Efficient Plant Regeneration and Genetic Fidelity Assessment of In Vitro-derived Plants of *Dendrobium nobile* – an Endangered Medicinal and Ornamental Herb

Changmei Song1,2‡, Jichuan Kang1,2,3†, Xiangbiao Ji1, Chunhua Fu1, Xiaopeng Wen1,2,4, Lifei Yu4, Guang Qiao†

1 Guizhou Key Laboratory of Agricultural Bioengineering, Guizhou University, Guiyang 550025, P. R. China
2 Education Ministry Center of Biochemical Engineering, Guizhou University, Guiyang 550025, P. R. China
3 College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, P. R. China
4 Forestry College, Guizhou University, Guiyang 550025, P. R. China
‡ These authors contributed equally to the work

**ABSTRACT**

*Dendrobium nobile* is of great interest because of its high value as a floricultural commodity and a kind of elite herbage plant in Chinese traditional medicine. An efficient *in vitro* propagation system via protocorm-like body (PLB) induction has been developed using one-year-old axillary node explants obtained from field-grown plants in the present research. The excised nodal buds began to sprout on Murashige and Skoog (MS) basal medium containing 4.0 μM NAA after 20–25 days in culture. Axenic secondary explants were then transferred into new MS medium to which 4.0 μM NAA was added for callus induction. The highest callus differentiation index (CDI) was scored from MS medium containing 1.5 μM gibberellic acid (GA). Subsequently, the well-developed PLBs were transferred into a new MS medium containing 4.0 μM BA and 2.0 μM NAA, from which vigorous shoots were obtained on the 60th day after transfer. The regenerated shoots rooted well in ½MS basal medium when NAA (2.5–5.0 μM) and 3-indole butyric acid (IBA, 2.5–5.0 μM) were added. Genetic fidelity of callus-derived shoots was assessed by performing PCR using 38 arbitrary primers. It was observed that all the primers displayed the same profiles between the stock plant and the randomly-selected shoots regenerated from the callus that had been subcultured within 15 cycles in MS medium containing 4.0 μM NAA.

**Keywords:** *Dendrobium nobile*, genetic stability, *in vitro* propagation, protocorm-like body (PLB), RAPD

**INTRODUCTION**

Today, it is sad but true that the fine balance that once linked man and nature is getting exacerbating at an unthinkable speed in our lifetime. *Dendrobium*, the second largest genus from the Orchidaceae exhibits a vast diversity in vegetative and floral characteristics, and is of considerable interest due to its high value as a floricultural commodity (Jones et al. 1998). Moreover, the stems of five *Dendrobium* species, namely, *D. chrysanthum*, *D. fimbriatum*, *D. lodigesi*, *D. nobile* and *D. candidum*, also known as Shi-hu are used as a tonic to improve digestion and for promoting the production of body fluid, nourishing ‘yin’ and dispelling ‘evil-heat’ in Chinese traditional medicine (Shiau et al. 2005). The main classes of chemical compounds in *Dendrobium* are a group of sesquiterpene alka- loids (Tang and Eisenbrand 1992). In the local markets in Taiwan and mainland of China, the dried plant costs about 4,000 US$ kg⁻¹ (Shiau et al. 2005). Conventionally, these species were propagated by separation of bulbs or through stem cuttings in which the proliferation rate was considerably low. *D. nobile* is native to the regions of Southern and Southwestern China. Unfortunately, this species is encoun- tering an unprecedented disaster because of the difficulty in propagation and the exorbitant exploitation of wild germ- plasm. Therefore, it is legally listed as a strictly protected species in China. The use of *in vitro* techniques for plant propagation and germplasm conservation has strikingly increased over the past decade (Wen et al. 2005; Hao and Deng 2005; Teixeira da Silva 2006). Reliable and efficient protocols for *in vitro* plant regeneration have also been developed in many species of Orchidaceae (Chen et al. 2005). In *in vitro* propagation of *D. antennathum*, *D. phalaenopsis*, *D. candidum* and *Dendrobium* hybrids have previously been reported (Ku- kuczanka and Wojciechowska 1983; Shiau et al. 2005; Martin and Madassery 2006), and a high rate of shoot proliferation could also be induced from shoot segments of three epiphytic orchids (Nayak et al. 1997). In *in vitro* plant regeneration from seeds was reported in *D. nobile* (Nayak et al. 2002). Also, successful shoot regeneration of this species using thin shoot tip sections and triacontanol was documented recently (Malabadi et al. 2005). Although there are these successful reports about *in vitro* culture of *Dendro- bium*, there exist some problems e.g. inefficient multiplica- tion, difficult rooting induction and lower planting survival, etc. To the best of our knowledge, there is no report on the clonal propagation of *D. nobile* using axillary nodal seg- ments. An extensive expansion of micropropagation re- quires a more efficient regeneration and acclimatization protocol in *D. nobile*. Furthermore, no information about the genetic fidelity of *in vitro*-derived plants has yet ob- tained from this species by now, which is crucial for both micropropagation and germplasm conservation. To ensure true-to-type, the genetic stability of *in vitro* derived *D. nobile* plants should be detected. Several strategies, such as phenotypic investigation, karyological analysis or isozyme markers, may be used to assess the genetic stability of *in vitro*-derived plants (Al-Zahim et al. 1999; Soneji et al. 2002; Hao and Deng 2005), but they have their own limitations. During the past decade, DNA markers were widely employed to detect somaclonal variation in many plants.

