang nguy cấp EN A1a,c,d trong Sách đỏ Việt Nam (2007, phần thực vật). Để bảo tồn nguồn gen quý này, chúng tôi đã tiến hành phân lập và nuôi cấy. Hai môi trường MS và Knud đã được dùng để nhân nhanh chồi, kết quả sau 8 tuần nuôi cấy, số lượng chồi bất định hình thành và phát triển tốt trên cả 2 loại môi trường. Hai loại cytokinin (BAP và Kin) đã được bổ sung vào môi trường rắn MS để nghiên cứu khả năng tạo chồi và sự hình thành rễ bất định, kết quả thu được trên môi trường MS có bổ sung 0.6 mg/l BAP là thích hợp cho sự hình thành chồi bất định với 3,8 chồi/mẫu cấy, trong khi đó Kin thu được ở nồng độ cao hơn 1.0 mg/l số lượng chồi thu được 3,2 chồi/mẫu cấy. Thể chồi mập, xanh, xuất hiện một số lông tơ từ gốc thể chồi.

Từ khóa: Lan kim tuyến (*Anoectochilus setaceus*), hoóc môn thực vật, hệ số nhân nhanh chồi.