

Full Length Research Paper

Establishment and optimization of high efficiency embryogenic callus induction system in *Dendrobium candidum*

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The wild resource of *Dendrobium candidum*, a well-known epiphytic orchid, is limited. Plant cell culture technology is a promising alternative for production of high-value secondary metabolites. In this research, factors affecting the induction, maintenance, and multiplication of callus from protocorm-like bodies (PLBs) of *D. candidum* were systematically studied, and a technical callus inducing system with 100% rate was established which higher than previously reported values. The result showed that 2,4-dichlorophenoxy acetic acid (2,4-D) and 1-naphthalene acetic acid (NAA) could be of benefit to the callus induction. The optimized medium for callus induction was MS medium containing 0.5 mg/L 2,4-D and 0.25 NAA or 0.5 mg/L kinetin (KT). The callus clump enrichment method was used for directional screening and cell adaptation on MS medium supplied with plant growth regulators (PGR) such as 5 mg/L NAA and 0.5 mg/L 6-benzyladenine (6-BA) or KT for 30 days per cycle. After 6 to 9 months, *D. candidum* cell lines were obtained and the histological analysis showed that the main differences between callus and PLBs were meristematic cell content and internal cell differentiation degree, and the meristematic cells distributed in the external strip were contributed to the formation of callus. At last, the amount of polysaccharides and total amino acids were compared between the obtained cell lines, tissue culture seedlings and PLBs. The results showed that polysaccharides was 24.67% in callus and higher than that in tissue culture seedlings and PLBs. The amount of total amino acids was 5.94% in callus and higher than that in tissue culture seedlings. So the callus is considered a good choice for the expanding of *D. candidum* medicine sources.

Key words: *Dendrobium candidum*, Callus induction, tissue culture, natural products, plant growth regulators.

INTRODUCTION

Dendrobium candidum Wall. ex Lindl is a species that belongs to the Orchid family and a well-known orchid and epiphytic herb in South and Southeast Asian countries,

which has been used as a Chinese traditional herb for over 1500 years (Leung, 2006). Modern pharmacological research has shown that *D. candidum* is an important

Table 1. Effects of single plant growth regulators (PGRs) on callus induction of *D. candidum*.

| Medium | PIC | 2,4-D | NAA | Callus induction rate (%) | Callus color | Callus status |
|--------|------|-------|------|---------------------------|--------------|---------------|
| M1 | 0.25 | | | 2.1±0.35 ^{d*} | Brown | Died |
| M2 | 0.5 | | | 9.4±0.85 ^d | Brown | Died |
| M3 | 1.0 | | | 0.9±0.28 ^d | Brown | Died |
| M4 | | 0.5 | | 92.5±3.5 ^{ab} | Yellow | Growth well |
| M5 | | 1.0 | | 100 ^a | Yellow | Growth well |
| M6 | | 1.5 | | 47.5±10.6 ^c | Brown | Died |
| M7 | | | 1.0 | 27.3±5.9 ^d | Brown | Died |
| M8 | | | 5.0 | 89.5±6.2 ^b | Yellow | Growth well |
| M9 | | | 10.0 | 83.2±8.1 ^b | Yellow | Growth well |

*All values are means ± SE (n = 3). Mean values in each column followed by the same letter are not significantly different (P < 0.05) by the one-way ANOVA analysis.

candidate for modern drug development because it enhances immunity, exhibits antitumor activity, and other regulatory roles (Li et al., 2009; Luo et al., 2010; Xiao et al., 2011; Xing, 2013; Bian et al., 2002). The production of *D. candidum* in Yunnan was estimated to be lower than 1000 kg in 1998, which cannot meet the market (Huang et al., 1996), the development of *D. candidum* cultivation, is therefore necessary.

Plant cell, tissue, and organ cultures, such as callus, embryo (protocorm-like bodies, PLBs), and tissue culture seedlings, are complementary systems used to enhance the beneficial bioactivities of *D. candidum* (Kolewe et al., 2008). *D. candidum* can be micropropagated using explants, such as PLBs, protocorms, immature seeds, and shoot tips (Paulsen, 2001; Chen and Wang, 2005; Su and Yang, 2006) and there are also some studies focused on the induction, proliferation, and liquid culture of PLBs (Men et al., 2003; Shi and Zhao, 2012; Jasim et al., 2015). There is recently study on the PLBs suspension cultures of *D. candidum* in balloon type bubble bioreactors for the production of biomass and bioactive compounds such as polysaccharides, coumarins, polyphenolics, flavonoids, vitamin C and vitamin E (Cui et al., 2014). Plant cell culture is also a promising alternative for production of high-value bioactive compounds (Rao and Ravishankar, 2002; Ramawat and Merillon, 2008; Rischer et al., 2013). And there are only some report on the callus induction, but it is easy to differentiation into protocorm or PLB (Roy and Banerjee, 2003; Chen and Chang, 2000; Zhao et al., 2008), and there is no research on callus maintain or scale-up process for the production of bioactive compounds from *D. candidum* suspension cultures. The present study established an effective method for embryogenic callus induction and maintain, then the

