Micropropagation of the Endangered Medicinal Orchid, *Dendrobium officinale*

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Abstract: The endangered medicinal orchid, *Dendrobium officinale*, has low reproductive capacity in its natural environment and its wild resources are exceedingly scarce. Domestication, including development of rapid propagation methods, is needed urgently to satisfy human demand for its medicinal products. This study developed optimized micropropagation methods for *D. officinale* by testing the effects of tissue culture media and additives on shoot proliferation and rooting. The optimal proliferation medium for *D. officinale* was ½ Murashige and Skoog (MS) with 2.0 mg L^{-1} benzyladenine (BA) + 0.1 mg L^{-1} naphthaleneacetic acid (NAA) + 100 g L^{-1} potato extract. A rooting medium composed of ½ MS + 0.2 mg L^{-1} BA + 1.0 mg L^{-1} NAA provided 100% rooting. These methods allow reliable mass-production of *D. officinale* for medicinal purposes.

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1. Introduction

Dendrobium officinale (black knotweed or Yunnan dendrobium) is a perennial epiphytic herbaceous plant that has been harvested extensively from the wild for medicinal purposes (Li *et al*., 2008). *D. officinale* has an important role in providing anti-neoplastic, anti-aging, enhanced-immunity and vasodilator functions (Liu *et al*., 2011; Hou *et al*., 2012; Xia *et al*., 2012; Xiang *et al*., 2013). Long-term human collection and logging of its natural habitat are gradually exhausting its wild resources, and the species has become an endangered plant in China (Li *et al*., 2008; Hou *et al*., 2012). Regeneration by seed is low in natural conditions, and it has a slow rate of natural clonal propagation by bud separation. Domestication of *D. officinale* is needed urgently to satisfy the market demand for medicine and to reduce the pressures on natural populations from wild harvesting.

Previous attempts to propagate *D. officinale* through tissue culture have provided low proliferation rate, low rooting frequency, poor plantlet quality and high cost. Full-strength Murashige and Skoog (MS) media are suitable for propagation of *D. candidum* (Zhou *et al*., 1999). Half-strength MS medium containing 0.5 mg L^{-1} naphthaleneacetic acid (NAA) and 0.5% activated charcoal (AC) has been used for protocorm propagation of *D. officinale*, and the addition of banana or apple extracts accelerates seedling root formation and growth (Lin *et al*., 2003). B5 medium with coconut milk has been used for shoot and protocorm propagation and rooting of *D. officinale* (Zhou *et al*., 2005). Optimal media in another study were full-strength MS for multiplication and ½ MS for rooting (Wang and Gai, 2006). Hyponex No. 3 has been used as a multiplication medium, with ½ MS with 0.5 g L^{-1} indole-3-butyric acid (IBA) and 2 g L^{-1} AC used as a rooting medium (Wang *et al*., 2007). Full-strength MS medium containing 0.5 mg L^{-1} benzyladenine (BA), 0.1 mg L^{-1} NAA and 10% potato extract has provided shoot proliferation of *D. officinale* (Zheng *et al*., 2008), and N6 has been adapted as a basal medium to induce callus (Wang *et al*., 2008). Half-strength MS medium supplemented with 1.5 mg L^{-1} BA, 0.2 mg L^{-1} NAA and 10% coconut milk is also suitable for propagation of *D. officinale* (Liu *et al*., 2012).

Recently, the use of banana extract as an additive has improved propagation and growth of *D. officinale* (Su *et al*., 2012), and the use of banana extract or potato extract has increased the proliferation rate and rooting frequency of *D. candidum* (Liu, 2012). No unified medium and additive formulations were reported in these studies.

In this study, we tested different basal media, additives and hormone concentrations for proliferation and rooting of *D. officinale*. Our objective was to improve the multiplication rate, shoot vigour and rooting frequency to provide a reliable technique for mass propagation.

