academicJournals

Vol. 7(28), pp. 2098-2110, 25 July, 2013
DOI: 10.5897/JMPR11.1777
ISSN 1996-0875 ©2013 Academic Journals
http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Micropropagation and *in vitro* flowering of *Dendrobium wangliangii*: A critically endangered medicinal orchid

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Accepted 13 April, 2012

Dendrobium wangliangii **is seriously threatened because of anthropogenic and environmental pressures. A protocol with seed asymbiotic germination was developed to conserve the orchid *in vitro*. The germination rate was 92% under 16/8 h (light/dark) photoperiod after 240 days from hand-pollination in a modified medium. Polyembryony was founded in *D. wangliangii,* with a percentage of 0.87%. Half MS promoted the protocorms proliferation well, with the protocorms differentiated at the frequency of 28.79% when cultured on half MS, supplemented with 2 mg L^{-1} 6-benzylaminopurine (BA) and 0.1 mg L^{-1} naphthaleneacetic acid (NAA). Additives, especially 100 ml L^{-1} coconut milk (CM), improved the protocorm differentiation greatly but led to a lethality of 49.92%. The pretreatment of 1 mg L^{-1} GA_3 for 15 days, after inoculated to the medium amended with 2 mg L^{-1} BA, 0.1 mg L^{-1} NAA and 100 ml L^{-1} CM, increased the differentiation to 54.68%. The combination of 2 mg L^{-1} BA, 0.1 mg L^{-1} NAA, and 100 ml L^{-1} CM also showed an efficient role in adventitious bud multiplication. CM effectively controlled the albino phenotype. In addition, inflorescence induction (100%) was produced with 2 mg L^{-1} 1-phenyl-3-(1, 2, 3-thiadiazol-5-yl)-urea (TDZ) and the best flower development was obtained with 0.3 mg L^{-1} paclobutrazol (PP333), and 0.5 mg L^{-1} NAA.**

Key words: *Dendrobium wangliangii,* micropropogation, asymbiotic germination, adventitious buds multiplication, polyembryony, *in vitro* flowering.

INTRODUCTION

Dendrobium wangliangii is one of the largest genera in the Orchidaceae comprising approximately 1,100 species (Puchooa et al., 2004). About 80 species of *Dendrobium* are known from China, most occurring in tropical and subtropical regions of the south (Hu et al., 2008). *Dendrobium* is one of the most famous traditional herbs in China. *D. wangliangii* G. W. Hu, C. L. Long and X. H. Jin is a newly discovered and described species with stems closely attached to the host's bark, making it distinguishable from other species (Hu et al., 2008). The mucopolysaccharide-rich fleshy stem is similar to two species with important morphological and medicinal properties, *Dendrobium officinale* Kimura and Migo, and *Dendrobium huoshanense* C. Z. Tang and S. J. Cheng, suggesting *D. wangliangii* probably has high medicinal value which is confirmed by the fact that the locals collect

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it for medicinal uses. In addition, the plant possesses high ornamental value because the large and showy pink flowers are very attractive compared to the small fleshy stems.

Field surveys confirmed that *D. wangliangii* was very rare with a limited distribution area in the wild which was only less than 10 km^2 in the northern portion of Yunnan Province, China. According to the criterion of International Union for Conservation of Nature (IUCN), *D*. *wangliangii* should be in the category “critically endangered” (CR). With some populations adjacent to villages, the habitats available are becoming severely affected by human activities, such as collecting, grazing, and cutting down trees for baking tobacco. The largest known population was split into two by a newly built road for a hydropower station.

As a drought tolerant plant, a continuous drought from November 2009 to May 2010, along with abnormal changes in climate, caused a population decrease, and few plants were in bloom. Based on our field investigation, it is estimated that the number of wild populations will at least decrease to less than 50% in nearly 10 years, provided the impact of human activity and abnormal climate change continuously. Also, sexual reproduction is greatly inhibited when planted in the Kunming Botanical Garden because most plants died in a trial study, and only one of the surviving plants bloomed. Therefore*, ex-situ* conservation, at least in Kunming, is not suitable for sustainable protection. However, *in vitro* conservation is an alternative method for the preservation of the extraordinarily valuable and critically endangered orchid, *D*. *wangliangii.*

Although, species conservation is achieved most effectively through the management of wild populations and natural habitats *(in situ* conservation), *ex situ* techniques can be used to complement *in situ* methods, and in some instances, may be the only option for some species (Maunder et al., 1998; Ramsay et al., 2000). As more species face the risk of extinction, *in vitro* techniques have been increasingly used in the conservation of threatened plants in recent years (Fay, 1992; Arditti and Krikorian, 1996; Benson et al., 2000). Sarasan et al. (2006) considered that, future conservation biotechnology research and its applications must be aimed at conserving highly threatened, mainly endemic plants from conservation hotspots. Thus, the critically endangered *D. wangliangii*, found only in one of the world hotspots of biodiversity (Myers et al., 2000), is promising for *in vitro* study.

One of the integral parts of *in vitro* conservation is to produce plants in large numbers for reintroduction into the wild (Misic et al., 2005). Some orchids from *in vitro* culture have been successfully reintroduced into the wild including *Cypripedium calceolus* L. (Ramsay and Stewart, 1998), *Vanda spathulata* Spreng (Decruse et al., 2003), and *Anacamptis laxiflora* (Lam.) R. M. Bateman, Pridgeon and M. W. Chase (Wood and Ramsay, 2004). Furthermore, *in vitro* culture allows for the provision of plant materials for DNA analyses, autoecological studies, and commercial uses. The intrinsic value of plant biodiversity must be recognized and its conservation prioritized.

Propagation of orchids by *in vitro* germination of seeds allows for the maintenance of higher genetic variability than other methods, which is quite useful for orchids (Ávila-Díaz et al., 2009; Sarasan et al., 2006). The purpose of this study is to investigate the factors that are relative with the seed germination, to develop a highly efficient micropropagation protocol, and to assess the effects of hormones on the *in vitro* flowering.

MATERIALS AND METHODS

Plant materials and culture conditions

Green pods generated through hand-pollination were collected from a wild population in northern Yunnan Province, China (The exact locality of *D. wangliangii* is not given with the concerns that this beautiful species will be the target of illegal collection). Seed capsules were sterilized with 75% (v/v) ethanol for 45 s and then dipped into 0.1% $HgCl_2$ for 15 min, followed by a thorough rinsing in sterile distilled water. The seeds extracted from each capsule were uniformly scattered into approximately 4 to 8 containers with the volume of 500 ml.