formation and characteristics of the callus were confirmed by anatomy analysis, at last, the amount of polysaccharides and total amino acids among different explants (tissue culture seedlings, callus, and PLBs) of *D. candidum* were compared to determine the most suitable methods for expanding *D. candidum* sources.

MATERIALS AND METHODS

Plant materials and cultivation

Shoot explants were obtained from wild *Dendrobium* plants in Yunnan province (which have been confirmed by a plant taxonomists named Xiang Jun in Huanggang normal university) were maintained on MS medium supplied with 1 mg/L thidiazuron (TDZ), 0.5 mg/L 1-naphthalene acetic acid (NAA), and 0.5 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) at 25°C in the dark.

Callus induction and cell line formation of *D. candidum*

PLBs were used as explants and MS medium as the basic culture medium for callus induction. The effects of picloram, 2,4-D, NAA, kinetin (KT), and 6-benzyladenine (6-BA) and their combinations on callus induction were determined (Tables 1 and 2). Induction was performed in the dark at 25°C. Twenty explants were inoculated for each experiment. The Callus induction rate and Browning intensity were evaluated after 20 day of culture.

D. candidum cell lines were obtained using the callus clump enrichment method

The well-conditioned buff callus briquette was minced with forceps and inoculated in the subculture medium 1 (SCM1). This process was repeated several times, with the first three to four multiplication cycles for 15 days and multiplication cycles after 3 months were gradually extended to 20 day.

The subculture medium was adjusted to subculture medium 2

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Table 2. Effects of plant growth regulators (PGRs) combinations on callus induction.

| Medium | 2,4-D | NAA | KT | 6BA | Callus formation time (days) | Callus induction rate (%) | Browning intensity | Growth state | Re-differentiation |
|--------|-------|------|------|------|------------------------------|---------------------------|--------------------|----------------|--------------------|
| MC1 | 0.5 | | 0.25 | | 9 | 100 ^{a*} | - | Good condition | + |
| MC2 | 0.5 | | 0.5 | | 10 | 100 ^a | - | Good condition | + |
| MC3 | 1.0 | | 0.25 | | 8 | 100 ^a | + | Grow well | ++ |
| MC4 | 1.0 | | 0.5 | | 7 | 100 ^a | + | Grow well | ++ |
| MC5 | | 5.0 | | 0.25 | 12 | 100 ^a | - | Grow well | ++ |
| MC6 | | 5.0 | | 0.5 | 12 | 100 ^a | - | Grow well | ++ |
| MC7 | | 10.0 | | 0.25 | 14 | 85.0±7.1 ^b | ++ | Grow slowly | +++ |
| MC8 | | 10.0 | | 0.5 | 14 | 87.5±3.5 ^b | ++ | Grow slowly | +++ |
| MC9 | | 5.0 | 0.25 | | 12 | 100 ^a | - | Grow well | ++ |
| MC10 | | 5.0 | 0.5 | | 12 | 100 ^a | - | Grow well | ++ |
| MC11 | | 10.0 | 0.25 | | 13 | 85±7.1 ^b | ++ | Grow slowly | +++ |
| MC12 | | 10.0 | 0.5 | | 13 | 87.5±10.6 ^b | ++ | Grow slowly | +++ |

The Callus induction rate and Browning intensity were checked in 20th day after the culturing, and growth state, re-differentiation were checked after 3 months culture. Re-differentiation: “+” means the re-differentiation rate is lower than 10%; “++” means the re-differentiation rate is around 30%, and “+++” means the re-differentiation rate is around 50%. Browning intensity: “-” means no browning; “+” means a little browning; “++” means a heaven browning. * All values are means ±SE (n = 3). Mean values in each column followed by the same letter are not significantly different (P < 0.05) by the one-way ANOVA analysis.

(SCM2), and the multiplication cycle was gradually extended from 20 to 30 day to adapt to the final consumption demand of *D. candidum* cell culture.

