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Efficient Direct Protocorm-Like Bodies Induction of Dwarf *Dendrobium* using Thidiazuron

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Abstract

An efficient induction of protocorm-like bodies (PLBs) from seedlings of dwarf *Dendrobium* was accomplished in order to develop mass-scale propagation. The effect of N^6-benzyladenine (BA) and thidiazuron (TDZ) on the *in vitro* induction of PLBs was studied. TDZ was found to be a more effective inducer of PLBs and their proliferation than BA. The highest percentage for the induction of PLBs (86%) and the highest number of PLBs (3.6) per protocorm were observed after 9 weeks of culture in modified Murashige and Skoog (MS) liquid medium supplemented with 18 μM TDZ.

Keywords: *Dendrobium* hybrid, direct embryogenesis, micropropagation, protocorm, seed culture

Introduction

Orchids are popular horticultural and ornamental plants mostly because of their exquisite flowers. In Thailand, orchids are very important commercial plants and produce an annual income for Thailand of 2 billion Baht (Rojanawong *et al.*, 2006). *Dendrobium* orchids, that are the largest genus in the family Orchidaceae, have become a major cut flower crop for export. *Dendrobiums* are normally reproduced asexually by division of offshoots, but the multiplication rate is low (Martin and Madassery, 2006; Nasiruddin *et al.*, 2003). Sexual propagation to produce complete plants through orchid seeds is difficult because their seeds are minute and have no endosperm. Consequently, they need symbiotic fungi in order to germinate (Anjum *et al.*, 2006; Thomas and Michael, 2007). Dwarf *Dendrobium* (*Dendrobium* 'Queen Pink' x *Dendrobium* 'Phakbung'), a member in *Dendrobium* hybrids, is a sympodial epiphytic orchid, and it has small pseudobulbs. Also, its flowers have longevity and blooms many times a year. It is generally used as a flowering-potted for ornamental plants. In addition, it has potential commercial value as gifts, souvenirs, or decorations which are miniaturized orchid plantlets with flowers. Therefore, tissue culture has for many years played an important role as a means to propagate orchids and several *in vitro* cultural protocols have been developed in this genus (Arditti and Ernst, 1993; Chugh *et al.*, 2009).

The production of orchid seedlings from seeds involves three successive phases: germination, formation of protocorms, and seedling development (Mitra *et al.*, 1976). The development of protocorms from germinated seed and the subsequent induction of PLBs, from different tissues as explants has become a reliable method for breeding orchids. Propagation by formation of PLBs is a preferred option because of the large number of PLBs that can be obtained within a short period of time. The need for mass propagation has led to the development of *in vitro* methods such as the propagation of large-scale PLBs using: shoot tips (Malabadi *et al.*, 2005; Sheela *et al.*, 2004), leaf segments (Martin and Madassery, 2006; Park *et al.*, 2002a), protocorms (Sheelavanthmath *et al.*, 2005; Teng *et al.*, 2004), flower stalks (Chen and Chang, 2000; Chen *et al.*, 2002), stem segments (Luo *et al.*, 2008) and root tips (Manners *et al.*, 2010; Park *et al.*, 2003). PLBs can proliferate rapidly and can readily regenerate into complete plantlets, so they are also the most general target tissue for genetic transformation studies in orchids (Liau *et al.*, 2003; Sreeramanan *et al.*, 2008). Moreover, PLBs are well-differentiated tissues that are sometimes regarded as orchid embryos that can develop two distinct bipolar structures, namely, the shoot and root meristem. Thus, these structures are able to convert to plantlets easily when grown on plant growth regulator-free medium (Ng and Saleh, 2011).

Thidiazuron (TDZ: N-phenyl-N'-1,2,3-thidiazol-5-ylurea), a phenylurea derivative and a non-purine cytokinin compound, is not catabolized via cytokinin oxidase (Hare and Van, 1994; Kishor and Devi, 2009). Previously, TDZ was reported to be effective in the regeneration of a number of orchid species such as *Doritaenopsis* (Ernst, 1994), *Phalaenopsis* (Chen and Piluek, 1995), *Cymbidium* (Chang and Chang, 1998; Nayak *et al.*, 1997), *Oncidium* (Chen *et al.*, 1999; Chen and Chang, 2001), and *Dendrobium* (Roy *et al.*, 2007). BA (N^6-benzyladenine) is an adenine-type cytokinin. The beneficial effect of BA has also been described for the regeneration of several orchids, including the genera *Oncidium* (Kalimuthu *et al.*, 2007), *Geodorum* (Sheelavantmath *et al.*, 2000), *Vanda* (Decruse