The basal medium was half MS (Murashige and Skoog, 1962) amended with 5.6 g L^{-1} agar and 20 g L^{-1} sucrose. The pH of the medium was adjusted to 5.8 with 1N KOH or HCl. Prior to use, the medium was autoclaved for 20 min at 121°C. All other experimental media treatments are described separately. Except for the darkness treatment, other experiments were placed at light conditions under a 16/8 h (light/dark) photoperiod with light provided by 36-W cool-white fluorescent bulbs (PHILIP TLD, 36 µmol m^{-2} s^{-1}/827) at 22 ± 2°C.

Seed morphology and germination

Seed capsules were collected at 180, 210, 240, and 270 days after pollination. At each collection date, seeds were placed on the basal medium N1 containing microelement, organics, inositol, and ferric salt (the same as MS), plus 3 g L^{-1} Hyponex-1, 0.5 mg L^{-1} NAA, and 0.5 g L^{-1} activated charcoal (AC) under the light/dark and darkness treatments. Germination was observed under a microscope (Axioskop 40; Zeiss, Gottingen, Germany) and dissecting microscope (Canon A2000IS; Olympus SZX7) every 2 or 3 days. While recording, the seed morphologically changes with photography. The detailed morphology was observed with a scanning electron microscope (KYKY 1000B). The germination frequency was determined at 30 days after inoculation when green protocorms were formed.

Protocorm proliferation

To multiply rapidly, protocorms within 60 days of inoculation were transferred to new containers (90 protocorms each at least) with the following media: half MS; N1; half MS supplemented with 1 mg L^{-1} BA + 0.1 mg L^{-1} indole-3-acetic acid (IAA); and half MS supplemented with 0.2 mg L^{-1} PP333 + 0.5 mg L^{-1} NAA. Data relative to the multiplication was measured 90 days after inoculation into the new media.

Shoots differentiation

Experiments were performed for improving the shoot differentiation rate of protocorms. The protocorms that did not germinate after 70 days were transferred to half MS amended with various plant growth regulator combinations of four cytokinins [0.01, 0.05, 0.1 mg L^{-1} TDZ; 0.1, 1, 2, 3 mg L^{-1} BA; 1, 2, 3 mg L^{-1} N^6-[2-Isopentenyl]-adenine (2ip); and 0.1, 0.5, 1 mg L^{-1} Zeatin (ZT)] and the auxin 0, 0.1, 0.5,1 mg L^{-1} NAA. At least 90 protocorms were observed per container. Data was collected 90 days after inoculation into the new media.

Effect of additives on the protocorms growth and regulation

The protocorms with 30 or 60 days culture after seed inoculation were transferred to half MS (a minimum of 90 per container) supplemented with 20 g L^{-1} sucrose and 0.5 g L^{-1} AC, then amended with 100 g L^{-1} banana mash, 100 g L^{-1} potato mash, 100 g L^{-1} plum mash, 100 g L^{-1} tomato mash, 100 ml L^{-1} CM, and 4 g L^{-1} lactalbumin hydrolysate (LH), respectively. The sources of these additives were fresh banana, potato, plum and tomato that were skinned and homogenized in a blender. CM previously produced was stored in -20°C. Data was collected from 60 days after inoculation.

Effect of GA_3 on protocorms growth

Protocorms with 60 days inoculation were transferred to half MS with 0, 0.1, 1 and 2 mg L^{-1} gibberellic acid (GA_3) for 15 days, followed by inoculated to the 2 mg L^{-1} BA + 0.1 mg L^{-1} NAA + 100 ml L^{-1} CM with data recorded 60 days after inoculation.

Adventitious multiplication

The protocorms cultured in the half MS without subculture for 120 days differentiated into shoots. These shoots were used to evaluate the adventitious bud proliferation in half MS supplemented with: 0.5, 1, 2 mg L^{-1} TDZ; 0.5, 1, 2 mg L^{-1} BA; 0.5 mg L^{-1} BA + 0.5 mg L^{-1} NAA; 1 mg L^{-1} BA + 1 mg L^{-1} NAA; half MS + 4% potato mash + 0.5 mg L^{-1} NAA; C10 (half MS amended with 10 ml L^{-1} CCC; 0.2 mg L^{-1} BA, and 0.2 mg L^{-1}NAA); 2 mg L^{-1} BA + 0.1 mg L^{-1} NAA + 100 ml L^{-1} CM; and 0.3 mg L^{-1} PP333 + 0.5 mg L^{-1} NAA. Each container consists of 30 seedlings with every six seedlings planted close together to form a small community. Data was measured after 60 days. The frequency of inflorescence induction was collected at 90 days after inoculation.

***In vitro* flowering**

The protocorms cultured in the half MS without subculture for 120 days differentiated into shoots that were also used to evaluate *in vitro* flowering in half MS amended with: 0.5, 1, 2 mg L^{-1} TDZ; 0.5, 1, 2 mg L^{-1} BA; 0.5 mg L^{-1} BA + 0.5 mg L^{-1} NAA; 1 mg L^{-1} BA + 1 mg L^{-1} NAA, C10; and 0.3 mg L^{-1} PP333 + 0.5 mg L^{-1} NAA. The inflorescence induction frequency was recorded 60 day after the transfer to inflorescence induction medium. The inflorescence induction frequency was recorded 60 days after the transfer to the medium. Moreover, sixty days after planting of seeds on to medium, the protocorms were transferred to the half MS supplemented with 20 g L^{-1} sucrose and 0.5 g L^{-1} AC, then amended with 100 g L^{-1} banana mash, 100 g L^{-1} potato mash, 100 g L^{-1} plum mash, 100 g L^{-1} tomato mash, 100 ml L^{-1} coconut milk, and 1 mg L^{-1} GA_3 to produce seedlings for another 60 days. Then the seedlings were inoculated to the C10 medium. The inflorescence induction frequency was recorded 60 days after the transfer to C10.

Rooting and acclimatization

The roots were generated on half MS supplemented with 0.5 mg L^{-1} NAA. Two months old plantlets bearing over 3 roots were bound up by moss and then put into pots with the same rubble at the bottom. After that, the plantlets were transferred to a greenhouse with ca. 70% of shading of sunlight and the temperate 25 ± 2°C.

Data analysis

At least five replicates of each treatment were pooled for a t-test ($p < 0.05$), using the program statistical analysis system (SAS Institute Inc 2000 to 2004).