Received: 8 June, 2007. Accepted: 25 August, 2007.
(Wen and Deng 2005; Podwysynska et al. 2006; Joshi and Dhawan 2007).

In view of the high commercial value and worsening germplasm conservation situation of *D. nobile*, it is highly desirable to develop a protocol for the efficient and genetically stable *in vitro* multiplication. In the present work, we report a rapid propagation protocol using axenic nodal segments of *D. nobile*, and assess the undesirable somaclonal variation of the callus-derived plants, which may contribute to both *in vitro* micropropagation and conservation of this germplasm.

**MATERIALS AND METHODS**

**Plant materials**

Materials used for *in vitro* culture were initially collected from stock plants of *D. nobile* in the *Dendrobium* germplasm collection of Guizhou University (Guiyang, China). Plants 30–40 cm in height were grown in substrate (1:1, v/v) of tree bark (bottom) and moss (upper) in the greenhouse, and flowering plants were adopted in the present research because flowering ones have more stem nodes.

**Establishment of aseptic cultures**

One-year-old axillary node explants obtained from vigorous shoots (axillary buds covered with leaf sheaths) were surface disinfected with 70% ethanol for 30 s, followed by 0.1% **HgCl**$_2$ with 3–5 (approximately 0.1%, v/v) drops Tween 20 for 10 min, and rinsed five times with sterile distilled water. Sterilized shoots were then dissected under an aseptic hood, and excised nodal buds (about 2.0 cm in length) were inoculated into test tubes (20 × 170 mm) containing MS (Murashige and Skoog 1962) basal medium supplemented with 4.0 μM 6-benzyladenine (BA) and 2.0 μM naphthalene acetic acid (NAA) for germination.

About four to five weeks after regeneration, the contamination-free shoots were excised, and then transferred to new medium for further multiplication. Preliminary experiments indicated that depending on the culture condition, the regenerated shoots might multiply via direct shoot buds or protocorm-like bodies (PLBs). Compared with the former, however, the PLB pathway is more efficient. Therefore, detailed experiments were focused on the latter regeneration system.

**Callus induction and regeneration**

The axenic secondary explants were transferred into MS medium containing 4.0 μM of BA, kinetin (KT), 3-indole acetic acid (IBA) or NAA for callus induction. Then, the well-developed callus was selected to multiply on the same medium. Consequently, callus was used to induce PLBs on MS medium added with BA (4.5 μM), KT (4.5 μM) or gibberellin (GA). The callus differentiation index (CDI), which was defined as the number of PLBs derived from one gram of calli, was investigated after one-month culture.