2. Material and Methods

Seeds from *D. officinale* capsules were used as explants. Intact capsules were immersed in 70% ethanol for 1 min, rinsed in sterilized water,

transferred to 0.1% $HgCl_2$ for 12 min, and then rinsed in sterilized water four times. The capsules were carefully dissected and the seeds were transferred onto shoot initiation medium. The shoot initiation medium comprised hormone-free MS with 30 g L^{-1} sugar and 4 g L^{-1} agar (produced in Quanzhou, Fujian, China), pH 5.8. The seeds were incubated in the dark or under weak light, and they were transferred onto fresh initiation medium every 30 d for three passages.

The multiplication media comprised: (1) MS + 2.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA; (2) N6 + 2.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA; or (3) ½ MS + 2.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA, each containing 30 g L^{-1} sugar and 5.5 g L^{-1} carrageenan (produced in Quanzhou, Fujian, China), pH 5.8. These were supplemented with or without 0.5 g L^{-1} AC. In addition, a range of additives were tested in multiplication medium (3) without AC: (a) 3.0 g L^{-1} peptone; (b) 100 g L^{-1} apple juice (peel removed); (c) 100 g L^{-1} banana extract (skin removed); or (d) 25, 50 or 100 g L^{-1} potato extract (peel removed). The shoots were subcultured every 60 d for three passages. There were 30 jars of each medium. The shoots were maintained under 20–30 µmol m^{-2} s^{-1} irradiance (12 h d^{-1}) (Chen, 2009, 2012) with a room temperature of 26 ± 2°C. Shoot number and average shoot length were measured after each passage, and shoot vigour was observed macroscopically. Shoot multiplication rate was calculated as the average coefficient of multiplication (Sánchez and Vieitez, 1991; Hung and Trueman, 2011) per passage across the three passages.

Shoots of 1.5-cm length were then transferred to one of three rooting media: (1) MS; (2) ½ MS; or (3) ¼ MS, each supplemented with 0.2 mg L^{-1} BA + 1.0 mg L^{-1} NAA, and containing 15 g L^{-1} sugar and 5.5 g L^{-1} carrageenan, pH 5.8. The shoots were subcultured every 80 d for three passages. There were 30 jars of each medium. The shoots were maintained under 20–30 µmol m^{-2} s^{-1} irradiance (12 h d^{-1}) with a room temperature of 26 ± 2°C. Rooting percentage, root number, root length and plantlet height were recorded at the end of the three passages in rooting medium. Plantlets were transplanted into pots containing peat moss and then transferred under 40–70 µmol m^{-2} s^{-1} irradiance for a 10-day hardening period in a glasshouse with natural light.

Data were analysed by analysis of variance (ANOVA) (for 3–6 means) or t-test (for 2 means), with a post-hoc Tukey's test if the ANOVA was significant. Means are provided with standard errors, and means were considered significantly different at $P < 0.05$.

3. Results

Shoot multiplication rate was highest (6.7 ± 0.2 shoots per passage) in full-strength MS medium devoid of AC (Table 1). Shoot multiplication rate did not differ between N6 and ½ MS media in either the presence or absence of AC, but the addition of AC always reduced the multiplication rate. However, continuous culture in full-strength MS medium devoid of AC caused slight yellowing of the lower leaves and limited shoot elongation (2.5 ± 0.1 cm). Shoot length and vigour were greatest in ½ MS medium containing AC (5.4 ± 0.1 cm; green, vigorous shoots) but the shoot multiplication rate was low (2.9 ± 0.3 shoots per passage) in this medium. Therefore, ½ MS devoid of AC was selected as the basal medium for subsequent shoot proliferation, as this medium provided an effective combination of high multiplication rate (5.8 ± 0.3 shoots per passage), high shoot length (3.8 ± 0.3 cm) and green, vigorous shoots (Table 1).

The addition of peptone to ½ MS medium decreased shoot multiplication rate, length and vigour (Table 2). Addition of 100 g L^{-1} potato extract increased multiplication rate from 5.0 ± 0.2 to 6.0 ± 0.2 shoots per passage and increased shoot length from 0.6 ± 0.2 cm to 3.4 ± 0.2 cm (Table 3). Apple juice and banana extract did not affect multiplication rate significantly but they increased shoot length, albeit less than the potato extract (Table 3). Lowering the concentration of potato extract from 100 g L^{-1} (Figure 1) to 25 g L^{-1} did not affect shoot multiplication rate significantly, but it reduced shoot length from 2.5 ± 0.1 cm to 1.8 ± 0.3 cm and it resulted in smaller, yellow leaves (Table 4).