RESULTS

Seed morphology

The seeds of *Dendrobium* are fusiform (Swamy et al., 2007; Wang and Xiao, 2010). As in the case of *D. wangliangii,* seeds are light yellow-white and approximately 300 µm long with the embryos accounting for half of the total length (Figure 1a). The embryo is hermetically coated by a testa which retrenches sharply at two terminals of the seed, while a small part of the seeds elongate to one direction with the majority extending to both sides in identical lengths, shaping as the linear or unciform. We observed some testa rupture prior to germination while some seeds failed to develop fully. Electron microscope scanning showed the testa surface possessing line-fluent ridges or zonal salients that were densely covered with lots of large-volume floccules and small-volume phase and points structures (Figure 1b and c). Wang and Xiao (2010) indicated that the size and number of floccules differed in several of *Dendrobium,* and the floccules of section *Dendrobium* were sparse and small. Oppositely, the floccules of *D. wangliangii* belonging to section *Dendrobium* were very dense. Light ridge salients also existed in the testa valley covered with floccules, small-volume phase, and point structures as well.

Polyembryony

Polyembryony with two embryos was observed. Two embryos with equal or different volume (Figure 1d and e) with a pecentage of 0.87% of the observed seeds (n = 2069). Seeds with two embryos have smaller embryos than those seeds with one embryo (less than 300 µm long). Based on the available literature, polyembryony in *Dendrobium* was first observed here.

Seed germination

As the embryo grew within the testa, the color turned from

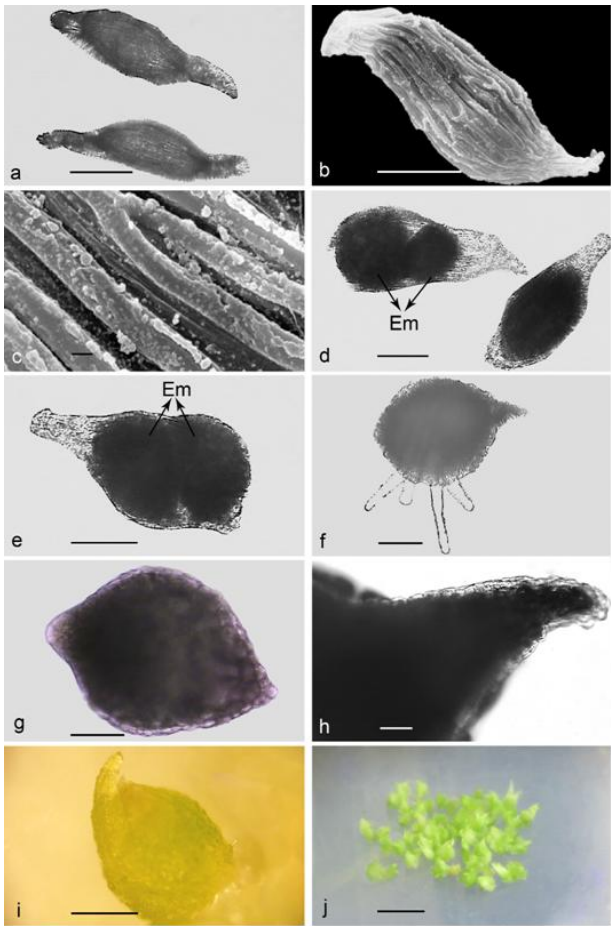

Figure 1. Morphology of seeds and germination stages of *D. wangliangii*. (a) Fusiform seeds (b) The seed under electron microscope scanning (c) Floccules and small-volume phase, and points structures on the testa (d) Different volume embryos (e) Equal volume embryos (f) Rhizoids formed on the protocorm (g) The protocorm completing polarity differentiation (h) The bud generated from a protocorm under microscope (i) The bud produced from a protocorm under the dissecting microscope (j) The shoots produced. Em: Embryo. Scale bar = 100 μm for a, b, d, e, f, g, and h; scale bar = 5 μm for c; scale bar = 500 μm for i; Scale bar =10^4 μm for j.

light yellow-white to yellowish green at 7 days after planting. The embryo altered its morphology from fusiform to ellipse due to the fast growth of the cells in the central portion after 9 days. The rhizoid emerged on the eleventh day (Figure 1f). As the embryo further developed, the proportion of the embryos with rhizoids increased in number and the length of rhizoids is enhanced as well. The embryo turned into spherical protocorms after 24 days, followed by a bulge generated at one end. The polarity differentiation is completed when another end is formed after 38 days (Figure 1g). Further development of the buds was recorded under the microscope (Figure 1h) and dissecting microscope (Figure 1i). Afterwards, the earliest shoots emerged after 70 days. The detachment time of testa from embryo varied greatly, with the earliest 23 days after inoculation, while the testa still adhered to the undifferentiating embryo even after 60 days. After inoculation for 30 days, the diameter of protocorms increased from 312.5 to 875.5 μm, among which the large-volume (≥ 687.5 μm) with a spherical shape accounted for 8.16%. The small-volume (≤ 500 μm) with testa and polarity differentiation accounted for 11.32%, and the rest occupied 80.52%. The earliest differentiated buds all originated from the small-volume protocorms; therefore, we were not certain if the earliest shoot differentiation resulted from polarity differentiation directly, without experiencing a globular embryo stage.

Effect of light on germination

Light significantly promoted the germination of *D. wangliangii* up to 98.33%. With light, seeds turned green and swollen on the seventh day, while seeds under darkness remained the same and did not germinate. Under microscope observation, the embryos became brown, and had broken testa with terminated growth. The seeds previously inoculated in darkness died in continuous darkness and under the transfer to light.

Effect of maturity on germination

Seed maturity improved germination frequencies (Table 1). The seeds of 180 and 210 days after hand-pollination aggregated in bulk and clung to the fruit inner wall, showing undeveloped embryos in small-volume under the microscope. Although grown to some extent, the seeds failed to germinate in the end. On the contrary, the seeds of 240 after hand-pollination dispersed easily and germinated with the highest rate. The shoots differentiated in different times, 70 and 50 days, respectively, for the seeds of 240 and 270 days after hand-pollination. Furthermore, the bud differentiating frequency of the seeds of 240 after hand-pollination was higher than that of the seeds after 270 days from hand-pollination (unpublished data).

Effect of hormones on the protocorms proliferation

Half MS was beneficial to the protocorm-like bodies (PLBs) proliferation with higher frequency, vigorous growth, and less differentiation (Table 2, Figure 2a). However, PLBs differentiated into buds when the culture continued, so it was necessary to promptly subculture the PLBs before their differentiation to keep their previous condition. N1 exhibited worse effect on PLBs proliferation with a lower frequency and more shoot production. The

Table 1. Effect of maturity on germination.

Days after pollination	Seed condition	Percentage of germination ± SD
180	Bulk-like	0
210	Bulk-like	0
240	Powdery	91.94 ± 1.80[a]
270	Powdery	88.70±1.30[a]

Values followed by the same letter were not significantly different at $p < 0.01$ by the SAS test. SD: standard deviation.