To get elongated and vigorous shoots, the regenerated PLBs were transferred to MS medium containing different concentrations of BA and NAA for further development (*Table 1*). About 60 d later, the shoot number derived from each PLB, and shoot height were measured. A set of 50 PLBs cultured on MS medium without BA served as the corresponding control. Subsequently, the well-developed shoots were used to induce roots.

**Root induction of elongated shoots**

For induction of root formation, well-developed shoots (about 3.0 cm in height) were consequently transferred to 250 ml flasks containing approximately 80 ml ½MS basal medium supplemented with IBA and NAA (*Table 2*). Around 60 d later, the increment in shoot height, rooting percentage, root number per explant, and root length were investigated. The efficiency (percentage) of root formation was defined as the number of rooting shoots/the number of total shoots ×100%.

**In vitro and ex vitro hardening and transplantation**

The well-rooted plantlets were firstly hardened by opening the cover of flask for approximately two weeks. Then, plantlets of 5~7 cm in height were carefully removed from the flasks, and the roots were washed thoroughly under tap water to remove medium. Plantlets were transferred to 200 ml plastic pots containing tree bark (bottom) and moss (upper) as substrate (1:1, v/v) and hardened in growth chambers. The plantlets were initially covered with a beaker to maintain high humidity (above 90%) and irrigated once a week with MS basal medium. After acclimatization, all plantlets were transferred to the field with the above-mentioned substrate.

With the exception of ½MS (2%), all the media contained 3% sucrose as carbon source. The media were solidified with 0.7% (w/v) agar after adjusting pH to 5.8, and then sterilized by autoclaving at 121°C for 15 min. Cultures were incubated at 25 ± 2°C under 33 μmol photon m$^{-2}$ s$^{-1}$ in a 14 h photoperiod.

All experiments were carried out in a completely randomized block design. For each treatment, ten explants with five replicates were used. Statistical analyses of the effects of different treatments on the shoot regeneration response, shoot growth and root length were performed using SAS software, taking $P <0.05$ as significant level. Significances in rooting efficiencies were estimated by the Student’s $t$-test between two percentages.

**RAPD analysis**

The randomly-selected shoots regenerated from the callus subcultured for 1~16 cycles, and a total of 20 randomly-selected shoots derived from the callus transferred for 15 cycles were used to assess genetic fidelity. The experimental system was slightly modified from that described by Wen and Deng (2005). Reaction mixtures (20 μl) contained 2.5 mM MgCl$_2$, 0.2 mM dNTP, 0.4 μM primer (Shanghai Sangon Co. Ltd, China), 0.8 units Taq DNA polymerase and 100 ng genomic DNA as template. Amplifications were performed in a PTC-200 Thermal Cycler (NJ, USA). PCR began with 94°C for 2 min, 37°C for 1 min and 72°C for 2 min. The next 40 cycles were 94°C for 1 min, 37°C for 2 min, and 72°C for 2 min, followed by a final elongation step at 72°C for 10 min. Amplified products were electrophoresed on 1.6% (w/v) agarose gels, stained with ethidium bromide, and visualized or photographed on a UV trans-illuminator.

**RESULTS**

**Establishment of aseptic shoots**

After being sterilized, one-year-old axillary node explants were dissected under an aseptic hood to remove leaf sheaths and wounded surfaces, and excised nodal buds were inoculated on MS basal medium supplemented with 4.0 μM BA and 2.0 μM NAA. The axillary buds began to sprout when cultured for 20–25 d. About 30 d later, all contamination-free regenerated shoots were then excised and transferred into new media for further experiments.

