

## MICROPROPAGATION OF *Orchis catasetum* – A RARE AND ENDANGERED ORCHID

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**Abstract.** The application of modern biotechnology for mass propagation of rare and endangered species needs to develop a proper *in vitro* protocol. Here, a protocol was developed for high frequency *in vitro* multiplication of an endangered orchid, *Orchis catasetum*. Protocorms, as explants were cultured on Murashige and Skoog (MS) medium fortified with different concentrations of N<sup>6</sup>-benzyladenine (BA),  $\alpha$ -naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) either individually or in combination. A combination of 0.5 mg·L BA and 0.5 mg·L NAA was found to be suitable for maximum protocorm-like bodies (PLBs) regeneration ( $20.40 \times \text{plantlet}^{-1}$ ). The largest number of root ( $7.16 \times \text{plantlet}^{-1}$ ) and leaf ( $10.10 \times \text{plantlet}^{-1}$ ), also the highest plant height ( $114.20 \text{ mm} \times \text{plantlet}^{-1}$ ) and root length ( $193.40 \text{ mm} \times \text{plantlet}^{-1}$ ) were obtained on MS medium supplemented with 0.5 mg·L BA along with 0.5 mg·L NAA. Plantlets with well-developed leaves and roots were transplanted to pots filled with perlite, wood pieces, ionolite and mineral cartridge shell (1:1:1:1), also perlite individually and transferred to the greenhouse. Upon *ex vitro* transfer, 100% of plants survived.

**Key words:** auxins, cytokinins, endangered species, *in vitro* propagation, protocorm like bodies (PLBs), tissue culture

## INTRODUCTION

Orchids are one of the most diverse of the flowering plant families, containing 800 genera and 25000 species [Chugh et al. 2009]. Orchids have beautiful flowers and exhibit an incredible range of diversity in size, shape and colour. Orchids are grown as ornamentals and are valued as cut flowers not only because of their exotic beauty but also for their long shelf life [Chugh et al. 2009]. Large-scale multiplication of orchids, espe-

cially rare hybrids and endangered species using tissue culture techniques has helped orchids occupy a position as one of the top ten cut flowers [Chugh et al. 2009]. Orchid propagation by seed caused to the production of heterozygous plants. Thus, *in vitro* proliferation is a suitable alternative procedure for propagation of orchids. Different protocols have been established for micropropagation of orchids species through *in vitro* culture of various parts consisting shoot tip, root tip, stem, leaf, node, bud, inflorescence and rhizome, as well somatic embryo, callus and thin cell layer [Geetha and Shetty 2000, Seeni and Latha 2000, Park et al. 2002 2003, Teixeira da Silva 2003, Bhadra and Hossain 2003, Wang et al. 2004, Wu et al. 2004, Ket et al. 2004, Sheela et al. 2004, Kuo et al. 2005, Kalimuthu et al. 2006, Sinha et al. 2007, Janarthanam and Seshadri 2008]. In comparison to plantlet development from seeds or adventitious shoots, the micropropagation through PLBs is more efficient because PLBs can be rapidly proliferated on solid or in liquid culture medium, and a large number of PLBs can be provided in a short period [Luo et al. 2003a]. Many studies have revealed that the optimization of medium composition was an important approach to improve the micropropagation process of orchids by culturing PLBs that is species-specific [Shimura and Koda 2004, Luo et al. 2009]. In order to stimulate efficient micropropagation PLB, much effort has been done to modify the culture media, mainly by inclusion of plant growth regulators [Nayak et al. 2002, Nge et al. 2006] such as BA, thidiazuron (TDZ), N<sup>6</sup>-benzylaminopurine (BAP), NAA, 3-indoleacetic acid (IAA) and gibberellic acid (GA<sub>3</sub>) [Prakash et al. 1996, Roy and Banerjee 2003, Subramaniam and Taha 2003, Saiprasad et al. 2004, Malabadi et al. 2005, Roy et al. 2011]. Cytokinins are the most important factors to improve the plant regeneration from PLBs [Nayak et al. 2002, Nasiruddin et al. 2003, Luo et al. 2009]. Many orchid species such as *Orchis catasetum* are threatened with the danger of extinction. In the work presented here, PLB multiplication and growth of *Orchis catasetum* have been studied under the controlled conditions of tissue culture in the absence and presence of BA, IBA and NAA.

