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

Changmei Song[1†] • Jichuan Kang[1,2†] • Xiangbiao Ji[1] • Chunhua Fu[3] • Xiaopeng Wen[1,2*] • Lifei Yu[4] • Guang Qiao[1]

[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

Corresponding author: * xpwensc@hotmail.com

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 6-benzyladenine (BA) and 2.0 μM naphthalene acetic acid (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.

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. loddigesii*, *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 alkaloids (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 encountering an unprecedented disaster because of the difficulty in propagation and the exorbitant exploitation of wild germplasm. 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 vitro* propagation of *D. antennathum*, *D. phalaenopsis*, *D. candidum* and *Dendrobium* hybrids have previously been reported (Kukulczanka 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 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 *Dendrobium*, there exist some problems e.g. inefficient multiplication, 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 segments. An extensive expansion of micropropagation requires a more efficient regeneration and acclimatization protocol in *D. nobile*. Furthermore, no information about the genetic fidelity of *in vitro*-derived plants has yet obtained 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

Original Research Paper

(Wen and Deng 2005; Podwyszynska *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 collectioin 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 in 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 butyric 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 μ mol 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.
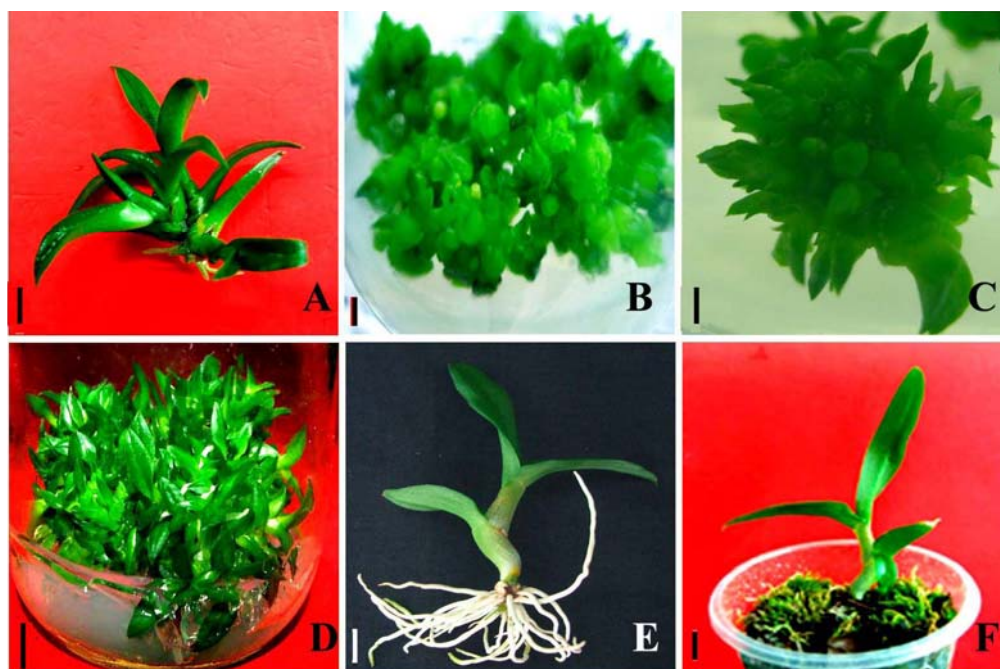
Fig. 1 *In vitro* **plant regeneration of *D. nobile* via shoot buds and PLBs.** (**A**) Regenerated shoots multiple through cluster buds; (**B**) Redifferentiating callus and the regenerating PLBs on the 30th day after transfer; (**C**) Well-developed PLBs regenerated from the callus on the 60th day after transfer; (**D**) Well-developed shoots regenerated from PLBs; (**E**) Regenerated plantlet with well-formed roots; (**F**) Plants after 20 d acclimatization in greenhouse. Bars = 1 cm.

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*.