**Factors affecting *in vitro* multiplication**

Depending on the culture condition, the regenerated shoots might multiply via direct shoot bud pathway by which two or three new shoots might be regenerated from each stock shoot per cycle (*Fig. 1A*), or via the PLB pathway with an initial callus phase (*Fig. 1B, 1C*). Compared with the former, however, the PLB pathway is much more efficient in multiplication since a large number of PLBs can be regenerated from callus. Type and concentration of PGRs showed significant effects on callus induction and callus quality. NAA or BA gave the highest callus formation percentage (70.0%), which was followed by KT (57.5%). Callus induction ability of IBA was the lowest (52.5%). In addition, friable callus could be obtained from medium added with 4.0 μM NAA, which might be sustainably subcultured and easily regenerated into PLBs.

In *Dendrobium nobile*, a rapid propagation protocol using axenic nodal segments of the plant was developed. The protocol involved a combination of callus induction, callus multiplication, and shoot regeneration. The protocol was validated using *D. nobile* germplasm collected from Guizhou University, China. The protocol involved the following steps:

1. **Callus induction and regeneration**: Axenic secondary explants were transferred into MS medium containing 4.0 μM of BA, kinetin (KT), 3-indole acetic acid (IBA) or NAA for callus induction. The callus was then used to induce PLBs on MS medium added with BA (4.5 μM), KT (4.5 μM) or gibberellin (GA).
2. **Root induction of elongated shoots**: For induction of root formation, well-developed shoots (about 3.0 cm in height) were transferred to 250 ml flasks containing approximately 80 ml ½MS basal medium supplemented with IBA and NAA.
3. **Ex vitro hardening and transplantation**: Rooted plantlets were hardened in growth chambers and then transferred to plastic pots containing tree bark (bottom) and moss (upper) as substrate. The plantlets were irrigated once a week with MS basal medium.

The protocol achieved high efficiencies in terms of callus formation, shoot regeneration, and root induction. The results indicated that the protocol is suitable for rapid propagation of *D. nobile* germplasm and could be used for germplasm conservation and agrobacterium-mediated genetic transformation.
Moreover, PGRs greatly influenced callus differentiation. Compared with 4.5 μM BA (45 ± 20) or 4.5 μM KT (48 ± 21), GA showed a predominant efficiency in CDI. Numerous PLBs might be derived from the callus cultured on MS medium containing GA for 30 d (Fig. 1B). The highest number (227 ± 11) was scored from the treatment with 1.5 μM GA. Unfortunately, hyperhydric and ill-developed PLBs, or occasionally deformed shoots were observed when the concentration was higher than 3.5 μM.

**PLB germination and strengthening of shoots**

To obtain vigorous shoots, well-developed PLBs should be transferred onto new medium. A suitable concentration of NAA and BA could obviously promote the germination of PLBs, effectively stimulate their multiplication, and substantially strengthen the development and elongation of shoot buds (Table 1). Among the treatments, the combination of 4.0 μM BA and 2.0 μM NAA gave the best germination effect (Fig. 1C), and the derived shoots had green leaves and vigorous growth (3.4 ± 0.4 cm in height) after 60 days in culture (Fig. 1D). Furthermore, a very high shoot number (3.2 ± 0.4) regenerated from each PLB could be obtained from this treatment. Consequently, these vigorous shoots could be transferred onto new medium for root induction.

**Rooting of in vitro-derived shoots and plantlet establishment**

With the addition of different combinations of IBA and NAA in ½MS basal medium, the rooting percentage, root number, root length, and shoot height increment of *in vitro* shoots were diverse (Table 2). Rooting percentages scored from the tested treatments were not as varied as other parameters. Taking all the parameters into consideration, NAA was more effective for root induction than IBA. The best response was achieved in medium containing NAA (2.5 μM) and IBA (2.5 μM), from which 100% of shoots could root, and the root length, root number per shoot and shoot height increment were optimal, followed by that obtained from the medium supplemented with 2.5 μM NAA, although the difference was not statistically significant. All of the treatments involving NAA showed very high rooting abilities, and 11 to 13 white roots with 2.5~4.5 cm in length were found from ½MS medium containing both NAA (2.5~5.0 μM) and IBA (2.5~5.0 μM) for 60 d (Fig. 1E). It indicates that NAA can proficiently stimulate root induction, which is consistent with the investigation in *D. candidum* (Shiau et al. 2005). It is noteworthy that the rooting medium also might promote the growth of an *in vitro* plant, and the shoot height could be remarkably increased (Table 2), which probably contributed to the improved absorption ability of the rooted plantlets.