SCM1: MS + 0.5 mg/L 2,4-D + 0.5 mg/L KT + 1% agar+3% sucrose
SCM2: MS + 5 mg/L NAA + 0.5 mg/L 6-BA + 1% agar+3% sucrose.

Anatomical analysis

Anatomical analysis of PLBs and callus of *D. candidum* was conducted using paraffin sections according to the methods of Ennajeh et al. (2010) and Zhao et al. (2007). Photographs were obtained using a microscope (AH2, Olympus, Japan).

Polysaccharide extraction and analysis

Approximately 0.5 g of samples were collected, dried in an oven at 55°C for 20 h, and then powdered using a mortar. The polysaccharides was obtained by water extraction and ethanol precipitation, and analyzed by phenol-sulfuric acid according to the method of Tong et al. (2015). Anhydrous glucose was used as a standard in determining the total polysaccharides content. From anhydrous glucose stock concentration of 0.09 g /L, several dilutions were made to prepare a series of concentrations at 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. A standard curve (Y=0.0082X+0.0036.R²=0.9992) was constructed with the optical density at 490 nm against the concentrations of glucose. The total polysaccharides content of the samples were expressed in milligram of glucose equivalent per gram of samples.

Amino acid extraction and analysis

Approximately 0.25 g of samples were dried in an oven at 55°C, powdered, and then transferred to tapered plug bottles. About 20 mL of 0.1 mol/L HCl solution was added, and the mixture was

ultrasonicated at 30°C for 30 min .The mixture was suctioned, and the pH was adjusted to 7 neutral with 15% hydroxide sodium solution. The samples were then transferred to 100 mL volumetric flasks and adjusted to 100 mL by adding distilled water. Briefly, 4.0 mL of the extract was transferred to a 10 mL tube and added with 1.0 mL of sodium acetate buffer salt solution (pH 6.5) with 1.0 mL of 2% ninhydrin solution. The mixture was heated in a water bath at 100°C for 40 min and cooled for 15 min before adjusting to 50 mL by adding water. Aspartic acid (HPLC≥98%, CAS: 5794-13-8) was used as a standard in determining the total amino acid content. From aspartic acid stock concentration of 0.1014 g /L, several dilutions were made to prepare a series of concentrations at 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. A standard curve (Y=13.93x-0.1799, R²=0.9996) was constructed with the optical density at 568 nm against the concentrations of aspartic acid. The total amino acid content of the samples were expressed in milligram of aspartic acid equivalent per gram of samples.

Statistical analysis for callus induction and cell line formation

Each treatment was repeated three times, for polysaccharide and for amino acids analysis, each sample were extracted three times and measurements were repeated three times. Data are the mean values of three replicates, and are expressed as mean±standard error (SE). Statistical analysis was performed using one-way ANOVA using an SAS software package.

RESULTS

Effects of different plant growth regulators on the callus induction and cell line formation of *D. candidum*

Since the auxin could benefit to the callus induction in many genus, so the effect of the auxin analogues