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et al., 2003), *Dendrobium* (Martin and Madassery, 2006; Nayak *et al.*, 2002), *Vanilla* (Geetha and Shetty, 2000), and *Cymbidium* (Paek and Yeung, 1991; Teixeira da Silva *et al.*, 2006). However, along with changing of plant materials, response of plant growth regulators (PGRs) is greatly changed. Thus, functions of the exogenous PGRs are quite different from orchid species to species. In this study, it has been described an efficient and rapid method for induction of PLBs from protocorms of a dwarf *Dendrobium* using TDZ.

Materials and methods

Seed culture

Healthy plants of dwarf *Dendrobium* were planted in pots and grown under greenhouse at Department of Biology, Prince of Songkla University, Hat Yai, Songkhla, Thailand. Flowers were hand pollinated and a pair of bipartite pollinia was picked and deposited on the stigma of a flower during pollination. The pollinated plants were maintained in the greenhouse. The 3-month-old mature and well-developed seedpods were collected for seed germination experiments. Seeds of mature seedpods were cultured immediately after collection. Seedpods were surface sterilized by immersion in 95% ethanol and flamed then they were dissected longitudinally and the seeds were cultured on MS (Murashige and Skoog, 1962) medium.

Culture medium and conditions

MS medium containing 3% (w/v) sucrose, 8.2 g/L agar, and 15% (v/v) coconut water (CW) was used throughout the experiments as the basal medium. The pH of the medium was adjusted to 5.5 prior to autoclaving at 121°C at 1 kPa for 20 min. All cultures were incubated at 25±1°C under a 16/8 (day/night) photoperiod provided with white fluorescent tubes at an intensity of 1,960 lux.

Induction of PLBs

The 2-month-old green protocorms were then used for induction of PLBs by transferring to 40 mL of the basal MS liquid medium supplemented with different concentrations of BA (4.4, 8.8, 13.2, 17.6 or 22 μM) and TDZ (4.5, 9, 13.5, 18 or 22.5 μM) in 100 mL Erlenmeyer flasks on a rotary shaker at 120 rpm for proliferation and multiplication.

Plantlet regeneration and transplantation of plantlets

PLBs were transferred to the basal MS medium for plantlet regeneration. Plantlets with three or four well-developed roots were taken out from the culture medium and washed thoroughly under running tap water to remove agar. They were acclimatized in pots containing wetted coconut husk. After hardening, the transplanted plantlets were grown in the greenhouse.

Experimental design and data analysis

Five protocorms were implanted per flask and subcultures of these protocorms were carried out every 3 weeks. Experiments were carried out in a completely randomized design and repeated twice with each treatment having twenty-five replicates. After 9 weeks of culture, explants were evaluated in terms of the percentage of PLBs formed and the number of PLBs per explant. The data were analyzed statistically using one-way analysis of variance (ANOVA). The mean values were compared using Duncan's multiple range test at $P \leq 0.05$.

Results

Seed germination occurred after 2 weeks of culture. The germinating seeds increased in size, became swollen, and later turned green. Seeds grew into protocorms after 2 months (Fig. 1A). Subsequently, the protocorms developed into PLBs after 6 weeks of culture on the PLB induction media. Formation of PLBs occurred directly on the surface of protocorms in the growth regulator medium. The efficiency of protocorms producing PLBs was dependent on the types and concentrations of cytokinins in the medium. The highest percentage of forming PLBs (86%) and the highest number of PLBs per explant (3.6) was found on modified MS medium supplemented with 18 μM TDZ (Fig. 1B, Tab. 1). Of the various concentrations of BA tested, the best response was recorded for the medium containing 4.4 μM BA, when 56% produced PLBs with an average of 0.9 PLBs per explant. TDZ was therefore more effective than BA in inducing formation of PLBs. Direct formation of PLBs was observed from protocorms without the intermediate formation of callus.

PLBs were transferred to the basal MS medium upon which they developed into shoots and roots in 6 weeks (Fig. 1C). The regenerated plantlets were then potted to wetted coconut husk and kept in greenhouse (Fig 1D). They grew well and developed into normal plants after 12 weeks of transplantation.