Table 2. Effect of hormones on the protocorms proliferation and protocorm growth status.

Medium components (mg L^{-1})	No. of protocorms ± SD	Growth status
1/2MS	8.32 ± 0.16[a]	Yellowish green, fewer differentiation
N1	5.44 ± 0.19[c]	Yellow, white, somewhat differentiation
1BA + 0.1I AA	6.48 ± 0.11[b]	Light yellow, somewhat differentiation
0.3PP333 + 0.5 NAA	6.74 ± 0.19[b]	More differentiation

Values followed by the same letter were not significantly different at $p < 0.01$ by the SAS test. SD: standard deviation.

influence of 1 mg L^{-1} BA + 0.1 mg L^{-1} IAA on PLBs multiplication was not so effective compared with half MS, owing to the less proliferation frequency and more differentiation. 0.3 mg L^{-1} PP333 + 0.5 mg L^{-1} NAA showed an efficient impact on differentiation but a lower frequency (Table 2).

Effect of hormone on shoot germination

In view of lower shoot differentiation, the effect of the combination of cytokinin and auxin on *D. wangliangii* protocorm shoot differentiation was investigated (Table 3). Experiments indicated different effects of the hormone combinations on protocorms differentiation. On the cytokinin aspects, TDZ had a negative effect on shoot emergence from PLBs with the highest frequency of only 9.20%, while the 2 mg L^{-1} BA + 0.1 mg L^{-1} NAA exhibited the best performance with the maximum of 28.79% (Figure 1j). Furthermore, the treatment of 0.5 mg L^{-1} ZT, 2 mg L^{-1} 2-ip, and 2 mg L^{-1} BA + 0.1 mg L^{-1} NAA also displayed a higher shoot differentiation frequency of 22.05, 24.00 and 25.08%, respectively. In general, the high ratio of the combination of cytokinin to auxin promoted bud differentiation. However, as for *D. wangliangii,* only 2 mg L^{-1} BA + 0.1 mg L^{-1} NAA manifested better result than 2 mg L^{-1} BA alone (28.79%/16.96%). 0.05 mg L^{-1} TDZ, 2 mg L^{-1} 2-ip, and 0.5 mg L^{-1} ZT showed a worse impact on shoot production compared to cytokinin alone. Moreover, the combination of several cytokinins did not improve the shoot differentiation (Table 3). The influence of hormone categories and dose on the protocorm volume was different as well. 0.5 mg L^{-1} NAA and 0.01 mg L^{-1} TDZ + 0.1 mg L^{-1} NAA enhanced the diameter to 2.3 and 2.2 mm, respectively. The albino emerged in every media after transfer from the germination medium. The frequency of albino was lower than 1% in the second month but sharply increased to 87.03% in the third month. Brown protocorms were also observed in some media. All the albino protocorms nearly turned brown in the subculture to the same medium at the 90 days, but became green gradually on the medium consisting of 2 mg L^{-1} BA + 0.1 mg L^{-1} NAA + 100 ml L^{-1} CM.

Effect of additives on shoot differentiation

Additives remarkably promoted the brown frequency which was particularly related with seed inoculation time. The protocorms with 30 days inoculation reached a brown frequency over 90% in the media supplemented with additives and all became brown and died in LH. However, protocorms with 60 days inoculation did not display such a high brown frequency. Among all the additives, CM gave rise to the lowest brown frequency (49.92 ± 1.32%) and possessed the highest differentiation frequency (50.08 ± 1.32%). Almost all the protocorms without browning generated shoots (Table 4). In addition, additives promoted vigorous seedlings such as green and large-area leaves.

Effect of GA_3 on the protocorms browning, differentiation and multiplication

The array to test GA_3 effect on shoot differentiation was

Figure 2. Micropropagation and *in vitro* flowering of *D. wangliangii.* (a) PLBs multiplication; (b) Adventitious buds; (c) The inflorescence emerged; (d) The flower without full development; (e) The flower only with the labellum fully developing; (f) The flower formed; (g) The wild flower; (h) Roots produced. Fl: flower bud; In: inflorescence: Po: pollinia. Scale bar = 1 cm.

conducted, as it could promote the stem elongation. Differentiation frequency is enhanced despite the presence of browning (Table 5). It is clear that GA_3 increased the differentiation frequency and at the dose of 0.1 and 1 mg L^{-1} but lowered at 2 mg L^{-1}, while obviously promoting the number of shoots at the concentration of 1 and 2 mg L^{-1}. With the synthetic action of 1 mg L^{-1} GA_3, 2 mg L^{-1} BA + 0.1 mg L^{-1} NAA, and 100 ml L^{-1} CM, the highest buds (7.32 ± 0.17) per protocorm was achieved.

Effect of hormones on adventitious multiplication

2 mg L^{-1} BA + 0.1 mg L^{-1} NAA + 100 ml L^{-1} CM was efficient on adventitious bud multiplication (Figure 2b), as high as 7.71 ± 0.42 (Table 6). The combination also produced sturdy seedlings with healthy green leaves and buds, while recovering 90% albino buds to the green ones.

The adventitious bud multiplication frequency reached 7.63 ± 0.60 on the half MS supplemented with 1 mg L^{-1} BA + 1 mg L^{-1} NAA generating vigorous shoots. In C10, the plantlets consisted of some with vitrification, and PLBs were also observed. Seedlings with robust growth were obtained on the medium half MS + 4% potato mash + 0.5 mg L^{-1} NAA. Half MS represented a lower effect of multiplication frequency and yellow-white, slender seedlings.

Effect of hormones on inflorescences induction

2 mg L^{-1} TDZ was effective on inflorescence induction, up to 100% (Table 6 and Figure 2c). 0.5 mg L^{-1} TDZ and 1 mg L^{-1} BA showed a high frequency, 85 ± 8 and 81 ± 10%, respectively. As for the pretreatment with additives or GA_3, 10% potato mash had the highest inflorescence induction with 71%. However, half MS and C10 failed to induce an inflorescence. The inflorescence of *D. wangliangii* is one-flowered, borne from the upper nodes of older leafless stems in the wild. The flower is red–lilac, lip white, red–lilac distally, disc with two greenish yellow patches on either side, column and anther-cap white (Figure 2g). However, the inflorescences of the *in vitro* seedling were generated from the apical bud. The treatment with 0.3 mg L^{-1} PP333 + 0.5 mg L^{-1} NAA produced usually 2 flowers per inflorescence while 2 to 8 flowers per inflorescence for 2 mg L^{-1} TDZ was observed. However, TDZ showed bad effects, such as the harm to subsequent flower development, morphological variation and short duration of the flower that withered prior to fully blooming. Among all the treatments, the labellum, sepal, and pollinia of *D. wangliangii* flower with a diameter of 15 mm developed normally in the medium amended with 0.3 mg L^{-1} PP333 + 0.5 mg L^{-1} NAA; however, the color was somewhat different from the wild morphotype (Figure 2f). Other treatments showed large range of variations such as small volume flowers with the diameter of 1 to 2 mm (Figure 2d) or the flowers only with labellum fully developed (Figure 2e). Furthermore, we found the continuous inflorescence generated during the subculture with the 0.3 mg L^{-1} PP333 + 0.5 mg L^{-1} NAA, but the inflorescence did not emerge in the subculture on the medium with C10.