### Table 1 Effect of plant growth regulators (PGRs) on shoot multiplication and development of *D. nobile*.

<table>
<thead>
<tr>
<th>PGR (μM)</th>
<th>Shoot number per explant a</th>
<th>Shoot height (cm) b</th>
<th>Morphogenetic response</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>NAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1.0 ± 0.1</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>2.0</td>
<td>0</td>
<td>2.5 ± 0.3</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>4.0</td>
<td>0</td>
<td>2.7 ± 0.1</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>6.0</td>
<td>0</td>
<td>2.9 ± 0.4</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>8.0</td>
<td>0</td>
<td>2.6 ± 0.4</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>0</td>
<td>2.0</td>
<td>1.4 ± 0.1</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>2.0</td>
<td>2.0</td>
<td>2.5 ± 0.2</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>4.0</td>
<td>2.0</td>
<td>3.2 ± 0.4</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>6.0</td>
<td>2.0</td>
<td>3.5 ± 0.5</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>8.0</td>
<td>2.0</td>
<td>2.8 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>0</td>
<td>4.0</td>
<td>1.2 ± 0.1</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>2.0</td>
<td>4.0</td>
<td>2.2 ± 0.2</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>4.0</td>
<td>4.0</td>
<td>3.3 ± 0.7</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>6.0</td>
<td>4.0</td>
<td>3.3 ± 0.5</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>8.0</td>
<td>4.0</td>
<td>3.0 ± 0.5</td>
<td>1.9 ± 0.3</td>
</tr>
</tbody>
</table>

Values within a column followed by the same letters are not significantly different at *P* = 0.05; values are means ± SE.
Table 2 Influence of NAA and IBA concentrations on the root formation and shoot growth of in vitro *D. nobile* shoots.

<table>
<thead>
<tr>
<th>NAA (µM)</th>
<th>IBA</th>
<th>Shoot height increment (cm)</th>
<th>Rooting rate (%)</th>
<th>Root length (cm)</th>
<th>Number per shoot</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2.7 ± 0.1 b</td>
<td>92.5 c</td>
<td>2.4 ± 0.3 cd</td>
<td>7.0 ± 0.6 bc</td>
</tr>
<tr>
<td>0</td>
<td>2.5</td>
<td>2.8 ± 0.3 ab</td>
<td>97.5 ab</td>
<td>2.9 ± 0.3 bc</td>
<td>7.8 ± 1.5 c</td>
</tr>
<tr>
<td>0</td>
<td>5.0</td>
<td>2.6 ± 0.2 b</td>
<td>95.0 bc</td>
<td>2.7 ± 0.3 bc</td>
<td>7.9 ± 1.0 c</td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td>3.0 ± 0.2 ab</td>
<td>100.0 a</td>
<td>3.9 ± 0.6 a</td>
<td>12.6 ± 2.1 a</td>
</tr>
<tr>
<td>2.5</td>
<td>2.5</td>
<td>3.2 ± 0.2 a</td>
<td>100.0 a</td>
<td>4.1 ± 0.3 a</td>
<td>13.0 ± 2.1 a</td>
</tr>
<tr>
<td>2.5</td>
<td>5.0</td>
<td>2.9 ± 0.2 ab</td>
<td>100.0 a</td>
<td>2.5 ± 0.6 cd</td>
<td>11.7 ± 3.2 a</td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td>2.9 ± 0.1 ab</td>
<td>97.5 ab</td>
<td>3.8 ± 0.6 a</td>
<td>11.5 ± 1.8 a</td>
</tr>
<tr>
<td>5.0</td>
<td>2.5</td>
<td>2.7 ± 0.4 b</td>
<td>97.5 ab</td>
<td>3.3 ± 0.5 ab</td>
<td>11.9 ± 1.4 a</td>
</tr>
<tr>
<td>5.0</td>
<td>5.0</td>
<td>2.6 ± 0.2 b</td>
<td>97.5 ab</td>
<td>2.7 ± 0.3 bc</td>
<td>11.1 ± 2.4 a</td>
</tr>
</tbody>
</table>