## MATERIAL AND METHODS

**Explant collection and surface sterilization.** Healthy and sterilized protocorms of *Orchis catasetum* was prepared from a plant tissue culture, Mahmoudabad, Iran, as a source of explants.

**Culture media and conditions for protocorm germination.** Once the micropropagation system had been established, protocorms were cultured in MS [1962] medium supplemented with 3% (w/v) sucrose and 0.8% agar-agar. All media were adjusted to a pH of 5.7 ± 0.02 with HCl and NaOH prior to autoclaving at 121°C and 105 kg · cm<sup>-2</sup> for 20 min. All the cultures were incubated at 24 ± 2°C under cool white fluorescent light (56 μmol · m<sup>-2</sup> s<sup>-1</sup>) with a 16-h photoperiod.

**Plant growth regulators and protocorm multiplication.** The effect of plant growth regulators added to MS medium on protocorm multiplication and subsequent plantlets growth and development was evaluated. The protocorms were cultured in MS medium containing BA (0.0, 0.2, 0.5, 1.0, 1.5 and 3.0 mg · L), IBA (0.0 and 0.5 mg · L) and NAA (0.0 and 0.5 mg · L). Each treatment consisted of three Petri dishes and in each

Petri dishes four protocorms were inoculated. Explants secrete phenolic compounds into the media, therefore, 0.5 mg·L activated charcoal was added to the media. Activated charcoal absorbs phenolic compounds. Observations on protocorms regeneration, number of root, number of leaf, plant height and root length were recorded 60 days after the culture initiation.

**Statistical analysis.** The experimental units were setup in a completely randomized block design. Each experiment was carried out in three replicates and each replicate includes four specimens (totally; 12 specimens for each treatment). The data were analyzed by analysis of variance (ANOVA) by using MSTAT-C software and the mean values were compared using Duncan multiple range test (DMRT) at  $P = 0.05$ .

## RESULTS

The results of this research present a simple and reliable protocol for rapid micro-propagation of *Orchis catasetum*, an endangered orchid species. This method may be applied to produce large number of plantlets during a short time.

**Influence of BA and IBA or NAA on protocorm regeneration.** Protocorm-like bodies (PLBs) number is affected by the presence of BA, IBA and NAA in MS medium. The effect of BA, IBA and NAA, individually or in combination with each other on protocorm regeneration and growth are shown in Table 1. A combination of 0.5 mg·L BA and 0.5 mg · L NAA induced maximum PLBs regeneration ( $20.40 \times \text{plantlet}^{-1}$ ). Among all treatments of BA, highest PLBs regeneration ( $12.30 \times \text{plantlet}^{-1}$ ) was obtained in medium containing 0.2 mg · L BA. Higher concentrations of BA did not yielded more PLBs. Minimum PLBs number was observed in media supplemented with 1.5 mg · L BA along with 0.5 mg · L NAA ( $4.30 \times \text{plantlet}^{-1}$ ) and 3.0 mg · L BA along with 0.5 mg · L NAA ( $4.50 \times \text{plantlet}^{-1}$ ). There is no positive correlation between the increases of BA concentration and enhance of PLBs number (tab. 1). DMRT showed significant differences among different concentrations of BA, also reciprocal effect of BA and IBA or NAA for PLBs number ( $p \leq 0.01$ ). DMRT showed that the effect of IBA and NAA was no significant on PLBs number.

**Influence of BA and IBA or NAA on plant height.** The effects of auxins and cytokinin on the plant height were significant. Among different concentrations of BA, 0.2 mg · L was found to be the most effective on enhancing the plant height ( $100.10 \text{ mm} \times \text{plantlet}^{-1}$ ) (tab. 1). Maximum plant height ( $114.20 \text{ mm} \times \text{plantlet}^{-1}$ ) was obtained on medium enriched with 0.5 mg · L BA along with 0.5 mg · L NAA (tab. 1). 0.5 mg · L BA and 0.5 mg · L NAA were not proper for inducing the plant height, lonely, because those stimulated only 40.00 and 49.00 mm long per plant (tab. 1). Minimum plant height ( $39.97 \text{ mm} \times \text{plantlet}^{-1}$ ) was recorded on medium supplemented with 0.2 mg · L BA along with 0.5 mg · L IBA.