Rooting and acclimation

Roots developed well on the half MS supplemented with 0.5 mg L^{-1} NAA (Figure 2h). Keeping with the careful maintenance, most of the plantlets acclimatized successfully, with an average survival rate of 80%.

DISCUSSION

Plants are the basis of sophisticated traditional medicine approaches that have been used for thousands of years and continue to provide mankind with new remedies and many other pharmaceutical products (Nalawade and Tsay, 2004; Teixeiraa et al., 2011) With the increase in the demand for the crude drugs, the plants are being

Table 3. Effect of hormones on protocorm size, browning, albino and differentiation.

Hormones (mg L^{-1})					Diameter (mm)	Percentage of browning	Percentage of albino	Percentage of differentiation
TDZ	BA	2-ip	ZT	NAA				
0.01	-	-	-	0.1	2.2	10.3	59.22	9.20
0.05	-	-	-	-	0.80	0	78.51	3.74
0.05	-	-	-	0.1	1.1	0	33.64	0.86
0.1	-	-	-	0.1	2.0	0	49.36	8.33
-	1	-	-	0.1	1.6	0	76.19	16.40
-	2	-	-	-	1.9	0	77.78	16.96
-	2	-	-	0.1	1.7	0	50.70	28.79
-	3	-	-	0.1	2.0	0	87.03	17.83
-	-	1	-	0.1	1.2	0	73.45	5.56
-	-	2	-		1.8	0	81.71	24.00
-	-	2	-	0.1	1.5	0	77.59	13.79
-	-	3	-	0.1	1.6	6.35	71.96	12.17
-	-	-	0.1	0.1	1.1	0	69.00	10.00
-	-	-	0.5		1.8	0	68.71	22.05
-	-	-	0.5	0.1	1.2	0	80.81	5.05
-	-	-	1	0.1	1.5	0	28.28	14.48
-	1	1	-	0.1	1.5	0	79.27	7.41
0.05	1	-	-	0.1	1.1	0	66.67	9.88
-	1	-	0.5	0.1	1.9	0	19.63	16.56
0.01	1	1	0.1	0.1	1.3	0	50.00	10.00
-	-	-	-	0.5	2.3	6.38	70.21	10.64
-	0.1	-	-	0.5	1.2	9.68	58.06	3.23
-	2	-	-	1	1.8	9.43	39.62	25.08

Values followed by the same letter were not significantly different at $p < 0.01$ by the SAS test. SD: standard deviation.

Table 4. Effect of additives on lethality and shoot differentiation.

Additives	Percentage of lethality ± SD	Percentage of differentiation ± SD
100 g L^{-1} potato	$62.15 \pm 1.49^{d*}$	36.85 ± 1.32^{b}
100 g L^{-1} carrot	66.45 ± 0.93^{c}	31.56 ± 1.58^{c}
100 ml L^{-1} CM	49.92 ± 1.32^{e}	50.08 ± 1.32^{a}
100 g L^{-1} tomato	72.20 ± 0.33^{b}	19.53 ± 1.20^{d}
4 g L^{-1} LH	90.46 ± 1.58^{a}	5.34 ± 1.15^{e}

Values followed by the same letter were not significantly different at $p < 0.01$ by the SAS test. SD: standard deviation.

Table 5. Effect of GA_3 on the protocorms browning, differentiation, and multiplication.

GA_3 (mg L^{-1})	Percentage of browning ± SD	Percentage of differentiation ± SD	No. of buds per protocorm ± SD
0	45.50 ± 0.82^{b}	51.50 ± 1.30^{b}	4.64 ± 0.05^{c}
0.1	42.32 ± 0.44^{c}	55.64 ± 0.65^{a}	5.64 ± 0.13^{b}
1	44.91 ± 0.50^{b}	54.68 ± 0.12^{a}	7.32 ± 0.17^{a}
2	51.78 ± 0.62^{a}	46.20 ± 0.63^{c}	7.15 ± 0.19^{a}

Values followed by the same letter were not significantly different at $p < 0.01$ by the SAS test. SD: standard deviation.

overexploited, threatening the survival of many rare species. Plant *in vitro* regeneration is a biotechnological tool that offers a potential solution to this problem (Afolayan and Adebola, 2004; Nalawade and Tsay, 2004). The use of plant cell and tissue culture methodology as a means of producing medicinal metabolites has a long

Table 6. Effect of hormones on adventitous bud differention and inflorescences induction.

Treatments (mg L^{-1})	Percentage of multiplication ± SD	Percentage of inflorescence induction ± SD
2 BA + 0.1 NAA + 100 ml L^{-1} CM	7.71 ± 0.42^{a}	-
0.5T DZ	4.83 ± 0.95^{cd}	85 ± 8^{ab}
1 TDZ	5.83 ± 0.79^{bc}	57 ± 1.8^{c}
2 TDZ	6.17 ± 0.60^{abc}	100^{a}
0.5 BA	6.13 ±1.03^{abc}	66^{d}
1 BA	4.50 ± 0.56^{cd}	81 ± 10^{abc}
2 BA	5.57 ± 1.09^{bcd}	7 ± 7^{d}
0.5 BA + 0.5 NAA	5.40 ± 1.17^{cd}	7 ± 7^{d}
1 BA + 1 NAA	7.63 ± 0.60^{ab}	10 ± 10^{d}
40 ml L^{-1} potato mash + 0.5 NAA	6.00 ± 0.73^{abc}	-
0.3 PP333 + 0.5 NAA	-	15 ± 6^{d}
C10	6.00 ± 0.58^{abc}	0
1/2MS	3.57 ± 0.57^{d}	0
100 g L^{-1} carrot/C10	-	27 ± 13^{d}
100 g L^{-1} potato/C10	-	71 ± 16^{bc}
100 g L^{-1} tomato/C10	-	0
1 GA_3/C10	-	19 ± 11^{d}
100 g L^{-1} banana/C10	-	6 ± 6^{d}

Values followed by the same letter were not significantly different at $p < 0.01$ by the SAS test. –; data not collected. SD: standard deviation

history (Rout et al., 2000; Verpoorte et al., 2002) and are currently being used in many medicinally valuable secondary metabolites production (Rout et al., 2000;Verpoorte et al., 2002; Guo et al., 2007; Matkowski, 2008; Shinde et al., 2010). As for the genetic stability of medicinal plants in tissue culture, Sliwinska and Thiem (2007) investigated the DNA content by flow cytometry for the seedlings and *in vitro* clones of six species which produce secondary metabolites, mainly phenolic compounds, with the exception of *Pueraria lobata*, there was no difference in genome sizes between seedlings and *in vitro* cultured plants from any species, indicative that they maintained their genetic stability during *in vitro* culture. This confirmed the usefulness of tissue culture for production of certified plant material to obtain herbal medicines.