Same letters within a column are insignificant (*P* < 0.05).

Hardening and transplantation

After 60 d of root induction, the cover of flasks was opened for about two weeks. Subsequently, the rooted plantlets were washed free of agar and transferred to plastic chambers containing pine bark (bottom) and moss (upper), covered with a beaker that was occasionally opened during the acclimatization period of about 30 d (Fig. 1F). The transferred plants were first grown in a chamber at 25 ± 2°C under 33 µmol m⁻² s⁻¹ at a 14 h photoperiod. After four weeks hardening plants were directly transplanted to field conditions, and more than 90% success might be achieved. Plants maintained under field conditions developed normal fasciculated roots. Regenerated plants were also free from any noticeable phenotypic variability.

Genetic fidelity detection of *in vitro* plants

To investigate the genetic stability of *in vitro* shoots regenerated from *D. nobile* callus, genomic DNA of the explant donor stock plant (the control), and *in vitro* shoots derived from the callus were used to perform PCR using 38 arbitrary primers. With an average of 3.7 scorable bands per primer, a total of 141 distinct markers were recorded. No aberrant profile were observed among the randomly-selected shoots regenerated from the callus subcultured for no more than 15 cycles (ca. 24 months), whereas three polymorphic markers (two absent and one aberrant) were scored from that derived from the 16th cycle callus (Fig. 2A). Also, no genetic variation was assayed among the 20 randomly-selected plants that regenerated from the 15th cycle of callus (Fig. 2B). This result indicates that with the markers yielded from the tested primers, no genetic variation in the DNA site was detected in the *in vitro* shoots derived from *D. nobile* callus that had been subcultured for as many as 15 cycles on MS medium containing 4.0 µM NAA, which is desirable for both micropropagation and *in vitro* conservation of this germplasm.

DISCUSSION

*Dendrobium* is one kind of important epiphytic orchid that is valued for its attractive flowers and striking use in Chinese traditional medicine. Unfortunately, this species was very difficult to propagate. Therefore, germplasm has encountered a disaster due to the over-exploitation of wild resources. The application of micropropagation may provide an efficient method for its propagation. There exist some successes in the *in vitro* propagation of *Dendrobium* (Nayak *et al.* 1997, 2002; Shiau *et al.* 2005; Martin *et al.* 2006). Nayak *et al.* (2002) first reported a procedure for the rapid *in vitro* multiplication of *D. nobile* through shoot proliferation from thin cross sections (TCs) of PLBs derived from seeds. Further, a successful shoot regeneration of this species was documented using thin shoot tip sections (Mala-badi *et al.* 2005). However, it was an arduous and tedious work because the PLBs or shoot tip should be cut into TCs before multiplication. Recently, *D. candidum* was micropropagated by *in vitro* culture of 120-day-seed (Shiau *et al.* 2005). In the present study, a micropropagation procedure was established and optimized by inducing the reproducible and differentiable callus from axenic secondary shoots, from which a huge number of PLBs might be substantially obtained under suitable conditions.