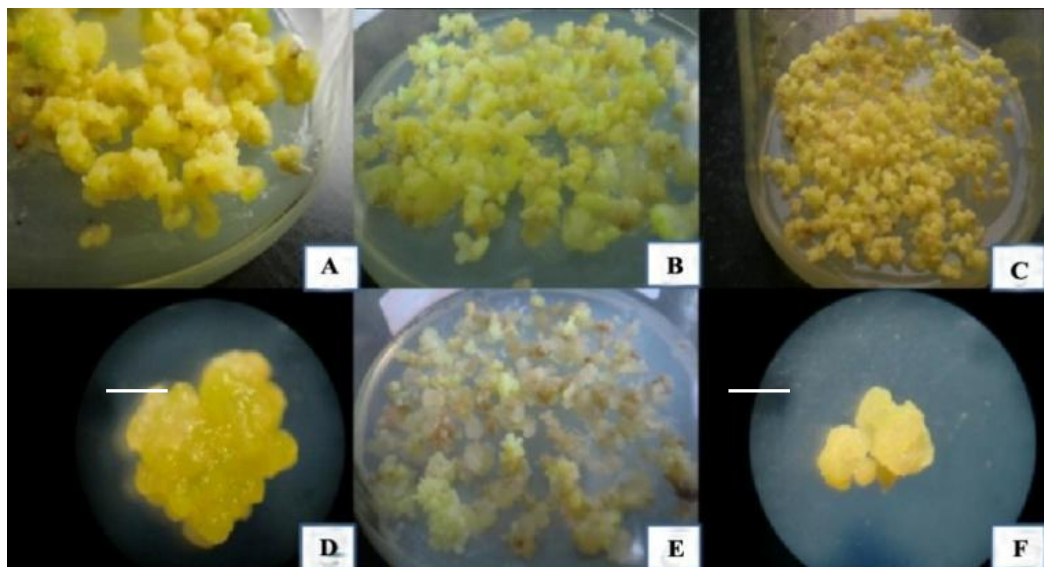


Figure 1. Cell acclimation of *D. candidum*. A, D: Initial subcultured cells; B, E: The differentiated cells during subculture; C, F: Cell lines formed after 3 months on SCM2 medium. The images in D, F were obtained under a stereo microscope at 20 \times , the bar represent 1 mm.

picloram, 2,4-D, NAA on the callus induction of *D. candidum* were examined. The results showed that PLB cultured on the medium containing picloram are all died, and callus could be induced on the medium containing 2,4-D (0.5 and 1.0 mg/L) with high induced rate above 90%, or NAA (5 and 10 mg/L) with a induced rate around 80 to 90% (Table 1). But the calli induced on the MS medium supplied with 2,4-D alone gradually became brown. Then the effects of MS medium supplied with 2,4-D, NAA and cytokinin on the callus induction were further carried out. The results showed that the supply of cytokinin could benefit to the callus induction and 2,4-D-treated group showed visible calli within 7 to 10 day. On day 20, MC1, MC2, MC3, and MC4 media containing 2,4-D and KT obtained 100% callus induction rate. The callus induced on MC1 and MC2 medium containing 0.5 mg/L 2,4-D showed no browning, and were in good conditions with slight re-differentiation rate when transferred to sub-cultured medium SCM1 to form cell line. The callus appeared a slight browning on MC3 and MC4 medium containing 1.0 mg/L 2,4-D, and grow well with middle re-differentiation rate on SCM1 medium. The callus mass gradually appeared after 12 to 14 day culture on medium containing NAA and 6-BA or KT. The callus induction rate on medium (MC5, MC6, MC9, MC10) containing 5 mg/L NAA was also 100%, the calli presented a pale yellow color with almost no browning, and grow well with no re-differentiation on SCM1 medium. The calli induction rate were around 85% on medium (MC7, MC8, MC11, MC12) containing 10 mg/L NAA, the calli showed some browning, and grow slowly with a heavy browning.

As differentiation into seedlings easily occurs during cell line establishment for *D. candidum*, culture conditions

must be controlled during domestication. The newly induced callus of *D. candidum* presented a dense structure with large clumps (Figure 1A and D). Some callus became brown and necrotic, whereas the other parts appeared to be differentiated during subculture (Figure 1B and E). A strong tendency toward callus differentiation was observed in the initial subculture. This finding indicated that short subculture periods (15 day) were necessary in the first three to four cycles. Clumps were crushed to granules during sub-inoculation. After 3 months of continuous screening and domestication on medium SCM1, the texture of the callus became visibly loose, granules became stronger and more evident, and the re-differentiation tendency gradually weakened on medium SCM2 (Figure 1C and F).

Comparison of anatomical characteristics between callus and PLBs of *D. candidum*

Callus and PLBs were selected for paraffin sectioning. The results showed the initial PLB contained a small number of meristematic cells distributed in a strip in the near surface zone of tissues, whereas the internal part was composed of non-meristematic cells with few inclusions and large particle sizes (Figures 2A to C). Callus was mainly composed of square or circular meristematic cells with strong division ability and large nuclei. Some cells do division and present a dual-core state (Figures 2D to F). That suggested the main differences between callus and PLBs were meristematic cell content and internal cell differentiation degree, and the meristematic cells distributed in the external strip