Figure 1. Proliferation of *Dendrobium officinale* shoots in ½ MS medium containing 2.0 mg L^{-1} benzyladenine (BA), 0.1 mg L^{-1} naphthaleneacetic acid (NAA) and 100 g L^{-1} potato extract

Table 1. Effect of basal medium and activated charcoal on proliferation, length and vigour of *D. officinale* shoots

Medium	BA (mg L^{-1})	NAA (mg L^{-1})	AC (g L^{-1})	Multiplication rate	Shoot length (cm)	Vigour
MS	2.0	0.1	0	6.7 ± 0.2a	2.5 ± 0.1c	Slight yellowing of lower leaves. Slow shoot growth. ++
N6	2.0	0.1	0	5.6 ± 0.2b	0.8 ± 0.1d	Serious yellowing of lower leaves. Slow shoot growth. +
1/2MS	2.0	0.1	0	5.8 ± 0.3b	3.8 ± 0.3b	All leaves green. Vigorous shoots. +++
MS	2.0	0.1	0.5	3.4 ± 0.1c	3.6 ± 0.2b	Slight yellowing of lower leaves. Slow shoot growth. ++
N6	2.0	0.1	0.5	2.8 ± 0.1d	1.2 ± 0.2d	Serious yellowing of lower leaves. Slow shoot growth. +
1/2MS	2.0	0.1	0.5	2.9 ± 0.3cd	5.4 ± 0.1a	All leaves green. Vigorous shoots. +++

BA: benzyladenine; NAA: naphthaleneacetic acid; AC: activated charcoal. Means (± SE) with different letters within a column are significantly different (ANOVA and Tukey's test; $P < 0.05$; n = 30 jars). '+++': good growth; '++': intermediate growth; '+': poor growth

Table 2. Effect of peptone on proliferation, length and vigour of *D. officinale* shoots

Additive	Concentration (g L^{-1})	Multiplication rate	Shoot length (cm)	Vigour
Peptone	3.0	2.4 ± 0.1b	2.2 ± 0.1b	Slow growth of shoots. +
Control	0	3.0 ± 0.2a	3.1 ± 0.3a	Vigorous shoots. +++

Means (± SE) with different letters within a column are significantly different (t test; $P < 0.05$; n = 30 jars). '+++': good growth; '+': poor growth

Table 3. Effect of fruit extracts on proliferation, length and vigour of *D. officinale* shoots

Additive	Concentration (g L^{-1})	Multiplication rate	Shoot length (cm)	Vigour
Potato	100	6.0 ± 0.2a	3.4 ± 0.2a	Tall vigorous shoots; green leaves. ++++
Apple	100	4.5 ± 0.2b	1.4 ± 0.1c	Less-vigorous small shoots; small yellow leaves. ++
Banana	100	5.0 ± 0.3b	2.2 ± 0.1b	Shoots with good vigour; small yellow leaves. +++
Control	0	5.0 ± 0.2b	0.6 ± 0.2d	Small shoots; some shoots chlorotic; small yellow leaves. +

Means (± SE) with different letters within a column are significantly different (ANOVA and Tukey's test; $P < 0.05$; n = 30 jars). '++++': excellent growth; '+++': good growth; '++': intermediate growth; '+': poor growth

Table 4. Effects of potato extract concentration on proliferation, length and vigour of *D. officinale* shoots

Additive	Concentration (g L^{-1})	Multiplication rate	Shoot length (cm)	Vigour
Potato	25	5.8 ± 0.2a	1.8 ± 0.3b	Small shoots; small yellow leaves. +
Potato	50	6.0 ± 0.3a	2.1 ± 0.1ab	Intermediate shoots; small yellow leaves. ++
Potato	100	6.2 ± 0.3a	2.5 ± 0.1a	Strong shoots; green leaves. +++

Means (± SE) with different letters within a column are significantly different (ANOVA and Tukey's test; $P < 0.05$; n = 30 jars). '+++': good growth; '++': intermediate growth; '+': poor growth

Conversion to plantlets was high (92.0 ± 2.0% – 99.9 ± 0.1%) in all rooting media (Table 5). However, the optimal combination of highest rooting frequency (99.9 ± 0.1%), root number (4.3 ± 0.2), root length (3.2 ± 0.1 cm) and plantlet height (2.8 ± 0.2 cm) was obtained with the ½ MS medium. This was also the only medium that provided large, green leaves (Figure 2) rather than small, yellow leaves. The plantlets acclimatized readily to glasshouse conditions, with 90% survival.