**Influence of BA and IBA or NAA on leaf number.** Explants cultured in the presence of 0.5 mg · L BA along with 0.5 mg · L NAA contained the largest number of leaf ( $10.10 \times \text{plant}^{-1}$ ) being more than 2.50-fold higher than that found in explants grown in the medium containing 0.5 mg · L BA ( $4.00 \times \text{plantlet}^{-1}$ ) (tab. 1). Among all treatments of BA, largest number of leaf ( $9.10 \times \text{plantlet}^{-1}$ ) was obtained in medium

supplemented with 0.2 mg · L. DMRT showed significant differences among different concentrations of BA ( $p \leq 0.01$ ), NAA and IBA ( $p \leq 0.05$ ) also reciprocal effect of BA and IBA or NAA ( $p \leq 0.01$ ) for leaf number.

Table 1. Interaction effect of BA and IBA or NAA on protocorms multiplication and growth of *Orchis catasetum*

| Phytohormones (mg/L) |     |     | PLBs number                     | Plant height (mm)     | Leaf number                      | Root                 |                        |
|----------------------|-----|-----|---------------------------------|-----------------------|----------------------------------|----------------------|------------------------|
| BA                   | IBA | NAA |                                 |                       |                                  | number               | length (mm)            |
| 0.0                  | 0.0 | 0.0 | 11.60 <sup>bcd</sup>            | 76.50 <sup>bcd</sup>  | 7.60 <sup>bcd</sup>              | 4.50 <sup>def</sup>  | 82.08 <sup>defg</sup>  |
| 0.2                  | 0.0 | 0.0 | 12.30 <sup>cde</sup>            | 100.10 <sup>a</sup>   | 9.10 <sup>ab</sup>               | 5.50 <sup>bcd</sup>  | 106.30 <sup>bcd</sup>  |
| 0.5                  | 0.0 | 0.0 | 10.80 <sup>cde</sup>            | 40.00 <sup>f</sup>    | 4.00 <sup>i</sup>                | 2.20 <sup>i</sup>    | 50.13 <sup>fg</sup>    |
| 1.0                  | 0.0 | 0.0 | 8.10 <sup>efg</sup>             | 64.50 <sup>cdef</sup> | 5.60 <sup>defghi</sup>           | 4.20 <sup>defg</sup> | 90.50 <sup>defg</sup>  |
| 1.5                  | 0.0 | 0.0 | 9.20 <sup>d<sup>efg</sup></sup> | 46.00 <sup>ef</sup>   | 7.00 <sup>bcd<sup>ef</sup></sup> | 5.10 <sup>cde</sup>  | 60.50 <sup>efg</sup>   |