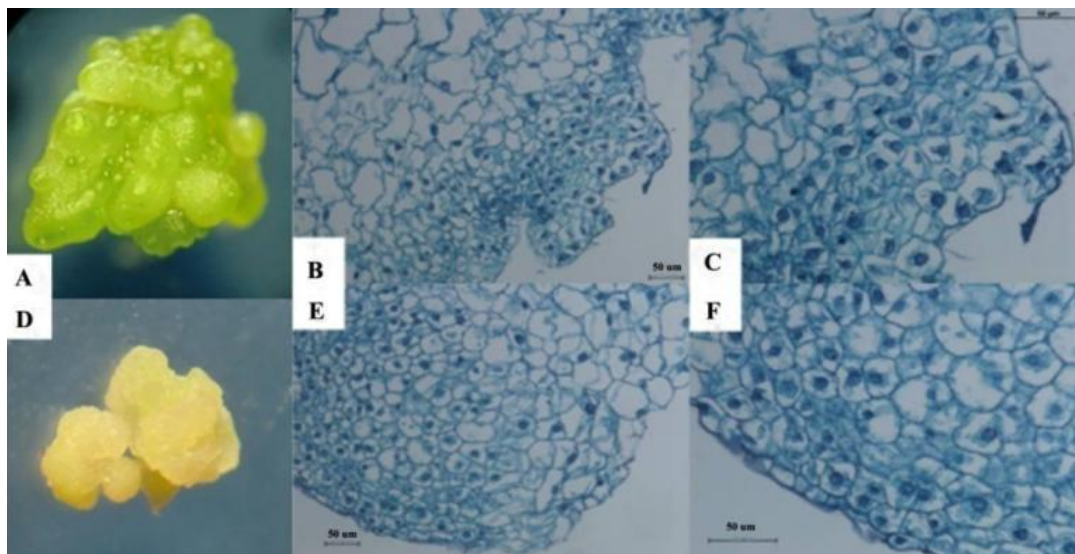


Figure 2. Comparison of the microstructure of protocorm-like bodies(A–C) and callus (D–F) of *D. candidum*.

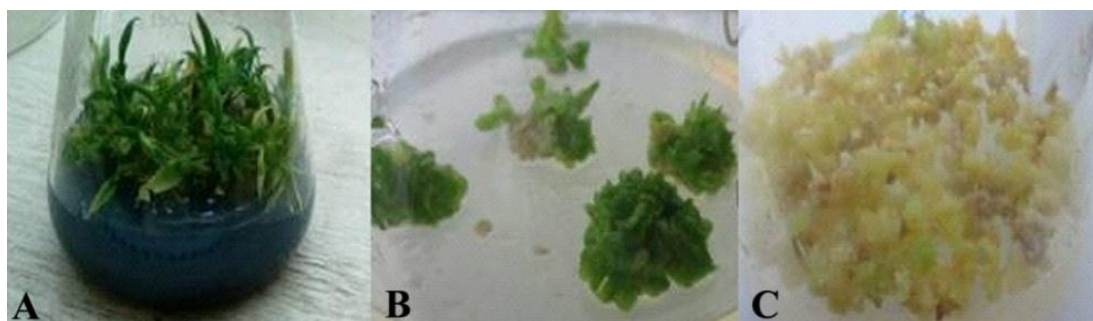


Figure 3. Three different *in vitro* cultures of *D. candidum*. A. Tissue culture seedlings; B. protocorm-like bodies (PLB); and C. callus.

Table 3. Polysaccharides and Amino acid compounds content in different *D. candidum* cultures.

| Parameter | Polysaccharides content (%) | Amino acid content (%) |
|-----------------------------|-----------------------------|--------------------------|
| Callus | 24.67±0.102 ^{a*} | 5.94±0.490 ^b |
| Protocorm-like bodies (PLB) | 17.01±0.716 ^b | 14.06±0.104 ^a |
| Tissue culture seedlings | 13.80±0.602 ^c | 3.34±0.180 ^c |

*All values are means ± standard error SE (n = 3). Mean values in each column followed by the same letter are not significantly different (P < 0.05) by the one-way ANOVA analysis.

were contributed to the formation of callus.

Quality analysis of different kinds of tissue cultures from *D. candidum*

The major bioactive compounds of *D. candidum* are polysaccharides (Liu et al., 1988; Yi et al., 1999). The

main active compounds (polysaccharides and amino acid) were compared among different *in vitro* cultures (tissue culture seedling, callus, and PLBs) of *D. candidum* (Figure 3) to evaluate the possibility of producing useful plant bioactivities using plant cell culture (Table 3). The results showed that polysaccharide content was significantly higher (24.67%) in callus than that in the PLBs and tissue culture seedlings (P < 0.05). Another

major compounds of total amino acids were also checked, the amount of total amino acids was $5.94 \pm 0.490\%$ in callus and higher than that in tissue culture seedlings, but lower than that in the PLBs (Table 3).