Discussion

The types and concentrations of PGRs play an important role in *in vitro* propagation of many orchid species (Arditti and Ernst, 1993). Differences in the induction rate for PLBs were observed between the treatments with TDZ and the BA. Comparatively, TDZ gave a superior response to BA for inducing PLBs in dwarf *Dendrobium*. TDZ has been previously used successfully to induce PLBs and the subsequent proliferation in *Dendrobium* 'Chiengmai Pink' (Chung *et al.*, 2005), *Vanda coerulea* (Malabadi *et al.*, 2004) and *Dendrobium chrysotoxum* Lindl (Roy *et al.*, 2007). In this study, TDZ at a particular concentration strongly stimulated the formation of PLBs. In a simi-

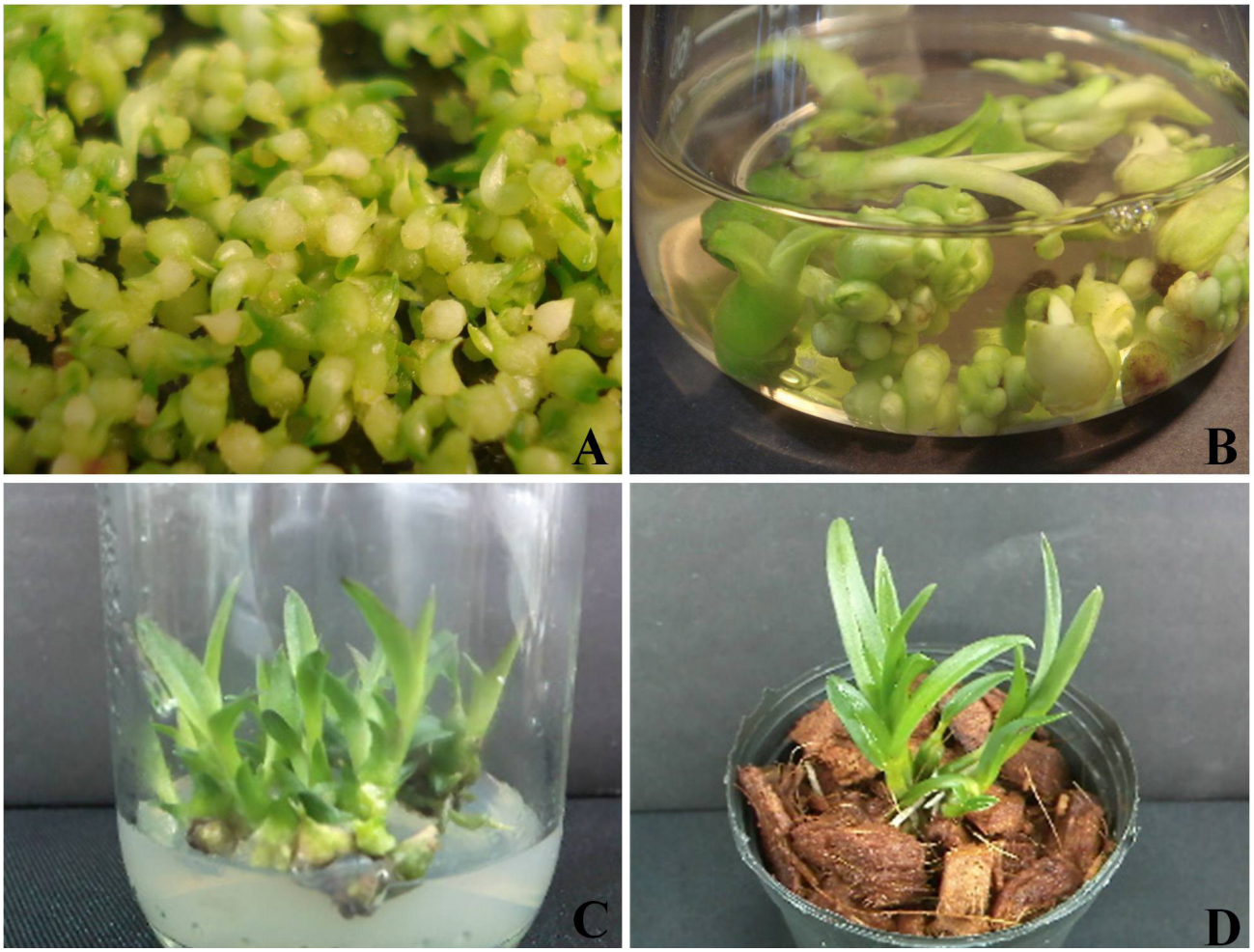

Fig. 1. (A) 2-month-old green protocorms. (B) PLBs development from protocorms after 9 weeks of culture in MS liquid medium with 18 μM TDZ. (C) Plantlets developed on MS medium. (D) Acclimated plants