| 3.0                  | 0.0 | 0.0 | 10.10 <sup>de</sup>             | 47.37 <sup>ef</sup>   | 5.70 <sup>cdefghi</sup>          | 4.70 <sup>def</sup>  | 101.40 <sup>bode</sup> |
| 0.0                  | 0.5 | 0.0 | 11.00 <sup>cde</sup>            | 42.00 <sup>f</sup>    | 6.80 <sup>ab</sup>               | 4.30 <sup>defg</sup> | 88.20 <sup>defg</sup>  |
| 0.2                  | 0.5 | 0.0 | 12.20 <sup>bcd</sup>            | 39.97 <sup>f</sup>    | 4.40 <sup>ghi</sup>              | 3.80 <sup>efgh</sup> | 74.35 <sup>defg</sup>  |
| 0.5                  | 0.5 | 0.0 | 14.50 <sup>bc</sup>             | 66.95 <sup>cdef</sup> | 8.08 <sup>abc</sup>              | 5.60 <sup>bc</sup>   | 93.12 <sup>cdef</sup>  |
| 1.0                  | 0.5 | 0.0 | 9.00 <sup>defg</sup>            | 49.25 <sup>def</sup>  | 4.70 <sup>fghi</sup>             | 2.90 <sup>ghi</sup>  | 48.92 <sup>fg</sup>    |
| 1.5                  | 0.5 | 0.0 | 9.60 <sup>defg</sup>            | 41.00 <sup>f</sup>    | 4.12 <sup>i</sup>                | 2.60 <sup>hi</sup>   | 47.50 <sup>g</sup>     |
| 3.0                  | 0.5 | 0.0 | 10.10 <sup>cde</sup>            | 57.75 <sup>cdef</sup> | 5.20 <sup>efghi</sup>            | 4.00 <sup>efgh</sup> | 73.67 <sup>defg</sup>  |
| 0.0                  | 0.0 | 0.5 | 12.50 <sup>bcd</sup>            | 49.00 <sup>def</sup>  | 6.88 <sup>ab</sup>               | 4.50 <sup>defg</sup> | 90.20 <sup>defg</sup>  |
| 0.2                  | 0.0 | 0.5 | 6.00 <sup>gh</sup>              | 51.58 <sup>def</sup>  | 4.40 <sup>hi</sup>               | 3.08 <sup>ghi</sup>  | 56.58 <sup>fg</sup>    |
| 0.5                  | 0.0 | 0.5 | 20.40 <sup>a</sup>              | 114.20 <sup>a</sup>   | 10.10 <sup>a</sup>               | 7.16 <sup>a</sup>    | 193.40 <sup>a</sup>    |
| 1.0                  | 0.0 | 0.5 | 15.60 <sup>b</sup>              | 84.13 <sup>bc</sup>   | 8.02 <sup>abcd</sup>             | 6.60 <sup>ab</sup>   | 135.10 <sup>bc</sup>   |
| 1.5                  | 0.0 | 0.5 | 4.30 <sup>h</sup>               | 64.73 <sup>cdef</sup> | 7.20 <sup>bcd<sup>e</sup></sup>  | 3.90 <sup>efgh</sup> | 58.67 <sup>bc</sup>    |
| 3.0                  | 0.0 | 0.5 | 4.50 <sup>h</sup>               | 53.92 <sup>def</sup>  | 5.60 <sup>defghi</sup>           | 3.50 <sup>fghi</sup> | 64.67 <sup>defg</sup>  |