PGR (μM)		Shoot number per explant [z]	Shoot height (cm) [z]	Morphogenetic response
BA	NAA			
0	0	1.0 ± 0.1 g	3.1 ± 0.4 ab	Vigorous, green leaves
2.0	0	2.5 ± 0.3 cd	2.4 ± 0.3 de	Vigorous, green leaves
4.0	0	2.7 ± 0.1 bc	2.5 ± 0.3 cd	Yellow leaves
6.0	0	2.9 ± 0.4 bc	2.6 ± 0.4 cd	Deformed and yellow leaves
8.0	0	2.6 ± 0.4 bc	2.5 ± 0.7 cb	Some yellow leaves
0	2.0	1.4 ± 0.1 f	2.9 ± 0.5 bc	Vigorous, green leaves
2.0	2.0	2.5 ± 0.2 cd	3.1 ± 0.5 ab	Vigorous, green leaves
4.0	2.0	3.2 ± 0.4 ab	3.4 ± 0.4 a	Most vigorous
6.0	2.0	3.5 ± 0.5 a	2.7 ± 0.5 bc	Yellow leaves
8.0	2.0	2.8 ± 0.2 bc	2.0 ± 0.2 de	Deformed and yellow leaves
0	4.0	1.2 ± 0.1 fg	2.8 ± 0.6 bc	Moderate vigorous, callus-forming
2.0	4.0	2.2 ± 0.2 de	2.9 ± 0.5 bc	Moderate vigorous, callus-forming
4.0	4.0	3.3 ± 0.7 ab	3.2 ± 0.6 ab	Weak, callus-forming
6.0	4.0	3.3 ± 0.5 a	2.3 ± 0.4 cde	Deformed leaves, forming callus
8.0	4.0	3.0 ± 0.5 ab	1.9 ± 0.3 e	Deformed leaves, many calli

[z] 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.

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

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. Subsquently, 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 hardened 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 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 (TCSs) of PLBs derived from seeds. Further, a successful shoot regeneration of this species was documented using thin shoot tip sections (Malabadi *et al*. 2005). However, it was an arduous and tedious work because the PLBs or shoot tip should be cut into TCSs 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
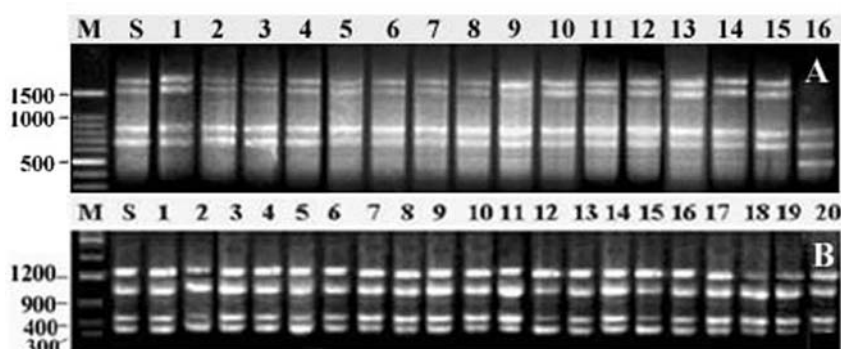
Fig. 2 DNA amplification profiles showing the genetic stability and variation of callus-derived shoots of *D. nobile*. (**A**) RAPD profiles (S-32, TCGGCGATAG) amplified with the genomic DNA of stock plant (S), and the shoots derived from callus subcultured for one (1) to 16 cycles; (**B**) Profiles (OPAC-02) amplified with the genomic DNA of stock plant (S), and the randomly selected 20 shoots derived from the callus subcultured for 15 cycle; M: DNA marker (Shanghai Sangon Co. Ltd, China).

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 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 occurence of somaclonal variation, and can be employed to micropropagate and conserve germplasm.