Tab. 1. Effects of TDZ and BA on the induction of PLBs from protocorms of dwarf *Dendrobium* after 9 weeks of culture

Cytokinin concentration (μM)	PLB formation (%) (Mean ± SE)	Number of PLBs per explant (Mean ± SE)
Control	14.4± 5.2^{e}	0.2± 0.1^{c}
TDZ		
4.5	43.2 ± 8.1^{bcd}	0.6 ± 0.2^{bc}
9.0	30.4 ± 7.3^{de}	0.3 ± 0.1^{c}
13.5	32.0 ± 6.8^{cde}	0.4 ± 0.1^{bc}
18	86.4 ± 6.1^{a}	3.6 ± 0.5^{a}
22.5	25.6 ± 8.0^{de}	1.5 ± 0.8^{b}
BA		
4.4	56.0 ± 8.5^{b}	0.9 ± 0.2^{bc}
8.8	41.6 ± 8.6^{bcd}	0.8 ± 0.3^{bc}
13.2	36.0 ± 8.5^{bcde}	0.5 ± 0.1^{bc}
17.6	54.4 ± 8.8^{bc}	0.9 ± 0.2^{bc}
22	25.6 ± 6.2^{de}	0.3 ± 0.1^{c}

Means within a column followed by the same letter are not significantly different by Duncan's multiple range test (P≤0.05)

lar way, TDZ was found to be suitable for production of PLBs from thin leaf sections of a *Doritaenopsis* hybrid (Park *et al.*, 2002b). This study also clearly shows that TDZ used alone was more effective than BA in PLBs induction and proliferation. This result is also in agreement with the observations in *Epidendrum radicans* (Chen *et al.*, 2002), *Doritaenopsis* (Park *et al.*, 2003), and *Phalaenopsis* (Kuo *et al.*, 2005).

Morel (1960) was the first to report the shoot tip culture in orchids for mass propagation. Normally, plant regeneration and mass propagation from various types of explants have been achieved by four protocols. The first is adventitious bud induction through direct organogenesis. The second is protocorm induction which eventually develops into plantlets. The third is PLB regeneration through direct embryogenesis. The fourth is the transverse thin cell layers culture. According to these protocols, bud formation via organogenesis and direct PLBs formation through embryogenesis are used as the cultural conditions from the explants (Zhao *et al.*, 2008).

Regeneration of PLBs is comparable to the somatic embryogenesis pathway in orchids (Morel, 1974). Formation of PLBs can be classified into two types. The first is the direct formation of PLBs from protocorms, shoot tips, root tips, and stem segments through direct embryogenesis (Luo *et al.*, 2008; Mayer *et al.*, 2010; Naing *et al.*, 2011). The second is indirect formation of PLBs from callus. (Hong *et al.*, 2008; Huang and Chung, 2010; Ng and Saleh, 2011). In the present experiment, PLBs developed directly from the protocorms without callus formation. Similar result has been demonstrated for *Aerides crispum* (Sheelavanthmath *et al.*, 2005). Lee and Phillips (1988) attributed this point as being of major importance because plants produced by direct regeneration will exhibit greater genetic stability than those produced by callus. Furthermore, regenerated plantlets, produced through the direct formation of PLB, produced fewer variants. Moreover, tissue cultures of orchids have not been focused on callus because of their slower growth rate and increased necrosis during culture (Zhao *et al.*, 2008). In the present study, the proficiency for inducing PLBs from protocorms is in agreement with the finding of Sheelavanthmath *et al.* (2005) who illustrated that juvenile explants like protocorms and young leaves were important for the efficient induction of PLBs and the subsequent regeneration of plants in *Aerides crispum*.

Conclusions

The efficient induction of PLBs and their proliferation from protocorms of dwarf *Dendrobium* was achieved for large scale propagation. It was found that 18 μM TDZ was the optimum concentration for inducing PLBs.

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