In each column, mean values followed by the same letters are not significantly different at 0.05 levels (DMRT)

**Influence of BA and IBA or NAA on root number and length.** Root number and root length are affected by the presence of BA, IBA and NAA in MS medium. The effect of BA, IBA and NAA, individually or in combination with each other on root number and root length are shown in Table 1. A combination of 0.5 mg · L BA and 0.5 mg · L NAA provoked the largest number of root ( $7.16 \times \text{plantlet}^{-1}$ ) and the highest length of root ( $193.40 \text{ mm} \times \text{plantlet}^{-1}$ ). A combination of 1.0 mg · L BA and 0.5 mg · L NAA was a suitable treatment for induction of root number ( $6.60 \times \text{plantlet}^{-1}$ ) and root length ( $135.10 \times \text{plantlet}^{-1}$ ). Among all treatments of BA, largest number of root ( $5.50 \times \text{plantlet}^{-1}$ ) and the highest length of root ( $106.30 \text{ mm} \times \text{plantlet}^{-1}$ ) were calculated in MS medium containing 0.2 mg · L (tab. 1). Higher concentrations of BA did not produced more root

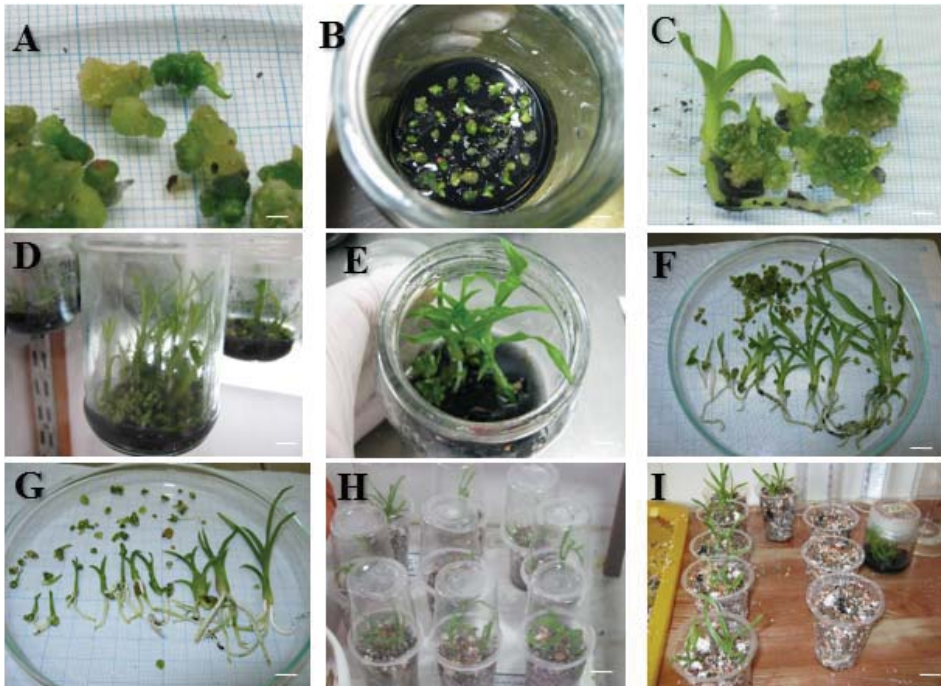


Fig. 1. Micropropagation of *Orchis catasetum* through protocorms-like bodies (PLBs). (A) PLBs formed from seeds used as explants (Bar = 1 cm). (B) PLBs cultured on MS medium containing plant growth regulators (Bar = 2 cm). (C) A developing PLBs (Bar = 1 cm). (D) Micropropagated shoots from PLBs on medium containing 0.5 mg·L BA along with 0.5 mg·L NAA (Bar = 1 cm). (E) Well-developed plantlets derived from PLBs (Bar = 2 cm). (F and G) Plantlets obtained from media enriched with different concentrations of growth regulators (Bar = 2 cm). (H and I) The process of plantlets acclimatization (Bar = 2 cm)

number and length. Minimum root number ( $2.20 \times \text{plantlet}^{-1}$ ) was observed in medium supplemented with 0.5 mg · L BA. Also, the least root length ( $47.50 \times \text{plantlet}^{-1}$ ) was calculated in the medium enriched with 1.5 mg · L BA along with 0.5 mg · L IBA (tab. 1). There is no positive correlation between the increases of BA concentration and enhance of root number and length (tab. 1). Analysis of variance showed significant differences among different concentrations of BA along with IBA or NAA for production of root ( $p \leq 0.01$ ).

## DISCUSSION

Protocorm-like bodies (PLBs) can be safely used for rapid propagation of orchids. Addition of low concentrations of BA and NAA promoted protocorms multiplication and growth of *Orchis catasetum* plantlets. The combined use of BA and NAA is propo-

sed in micropropagation of *Orchis catasetum*, an endangered orchid. However, this paper can be introduced 0.2 mg · L of BA as an individual plant growth regulator to produce proper shoot formation and root induction. Kalimuthu et al. [2007] obtained the results same as our findings, but with BAP on *Oncidium* sp. These researchers showed that the maximum PLBs formation, number of shoots and roots were observed in MS medium supplemented with 2.0 mg · L BAP. BAP individually was better than in combination with NAA. Of course, 2.0 mg · L BAP individually or in combination with 1.5 mg · L NAA induced the same roots on shoots (100%).

Since the seeds of orchid are without endosperm hence it needs specific nutritional and environmental conditions [Arditti et al. 1990]. Protocorm is a rudimentary organ that differentiate to a new shoot. Cells of protocorms are highly meristematic, thus can be applied to enhance proliferation and simultaneous production of orchid plantlets [Teixeira da Silva et al. 2005]. Protocorms are being applied by many researchers as explants for micropropagation of many rare and endangered orchid species [Seeni and Latha 2000, Sheelavantmath et al. 2000, Nagaraju and Mani 2005, Dev and Temjensangba 2006, Teixeira da Silva et al. 2006, Hossain et al. 2010, Roy et al. 2011].