A number of chemical compounds produced in wild plants are synthesized and accumulated in cultured plant cells, calli, tissues or shoot cultures (Matkowski, 2008; Kuźma et al., 2009; Shinde et al., 2010; Teixeiraa et al., 2011). In some cases, full development in natural conditions is useful for producing a considerable amount of secondary products, especially if the undifferentiated cell culture is either unable to or barely accumulating antioxidant compounds. Interestingly, two species used medicinally due to their anticancer alkaloids, Catharanthus roseus and Camptotheca acuminata, produce significant amounts of anthocyanins of over 200 µg/g fresh weight in cell cultures (Filippini et al., 2003; Pasqua et al., 2005). Producing the active secondary metabolites or not, and the content in tissue culture derivatives depended on various of factors such as medium styles, hormones or culture systems (Palacio et al., 2008; Savio et al., 2011). In general, shoot cultures are useful to produce medical secondary metabolites (Kuźma et al., 2009), ellagic acids present in shoot cultures was 3 times higher than in *Rubus chamaemorus* plants (Thiem and Krawczyk, 2003), and the abietane diterpene antioxidants (carnosol and carnosic acid) were present only in shoot cultures in *Salvia officinalis* (Grzegorczyk et al., 2007) and rosemary (Caruso et al., 2000) *in vitro* cultures. In this report, we also used buds as the material to propagate *D. wangliangii*. For *Habenaria edgeworthii*, a rare Himalayan medicinal orchid, its medicinal properties are possibly due to production of secondary metabolites, including phenolic compounds and an *in vitro* method was developed for the production of phenolic compounds (Giri et al., 2012), revealing its medical properties which are persistent through *in vitro* cultures. Although, the active substances of *D. wangliangii* were not detected due to, that the plant was just reported 4 years ago, it was sure that the plant could produce its medical substances and should keep its medical properties on the basis of above evidences.

Polyembryony is a typical representative of Orchidaceae, which has attracted attention from botanists and horticulturalists in the Orchidaceae for over 100 years. Polyembryony in orchids is observed in 31 genera (Yam et al., 2002). The genus with the most species with polyembryony is *Habenaria* Willd, which includes 8 species (Yam et al., 2002). Polyembryony, however, in *D. wangliangii* with approximately 1,100 species, has never

been observed. With this first report of polyembryony in *D. wangliangii,* polyembryony follows two patterns of development: sexual and asexual processes taken place side by side in one seed; and cloning of either maternal or daughter organism generations (Batygina et al., 2003). The formation of polyembryony in orchids is considerably complicated. Efforts should focus on the origin of polyembryony for *D. wangliangii.* Ganeshaiah et al. (1991) indicated that polyembryony could serve as a counter-strategy by the mother against brood reduction driven by sibling rivalry. Seeds of orchids with polyembryony are genetically heterogeneous (Batygina et al., 2003). The selected types can be fixed by asexual reproduction processes common to the species, and then the plantlets from those seeds could be more flexible to the dynamic environment. Therefore, though there is rare occurrence of polyembryony in the angiosperms compared with gymnosperms, it is hard to define if polyembryony is inferior or superior to angiosperm evolution. Can the polyembryony in *D. wangliangii* be an adaptive characteristic for its habitat, a climate within a xerothermic valley? The presence of polyembryony in orchids has been mentioned in tropical species (Batygina et al., 2003), however, this feature does occur rarely in species growing in the temperate zone (Bragina, 2001). *D. wangliangii* is distributed in neither the temperate or tropical zones but in the high elevation, subtropical areas. Though, the distribution of polyembryony in orchids may correlate with diversity of orchids in the tropics, results show that the polyembryony of orchids is widespread in a broad area and not only limited in tropical zones.

We have collected nearly 300 orchids species, including 42 *Dendrobium* (over one half of Chinese wild *Dendrobium*), that are deposited in the Southwest China Germplasm Bank of Wild Species located at the Kunming Institute of Botany, Chinese Academy of Sciences. Nearly all the *in vitro* materials of *Dendrobium* are originated from green capsules. According to our experiences, N1 medium is very efficient in seed germination of *Dendrobiums*. High germination over 90% is obtained, therefore, other factors, such as hormone additives, are not involved in the experimental design of germination rates and frequencies.

Establishment of protocols for *in vitro* culture of orchid seeds is species-specific and depends on several factors, such as capsule maturity, components of culture media, light, and temperature conditions (Wei et al., 2010). Salenda et al. (1976) published optimal harvest times for selected orchids and hybrids of many genera. In general terms, immature seeds are removed from pods, which have progressed approximately from 1/2 to 2/3 in their development from pollination to maturity. Zeng and Cheng (1996) reported promising results by culturing 120 day old capsules of *Dendrobium* but differed greatly among species: *Dendrobium candidum* Wall. ex Lindl.120 days (Shiau et al., 2005); *Dendrobium* Chao Praya Smile (a hybrid) 120 days (Hee et al., 2007); *Dendrobium tosaense* Makino 84 days (Lo et al., 2004); and *Dendrobium fimbriatum* Hook. 70 days (Sharma et al., 2005). It was believed that fully mature orchid seeds were difficult to germinate via asymbiotic germination (Lee et al., 2006). Vasudevan and van Staden (2010) reported that, seeds harvested 129 days after pollination showed a substantially greater germination on account of the presence of gaps created by cellular degeneration in the inner seed integument, highly discontinuous cuticle layer present in the inner integument, and absence of cutinisation at the suspensor region of the embryo, thus, enabling the embryo to access water and nutrition. Long et al. (2010) demonstrated that the germination frequency of *Paphiopedilum villosum* var. *densissimum* (Z. J. Liu and S. C. Chen) Z. J. Liu and X. Q. Chen was the highest when seed coat formation started about 200 days after pollination. Embryos with less than 200 days after pollination were seemingly too underdeveloped to absorb nutrients and turned brown soon after subculture. While the frequency of germination for 300 days after pollination was lower than that of 200 days after pollination and after the seed coat had already formed, possibly attenuating nutrient uptake. Nonetheless, *Dendrobium draconis* Rchb. f. achieved an efficient regeneration with fully mature seeds (Rangsayatorn et al., 2009). As the case in *D. wangliangii*, only the mature or near-mature seeds were able to germinate but not for the green seeds, suggesting the optimal harvest time for germination is not necessary during 1/2 to 2/3 maturity. Particularly for some *Dendrobium,* a higher germination rate is achieved only through mature seeds, indicating that seed coat or cuticle layer may not be highly relative to the germination capability.