ACKNOWLEDGEMENTS

REFERENCES

Al-Zahim MA, Ford-Llord BV, Newbury HJ (1999) Detection of somaclonal variation in garlic (*Allium sativam* L.) using RAPD and cytological analysis. *Plant Cell Reports* **18**, 473-477

Arditti J, Ernst R (1993) *Micropropagation of Orchids*, Wiley Press, New York, US, pp 210-211

Ashmore SE (1997) *Status Report on the Development and Application of In Vitro Techniques for the Conservation and Use of Plant Genetic Resources.* International Plant Genetic Resources Institute Press, Rome, Italy, 67 pp

Chen Y, Liu X, Liu Y (2005) *In vitro* plant regeneration from the immature seeds of *Cymbidium faberi*. *Plant Cell, Tissue and Organ Culture* **81**, 247-251

Engelmann F (1997) *In vitro* conservation methods. In: Callow JA, Ford-Lloyd BV, Newbury HJ (Eds) *Biotechnology and Plant Genetic Resources*, CAB International Press, Oxford, UK, pp 119-161

Hao YJ, Deng XX (2005) Cytological and molecular evaluation of strawberry plants recovered from *in vitro* conservation by slow-growth. *Journal of Horticultural Science and Biotechnology* **80**, 588-592

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

Joshi P, Dhawan V (2007) Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay. *Biologia Plantarum* **51**, 22-26

Kukulczanka K, Wojciechowska U (1983) Propagation of two *Dendrobium* species by *in vitro* culture. *Acta Horticulturae* **131**, 105-110

Malabadi RB, Mulgund GS, Kallappa N (2005) Micropropagation of *Dendrobium nobile* from shoot tip sections. *Journal of Plant Physiology* **162**, 473-478

Martin KP, Madassery J (2006) Rapid *in vitro* propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies. *Scientia Horticulturae* **108**, 95-99

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Plant Physiology* **15**, 473-497

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

Nayak NR, Sahoo S, Patnaik S, Rath SP (2002) Establishment of thin cross section (TCS) culture method for rapid micropropagation of *Cymbidium aloifolium* (L.) Sw. and *Dendrobium nobile* Lindl. (Orchidaceae). *Scientia Horticulturae* **94**, 107-116

Podwyszynska M, Niedoba K, Korbin M, Marasek A (2006) Somaclonal variation in micropropagated tulips determined by determined by phenotype and DNA markers. *Acta Horticulturae* **714**, 211-219

Salvi ND, George L, Eapen S (2001) Plant regeneration from leaf callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. *Plant Cell, Tissue and Organ Culture* **66**, 113-119

Shiau YJ, Nalawade SM, Hsia CN, Mulabagal V, Tsay HS (2005) *In vitro* propagation of the Chinese medicinal plant, *Dendrobium candidum* Wall Ex Lindl. from axenic nodal segments. *In Vitro Cellular and Development Biology – Plant* **41**, 666-670

Soneji JR, Rao PS Mhatre M (2002) Somaclonal variation in micropropagated dormant axillary buds of pineapple (*Ananas comosus* L., Merr.). *Journal of Horticultural Science and Biotechnology* **77**, 28-32

Tang W, Eisenbrand G (1992) *Dendrobium nobile* Lindl. In: Tang W, Eisenbrand G (Eds) *Chinese Drugs of Plant Origin, Chemistry, Pharmacology, and Use in Traditional and Modern Medicine*, Springer-Verlag, Berlin, pp 451-454

Teixeira da Silva JA (Ed) (2006) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol II), Global Science Books, London, 571 pp

Teixeira da Silva JA, Bolibok H, Rakoczy-Trojanowska M (2007) Molecular markers in micropropagation, tissue culture and *in vitro* plant research. *Genes, Genomes and Genomics* **1**, 66-72

Teixeira da Silva JA, Bolibok H, Rakoczy-Trojanowska M (2006) Embryogenic callus, PLB and TCL paths to regeneration in hybrid *Cymbidium* (Orchidaceae). *The Journal of Plant Growth Regulation* **25**, 203-210

Wen XP, Deng XX (2005) Micropropagation of chestnut rose (*Rosa roxburghii* Tratt) and genetic stability assessment of the *in vitro* plants using RAPD and AFLP markers. *Journal of Horticultural Science and Biotechnology* **80**, 54-60

Yang H, Tabei Y, Kamada H, Kayamo T, Takaiwa F (1999) Detection of somaclonal variation in cultured rice cells using digoxogenin-based random amplified polymorphic DNA. *Plant Cell Reports* **18**, 520-526