Orchids need auxins and cytokinins for plantlets development [Roy et al. 2011]. The type and concentration of plant growth regulators play an important role during micropropagation of many orchids [Arditti and Ernst 1993]. Our study showed the positive effect of BA for maximum protocorm multiplication. BA acts more efficiently when used in combination with NAA. This finding is in agreement with some other findings obtained in micropropagation of orchids [Seeni and Latha 2000, Roy et al. 2011]. Study of Luo et al. [2009] on micropropagation of *Dendrobium huoshanense* showed that a high frequency of shoot formation was recorded on medium with 5–15  $\mu\text{M}$  2-iP, when compared to growth regulator-free medium. Several studies demonstrated the positive effect of BAP, NAA, TDZ and KIN for plantlet regeneration from PLBs [Nasiruddin et al. 2003, Luo et al. 2008, Chugh et al. 2009]. BAP and NAA are most applicable plant growth regulators for micropropagation of most orchids [Chugh et al. 2009].

Luo et al. [2008] showed that 5.0 mg·L BAP was the best for induction of PLBs ( $15 \times \text{explant}^{-1}$ ) per stem segment within 6 weeks. 0.5 mg · L KIN was also good for PLB formation. BAP in combination with NAA had been suggested by some studies to obtain the maximum number of PLBs [Kim and Kim 2003, Puchooa 2004]. Our finding is in consistent with these findings. However, Luo et al. [2008] showed that NAA added to the medium containing optimal BAP did not significantly improve response of explants in *Dendrobium densiflorum* and even decreased production of PLBs at concentration of more than 1.0 mg · L. In some orchids IBA induced rooting [Nayak et al. 2002].

In the present study, NAA was found more effective than IBA for micropropagation of *Orchis catasetum*. Study of Roy et al. [2011] on *Vanda coerulea*, an endangered orchid, showed that a synergistic combination of NAA (5.36  $\mu\text{M}$ ) and BAP (3.80  $\mu\text{M}$ ) led to maximum protocorm proliferation.



## CONCLUSION

Micropropagation of rare and endangered orchids in large scale have to be developed further because of commercial value and conserve them.

A proper ratio of auxins and cytokinins is required for optimal protocorm multiplication. The efficiency of type and concentration of plant growth regulators varied with kind of species or varieties.

BA and NAA at a particular concentration are suitable for protocorm-like bodies (PLBs) multiplication and growth of *Orchis catasetum*.

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### MIKROROZMNAŻANIE *Orchis catasetum* – RZADKIEJ I ZAGROŻONEJ ORCHIDEI

**Streszczenie.** Zastosowanie nowoczesnej biotechnologii do masowego rozmnażania rzadkich i zagrożonych gatunków wymaga opracowania właściwej procedury *in vitro*. W niniejszych badaniach opracowano metodę wysokiej częstotliwości rozmnażania *in vitro* zagrożonej orchidei *Orchis catasetum*. Protokormy, jako eksplanty, były hodowane na pożywce Murashige and Skoog (MS) wzmocnionej różnymi stężeniami N<sup>6</sup>-benzylodeaniny (BA), kwasu 1-naftylooctowego (NAA) oraz kwasu indolilomasłowego (IBA), pojedynczo lub w kombinacji. Stwierdzono, że kombinacja 0,5 mg · L BA i 0,5 mg · L NAA była odpowiednia do maksymalnej regeneracji PLB (20,40 × sadzonka<sup>-1</sup>). Największą liczbę korzeni (7,16 × sadzonka<sup>-1</sup>) i liści (10,10 × sadzonka<sup>-1</sup>), a także największą wysokość roślin (114,20 mm × roślina<sup>-1</sup>) i długość korzeni (193,40 mm × sadzonka<sup>-1</sup>) uzyskano na pożywce MS uzupełnionej za pomocą 0,5 mg · L BA razem z 0,5 mg · L NAA. Sadzonki o dobrze rozwiniętych liściach i korzeniach były przeniesione do doniczek wypełnionych perlitem, trocinami, jonolitem i kompleksem minerałów (1:1:1:1), a także samym perlitem, po czym zostały przeniesione do cieplarni. Po przeniesieniu *ex vitro* 100% roślin przeżyło.

**Słowa kluczowe:** auksyny, cytonininy, gatunki zagrożone, rozmnażanie *in vitro*, PLB, hodowla tkanek

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