Figure 2. Rooting of *Dendrobium officinale* shoots in ½ MS medium containing 0.2 mg L^{-1} benzyladenine (BA) and 1.0 mg L^{-1} naphthaleneacetic acid (NAA)

4. Discussion

The optimal medium for *D. officinale* propagation was ½ MS + 2.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA + 100 g L^{-1} potato extract, which provided a multiplication rate of 6.0 shoots per passage during passages of 60-d duration. Annual shoot production was, therefore, approximately 6.0^6= 46,656, which allows mass-propagation of *D. officinale* in a tissue culture facility. The shoot multiplication rate increases, but shoot size diminishes, during long-term production possibly due to cytokinin accumulation. Thus, BA and NAA concentrations could be adjusted during long-term propagation to maximize shoot production and quality.

The high inorganic salt and nitrogen concentrations of full-strength MS medium, while providing high shoot multiplication, reduced shoot elongation and caused yellowing of lower leaves. The high inorganic salt concentration and low ratio of nitrate to ammonium of N6 medium, also slowed shoot elongation and caused yellowing of lower leaves. Potato extract was much better than banana extract or apple juice as an additive to increase shoot multiplication and elongation in ½ MS medium, while peptone reduced multiplication and elongation. Potato extract has been used previously for micropropagation of *D. tosaense*, *D. moniliforme* and *D. officinale* (Lo *et al*., 2004; Su *et al*., 2012). The addition of 0.5 g L^{-1} activated charcoal to MS or ½ MS media produced longer shoots but it always reduced multiplication rate by approximately 50%. Similarly, activated charcoal increases shoot length but reduces shoot multiplication of *Cymbidium forrestii* and *Musa* sp. (Paek and Yeung, 1991; Costa *et al*., 2006) and it reduces bud induction in *Cattleya bicolor* (Prizão *et al*., 2012).

The use of ¼ MS as the basal medium also provided virtually 100% rooting but it reduced root number to 2.6 per plantlet, it caused leaf yellowing, and it reduced plantlet height. The use of full-strength MS medium led to a slightly reduced rooting rate of 92%, only 1.9 roots per plantlet, and leaf yellowing. Increasing root number from 1.9 to 4.3 may be critical for enhancing root system symmetry and improving plantlet growth because stability, survival and growth are sometimes limited by low numbers of adventitious roots (Haines *et al*., 1992; Goldfarb *et al*., 1998; Foster *et al*., 2000; Mokotedi *et al*., 2010).

Table 5. Effect of basal medium on rooting frequency, root number, root length and plantlet height of *D. officinale*

Medium	BA (mg L^{-1})	NAA (mg L^{-1})	Rooting (%)	Root number	Root length (cm)	Plantlet height (cm)	Vigour
MS	0.2	1.0	92.0 ± 2.0b	1.9 ± 0.1c	0.7 ± 0.2c	2.4 ± 0.3a	Most leaves yellow; minority of leaves large and elongating. +
1/2MS	0.2	1.0	99.9 ± 0.2a	4.3 ± 0.2a	3.2 ± 0.1a	2.8 ± 0.2a	Most leaves green; all leaves large and elongating. +++
1/4MS	0.2	1.0	99.9 ± 0.1a	2.6 ± 0.3b	1.6 ± 0.3b	1.8 ± 0.1b	Minority of leaves yellow; most leaves large and elongating. ++

Means (± SE) with different letters within a column are significantly different (ANOVA and Tukey's test; $P < 0.05$; n = 30 jars). '+++': good; '++': intermediate; '+': poor

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