DISCUSSION

In the present research, factors affecting the induction, maintenance, and multiplication of callus of *D. candidum* were systematically studied. An efficient method was established for callus induction and cell line construction and maintenance, and major bioactive compounds were compared among different *in vitro* cultures (tissue culture seedling, callus, and PLBs) of *D. candidum*. The results showed that calli exhibit the highest potential as an alternative medicine source. But for the plant cell culture, plant tissue de-differentiation is the key to callus formation and the induction efficiency was affected by several factors such as explant, plant growth regulators (Tao et al., 2011; Prakash et al., 1996; Nayak et al., 2002). In the de-differentiation induction of *D. officinale*, the highest callus induction rate reached 82% and was achieved using seed-induced PLBs as explants (Zhao et al., 2008). The highest callus induction rate reached 86.67% when tender stem segments were used as explants (Wang and Liang, 2010), and there is a novel study illustrating shedding light on the effect of various plant growth regulator 2,4-D and NAA on callus induction of *ricinus communis*. Callus culture was initiated from cotyledonary leaf and root segments explants from *in vitro Ricinus communis* L seedling. The results showed that 2,4-D and NAA effecting the callus induction, but 2,4-D was proved to be more efficiency for induction of callus. Individual treatment of NAA reveals low effectiveness for callus induction (Khadiga et al., 2015). In the current study, PLBs (which was defined as somatic embryos in orchids species, Lee et al., 2013) was used as explants and the callus induction rate reached 100% under the optimized condition which was MS medium containing 0.5 mg/L 2,4-D and 0.25 NAA or 0.5 mg/L kinetin (KT). The callus clump enrichment method was used for directional screening and cell adaptation on MS medium supplied with plant growth regulators (PGR) such as 5 mg/L NAA and 0.5 mg/L 6-benzyladenine (6-BA) or KT for 30 days per cycle. While in another study for *Dendrobium Broga* Giant, the percentage of PLBs induced was the highest on half strength MS semi-solid medium supplemented with 1.0 mg/L BAP and 0.5 mg/L NAA. The highest proliferation rate of 8.7% of PLBs was obtained in 1.0 mg/L BAP and 0.5 mg/L NAA (Jasim et al., 2015).

But the establishment of *D. candidum* cell line is challenging, and requires a long period of acclimation. At the beginning of subculture, *D. candidum* cells exhibit a strong ability to re-differentiate and gradually gain the

ability to maintain the dedifferentiation state. Plant growth regulator is the key factor to regulate the cell differentiation state. The early subculture cycle of *D. candidum* cell lines is insufficient to maintain the hormone levels in the culture medium above the threshold and thus cannot maintain the cell dedifferentiation state. Meanwhile, plant cell differentiation is controlled by the position effect, changing the cell position appropriately in the critical period before cell division could change cell development mode (Wolpert, 1969). In the subculture of *D. candidum*, cell granulation could be induced by changing the cell position by using tweezers to constantly mince callus.

Polysaccharides are the main components of *D. candidum*, and polysaccharide content is positively correlated with physiological function. Hence, the content of polysaccharides should be the widely accepted evaluation criterion for *D. candidum* and its use as a medicine source. Controlled cell culture conditions are conducive to the implementation of good manufacturing practices and short production cycles to generate medicines from *D. candidum*. Plant cultivation for medicinal purposes will also minimize wild collection, thus supporting plant conservation in the wild. However, total polysaccharide content of cultivated plants must be comparable with the wild plantlets from Yunnan, in which the total polysaccharide ranges from 18 to 46% (Jiang et al., 2014). In the current study, we found that calli possessed the highest polysaccharide content of 24.67%, and exhibit the highest potential as an alternative medicine source for *D. candidum*.

Conclusions

An effective method was established for embryogenic callus induction and maintenance of *D. candidum*. The proposed method included PLB induction under the optimized condition, callus induction and growth, and callus maintenance. The callus induction rate under the optimized conditions was 100% and higher than previously reported values. And the major bioactive compounds of polysaccharides in callus are higher than in tissue culture seedling and protocorm-like bodies. Therefore, callus maybe a suitable material for utilization of *D. candidum* as a medicine source.

Conflict of interests

The authors hereby declare that no conflict of interest exists among them.

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Abbreviations

6-BA, 6-benzyladenine, 2,4-D 2,4-dichlorophenoxyacetic acid; **Kin**, kinetin; **MS**, Murashige and Skoog (1962) medium; **NAA**, Naphthaleneacetic acid; **PLBs**, Protocorm-like bodies; **TDZ**, thidiazuron; **PIC**, picloram.

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