The role of the photoperiod in germination of orchid seeds was reported to vary for different conditions and species. Previously, it has been documented that in many terrestrial species, light inhibits germination (Takahashi et al., 2000). Continuous dark or light conditions were inhibitory for immature seed germination in *Dendrobium fimbriatum,* and darkness prior to light condition was necessary (Sharma et al., 2005). However, continuous light favored the germination of *D. candidum* (Luo et al. 2006). In this experiment, light was necessary for *D. wangliangii*; otherwise, it would fail to germination in persistent darkness. The darkness inhibiting seed germination in other epiphyte orchids was reported previously (Luo et al., 2006). *D. wangliangii* is generally distributed in the sparse *Quercus* (Fagaceae) hurst near the mountaintop with an abundant supply of light, so the dependence on light may reflect the adaption to the special habitat to some extent. The light requirements in germination of *Laelia speciosa* (Kunth) Schltr. also might show an adaption to habitat (Ávila-Díaz et al., 2009).

Albino, despite of protocorms or seedlings, was everywhere in *D. wangliangii* culture that turned brown and eventually died when subcultured. CM was considerately efficient to deal with the albino, with the

albino protocorms or buds turning green progressively. For *D. huoshanense*, albinos could be obviously prevented with potato extract (Wen et al., 1999). The reason why albinos persist is complicated, involving diverse factors. Albinos emerged in each media and green pod in *D. wangliangii* but not in some *Dendrobium*, suggesting that it probably is in relation with genotype. Albino phenotype was severely numerous in medium with high dose ZT or KT in *D. huosanase* protocorms (Wen et al., 1999). Lo et al. (2004) associated the albino with capsule maturity and the style of medium in *D. tosaense*. High ratio of cytokinin(s) to auxin(s) favored adventitious shoots origination and differentiation (Sriskandarajah et al., 2006). Furthermore, the type and dose of cytokinin varies depending on the species of *Dendrobium* (Luo et al., 2009). Our study revealed that *D. wangliangii* protocorms differentiated better with 2 mg L^{-1}BA + 0.1mg L^{-1}NAA. Nasiruddin et al. (2003) also reported that BA was most effective in PLBs differentiation of *Dendrobium formosum,* but showed a negative effect on PLBs differentiation of *Dendrobium nobile.* Despite necessary buds producing with this experiment's hormone combinations, differentiation rate was very low, 28.79%. Shoot differentiation from PLBs was observed within 120 days after subculture to half MS, but the frequency was not over 50%. Therefore, other methods should be explored to promote bud production in *D. wangliangii*. For example, *D. huoshanense* PLBs increased buds generation with 10 g L^{-1} maltose (Luo et al., 2009), and the differentiation rate of *D. huoshanense* and *D. nobile* was improved with the treatment of polyamines (Wei et al., 2010).

Clump bud generation is an important approach in orchid micro-multiplication, controlled by the cytokinin to auxin ratio, especially the cytokinin type and concentration. BA alone or in combination with other hormones is widely used in adventitious buds multiplication, and BA with NAA is most popular in *Dendrobium* for shoot induction (Nasiruddin et al., 2003; Shiau et al., 2005; Luo et al., 2006). The present experiment confirmed that, BA displayed a positive effect on *D. wangliangii* shoot induction when combined with 0.1 mgL^{-1} NAA + 100 ml L^{-1} CM or with 1 mg L^{-1} NAA. With CM enabling albino buds to turn green and stimulated more buds generating, we found the highest multiplication frequency (Table 6). BA in combination with CM promoting the proliferation rate was also reported in *Paphiopedilum* Pfitzer hybrids (Huang et al., 2001) and *Vanilla planifolia* Andrews (Kalimuthu et al., 2006). TDZ was also proved to perform well on shoot produced in some *Dendrobium*, such as *Dendrobium aphyllum* (Roxb.) C.E.C. Fisch., *Dendrobium moschatum* (Buch.-Ham.) Sw. (Nayak et al., 1997), and *Dendrobium* Second Love (Ferreira et al., 2006). As for *D. wangliangii*, TDZ and BA showed no significant difference in their highest shoot induction. TDZ is more powerful than BA in adventitious buds induction. Surprisingly, TDZ and BA performed best with the doses of 2 and 0.5 mgL^{-1}, respectively. In summary, the performance of hormones probably depends on the species, but does not necessarily relate with the concentration effect of the hormone itself.

In vitro flowering of *Dendrobium* has been a research focus based on the application of cross-breeding and investigation on molecular mechanism of flowering. Conventional orchid breeding is time consuming because the entire breeding cycle could take at least three to five years. *In vitro* flowering protocols could shorten the time to 5 or 6 months (Hee et al., 2007; Sim et al., 2007; Wang et al., 2009), which could be used to get an earlier assessment of certain desired characteristics of the flowers, such as identity of color, shape and size (Sim et al., 2007). Once the desired characteristics are selected, the clone could be mass propagated through tissue culture.

Flowering is a unique developmental event in plants which involves the transition of vegetative shoot apex to form either an inflorescence or a floral meristem, followed by initiation and subsequent maturation of the floral organs. Most successful *in vitro* flowering in *Dendrobium* was obtained with green pods (Wang et al., 1993; Wang et al., 1997; Sim et al., 2007), except in *Dendrobium aurantiacum* var. *denneanum* (Kerr) Z. H. Tsi with flowers generated from stems of wild plants (Guan and Shi, 2009).