Although PGRs play a major role in shoot regeneration from different explants of orchids, their concentrations, combinations and the ratio between them are critically important (Arditti and Ernst 1993). The requirement of PGRs also varies from species to species and from explant to explant. Nayak *et al.* (2002) reported that, with the utility of BA, KT, NAA, or both BA and NAA, a lower efficiency of PLB induction is observed. Malabadi *et al.* (2005) revealed that tricontanol can be used as an effective growth regulator in the micropropagation of *D. nobile*. In the present investigation, the PGR combination alters the *in vitro* regeneration pathway of this species. With the combinations of suitable BA and lower NAA concentration (2.0 µM), the *in vitro* shoots may directly multiply via shoot buds, although the multiplication efficiency is considerably low. Moreover, this PGR combination may substantially promote the germination of PLBs and remarkably induce the multiplication of neo-regenerated shoots. The friable callus that was obtained from medium supplemented with 4.0 µM NAA can be sustainably subcultured on the same medium. This result, to a certain extent, is in disagreement with that of Nayak *et al.* (2002), and the discrepancy between these studies might be attributed to the difference in genotypes since the germplasm used by Nayak *et al.* is from India. Although compared with the callus pathway, the shoot bud pathway shortens the micropropagation cycle by about 40~50 d, and its
proliferation rate is much lower. Therefore, it is suggested that the PLB pathway should be adopted in multiplication, a now commonly adopted pathway for *Cymbidium* spp. (Teixeira da Silva and Tanaka 2006). To get *in vitro* shoots, two steps, PLB induction and germination are necessary, in which different medium is needed.

Genetic fidelity of micropropagated plants is of the most important in both commercial implications and germplasm conservation. All methods for germplasm conservation are based on the assumption that plant materials are conserved under conditions that can ensure genetic fidelity. Various factors associated with *in vitro* manipulation, e.g. PGR type and concentration, subculture cycle, and regeneration pattern etc. may lead to genetic instability (Ashmore 1997; Engelmann 1997). The source of explants and pattern of regeneration are known to play major roles in determining the presence or absence of variation (Salvi et al. 2001). Compared with the plants derived from callus, those regenerated from adventitious buds or other well-developed meristems show the least tendency of genetic variations (Salvi et al. 2001; Wen and Deng 2005).

In the present research, compared with the cluster bud pattern, the PLB pathway showed remarkably higher multiplication efficiency. Probably, the latter may produce more somaclonal variation because it undergoes a callus stage, and it is generally accepted that there are much more variations in DNA sites when callus multiples (Al-Zahim et al. 1999; Yang et al. 1999). It is therefore essential to monitor genetic stability during subculture. Many investigations over the past decade had focused on assessments using DNA markers. Utility of RAPD as a means to assess the genetic stability of *in vitro* regenerated plants has been well documented (Wen et al. 2005; Teixeira da Silva et al. 2007). Actually, investigation here had indicated that *D. nobile* in *in vitro* shoots derived from callus are genetically stable if the calluses are subcultured for no more than 15 cycles in MS medium containing 4.0 μM NAA. Therefore, the *in vitro* culture procedure provided here is optimized to minimize the occurrence of somaclonal variation, and can be employed to micropropagate and conserve germplasm.

**ACKNOWLEDGEMENTS**

The project was supported by grants from the National Natural Science Foundation of China (30660115), Natural Science Foundation of Guizhou Province Mayor (China, 2005185), Excellent Young Scientist Program of Guizhou Province (2007), National Science Core Program of Guizhou City (China), and 973 Project of China (2006CB5206-6). Our appreciation also extends to Drs J. Shao and J. H. Liu from National Key Laboratory of Crop Genetic Improvement (China) for their critical revision of the manuscript.

**REFERENCES**


Jones WE, Kuehnle AR, Arumutanathan K (1998) Nuclear DNA content of 26 orchids (Orchidaceae) genera with emphasis on *Dendrobium*. *Annals of Botany* 82, 189-194


Nayak NR, Rath SP, Patnaik S (1997) *In vitro propagation of three epiphytic orchids, Cymbidium aloifolium (L.) Sw., Dendrobium aphylum (Roxb.) Fisch. and Dendrobium mosschatum (Buch-Ham) Sw. through thidiazuron-induced high frequency shoot proliferation*. *Sciencea Horticulturae* 71, 243-250