In vitro flowering of *Dendrobium* is influenced by various hormones. BA plays an essential role in inducing inflorescence for *Dendrobium* (Hee et al., 2007; Sim et al., 2007; Tee et al., 2008; Wang et al., 2009). Plantlets grown in BA-free medium did not produce an inflorescence (Hee et al., 2007; Wang et al., 2009). In some cases, BA alone was effective for floral induction (Wang et al., 1995; Sim et al., 2007; Tee et al., 2008). Furthermore, BA played a more powerful function in flowering and in combination with other substances (Wang et al., 1997; Sim et al., 2007). Wang et al. (1997) indicated that auxin alone suppressed *D. candidum* flower formation, and Goh and Yang (1978) also demonstrated that IAA could suppress the promotive effect of BA on flowering in two *Dendrobium* hybrids. *Dendrobium denneanum* even showed no flower when supplemented BA with NAA (Guan and Shi, 2009). The same phenomenon was also observed in *D. wangliangii* that BA combined with NAA would sharply reduce the flowering frequency. Therefore, it is noted that auxin probably will lag the flowering induction frequency depending on the *Dendrobium* species. TDZ was also used for *in vitro* flowering induction in *Dendrobium*. It showed more effective than BA and PP333 at 0.1 mg L^{-1}, but as the dose increased to 0.5 or 1 mg L^{-1}, the frequency sharply decreased instead (Wang et al., 2009). On the contrary, TDZ induced the maximum flowering frequency (100%) of *D. wangliangii* at 2 mg L^{-1}, indicating that the effect of TDZ varied, resting with the dose and

the *Dendrobium* species. Growth retardant such as PP333 was also shown to promote *in vitro* flowering in *Dendrobium* (Wang et al., 2006, 2009). Duan and Yazawa (1994) held that banana, tomato, and CM were not effective for *Dendrobium in vitro* flowering as no higher flowering frequency emerged when supplied with those extracts. However, in *Dendrobium* Madame Thong-In, CM was necessary for the transition of vegetative shoot apical meristem to inflorescence meristem (Sim et al., 2007). Nadgauda et al. (1990) suggested that cytokinins might be involved in flowering perhaps with CM supplying the inositol and cytokinin oxidase inhibitors, which promoted cytokinin responses. For *D. wangliangii*, except tomato pretreatment, other natural additives was revealed to promote the inflorescence induction to some extent, and potato showed the most effective function making inflorescence induction to 71%.

Moreover, the pretreatment, mainly the transfer from a media to other media, was greatly helpful in promoting *in vitro Dendrobium* flowering (Wang et al., 1995; Wang et al., 1997; Wang et al., 2006), even reaching 100% in *D. nobile* (Wang et al., 2009). High phosphorus (P) and low nitrogen (N) content formed a higher inflorescences number compared with the half-strength MS medium without any modification of the P/N ratio for *Dendrobium* Sonia 17 (Tee et al., 2008). Duan and Yazawa (1994) studied the *in vitro* flowering in *Doriella*, *Phalaenopsis* and *Dendrobium*, and found that high N in the medium decreased or discouraged the floral buds formation. Two-layer culture was an effective protocol creatively used in the latest years, especially for promoting development of flower buds for some tropical *Dendrobium* that were unable to produce an inflorescence when cultured on Gelrite-solidified medium (Hee et al., 2007; Sim et al., 2007). Selection of morphologically normal seedlings would ensure a higher percentage of inflorescence induction from 45 to 72% (Hee et al., 2007).

Some *in vitro Dendrobium* seedlings produced lesser and smaller flowers than field grown plants (Hee et al., 2007), while some produce more flowers (Wang et al., 2006). Although, *D. wangliangii* generated smaller flowers than field plants, it produced inflorescences with more than one flower in some treatments, especially with TDZ that only grew one flower per inflorescence compared to those in the wild, suggesting that TDZ altered the biological characteristics from a single flower to an inflorescence with more than 2 flowers first reported for *Dendrobium*. Different morphologies of *in vitro Dendrobium* flowers including abnormal flowers were observed (Wang et al., 1997; Wen et al., 1999; Hee et al., 2007; Sim et al., 2007; Wang et al., 2009). Those abnormal flowers displayed variations in structure, color or size. The phenomenon also appeared in the flowering of *D. wangliangii*, especially in the size and color. Color changed a lot compared with wild flowers, and the diameter ranged from 2 to 15 mm. The emergence of abnormal flowers was in relation with some factors. Flower development of *D. nobile* was deformed under 25°C but developed fully when grown in a lower temperature regime (23°C/18°C, light/dark) for 45 days(Wang et al., 2009). Higher dose TDZ increased the rate of abnormal flowers, but PP333 enhanced the normal flowers in *Dendrobium moniliforme* (Wang et al., 2006). This was true in the *D. wangliangii* when seedlings cultured in the medium with PP333 produced flowers similar to the wild flowers to the maximum extent, while the plantlets on the medium with TDZ just generated very small flowers with light-green color. The presence of putrescine and abscisic acid (ABA) caused morphological abnormalities as well (Wang et al., 1997). Various abnormalities of *in vitro* flower buds are formed, indicating that different conditions might be required for the initiation and development of flowers (Wang et al., 2006).

Conclusion

This was the first report on the polyembryony, micropropagation and *in vitro* flowering of *D. wangliangii*. Polyembryony was also first observed in *Dendrobium.* An effective method was found to rapidly propagate the critically endangered medicinal orchid with which cryopreservation of *D. wangliangii* could be realized successfully. Furthermore, *in vitro* flowering was also investigated which can provide acknowledgement with respect to breeding of *D. wangliangii* while enriching the understanding about how to induce *in vitro* flowering for *Dendrobium.* Based on the literatures in the paper, the medical properties of *D. wangliangii* will be retained during the process of *in vitro* culture.

ACKNOWLEDGEMENTS

The authors thank Mrs Chunfang Li for her kind help in the experiment. Jeffrey Boutain and Dr Bo Long have critically read and edited the manuscript. This work was supported by the Key Research Direction Project of Knowledge Innovation Program for Chinese Academy of Sciences (KSCX2-YW-Z-0925), the Yunnan Provincial Foundation of Natural Science (No. 312009CD113), Key Laboratory of Biodiversity Conservation in Southwest China, FSA (No. BC2010F10), and the Ministry of Education, China through its 985 and 111 projects (MUC 98506-01000101 and B08044).

ABBREVIATIONS

MS, a general medium created by Murashige and Skoog for plant tissue culture; **BA,** 6-benzylaminopurine; **NAA**, naphthaleneacetic acid; **CM,** coconut milk; **TDZ,** 1-phenyl-3-(1, 2, 3-thiadiazol-5-yl)-urea; **PP333,** paclobutrazol; **AC,** activated charcoal; **IAA,** indole-3-acetic acid; **2ip,** N^6-[2-Isopentenyl]-adenine; **ZT,** zeatin; **LH,** lactalbumin hydrolysate; **GA_3,** gibberellic

acid; **CCC,** chlormequat chloride.

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