



# In vitro propagation of *Cymbidium goeringii* Reichenbach fil. through direct adventitious shoot regeneration

Han Yong Park<sup>1</sup> · Kyung Won Kang<sup>2</sup> · Doo Hwan Kim<sup>3</sup> · Iyyakkannu Sivanesan<sup>3</sup>

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**Abstract** The influence of 2,4-dichlorophenoxyacetic acid (2,4-D), benzyladenine (BA), and thidiazuron (TDZ) on direct rhizome induction and shoot formation from rhizome explants of *Cymbidium goeringii* was explored. Rhizome segments obtained from in vitro seed cultures of *C. goeringii* were placed on Murashige and Skoog (MS) medium incorporated with 5, 10, 20, or 40  $\mu\text{M}$  2,4-D and 1, 2, 4, or 8  $\mu\text{M}$  BA or TDZ alone or in combination with 20  $\mu\text{M}$  2,4-D. The explants developed only rhizomes on MS medium with or without 2,4-D. The highest percent of rhizome formation (100%) was obtained on MS medium incorporated with 20  $\mu\text{M}$  of 2,4-D. The morphology and number of rhizomes varied with the level of 2,4-D in the medium. Direct adventitious shoot formation was achieved on medium incorporated with BA or TDZ. The adventitious shoots produced per explant significantly increased with the supplementation of 2,4-D to cytokinin-containing medium. The highest mean of  $21.8 \pm 1.8$  shoot buds per rhizome segment was obtained in medium fortified with 20  $\mu\text{M}$  2,4-D and 2  $\mu\text{M}$  TDZ. The greatest percent of root induction (100%) and the mean of  $5.3 \pm 1.1$  roots per shoot were achieved on  $\frac{1}{2}$  MS medium incorporated with 2  $\mu\text{M}$  of  $\alpha$ -naphthaleneacetic acid. About 97% of the in vitro-produced plantlets acclimatized in the greenhouse.

An efficient in vitro propagation protocol was thus developed for *C. goeringii* using rhizome explants.

**Keywords** 2,4-D · Benzyladenine · Direct shoot regeneration · Orchidaceae · Rhizome explants · Thidiazuron

## Introduction

The genus *Cymbidium* (Orchidaceae) comprises of 52 species of both ornamental and medicinal importance. *Cymbidium goeringii* Reichenbach fil., commonly called as spring orchid, is distributed across the Himalayas and in China, Taiwan, Japan, and Korea. *C. goeringii* has been widely cultivated for its beautiful and fragrant flowers. In the recent times, *C. goeringii* has disappeared in the wild because of habitat deterioration. Moreover, these plants are over collected by plant sellers and enthusiasts (Chung and Chung 1999). *C. goeringii* was designated as an endangered species (Wu et al. 2010; Tsuji and Kato 2010). Thus, efficient method for mass propagation is required to meet the growing demand and conservation of this species. Although it is propagated by seeds, its germination rate is extremely low under natural conditions, and fungal infection is required for the germination of seeds (Kang and Yang 2003). Furthermore, the multiplication rate of this species by a sexual method of conventional division is also very low under natural conditions (Paek and Kozai 1998). In vitro culture technique has therefore been exploited for the extensive commercial propagation and conservation of rare orchids (Koirala et al. 2013; Devi et al. 2015; Bhat-tacharyya et al. 2016; Bembemcha et al. 2016; Diengdoh et al. 2017; Jiang et al. 2017; Kim et al. 2017). However, the Asiatic temperate orchids such as *Cymbidium* has

✉ Iyyakkannu Sivanesan  
isivanesan@yahoo.com

<sup>1</sup> Department of Bioresource Engineering, Sejong University, Seoul 143-747, South Korea

<sup>2</sup> Babo Orchid Farm, Namyangju-si, Gyeonggi-do 472-831, South Korea

<sup>3</sup> Department of Bioresources and Food Science, Konkuk University, Seoul 143-701, South Korea

received less attention for commercial propagation using in vitro culture methods than other orchid species because of the rareness and shortage of plant materials (Chugh et al. 2009).

In vitro propagation methods have been established using seeds, flower buds, rhizomes, and shoot tips explants of *C. goeringii* (Ueda and Torikata 1969; Hasegawa and Goi 1987; Shimasaki and Uemoto 1991; Kang and Yang 2003). In most of these studies, rhizome segments obtained from in vitro cultures of flower buds, shoot tips, and seeds were used for regeneration of temperate *Cymbidium*. However, species, cultivars, culture media, plant growth regulators (PGRs), and culture environment affected the shoot regeneration ability of rhizome explants (Paek and Kozai 1998). Previous reports have shown that supplementation of cytokinin to the culture media alone or in combination with auxin required for shoot formation in vitro from rhizome explants of *C. goeringii* (Ueda and Torikata 1969; Shimasaki and Uemoto 1991). The authors studied the effect of N<sup>6</sup>-benzyladenine (BA), kinetin (Kin) and  $\alpha$ -naphthaleneacetic acid (NAA). However, none of these authors used thidiazuron (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D) for micropropagation.

The positive effect of TDZ in callus induction, shoot regeneration, somatic embryo formation and protocorm-like bodies (PLBs) induction have been reported in orchids, including in *Cymbidium* (Nayak et al. 1997; Chang and Chang 2000a; Huan et al. 2004; Chugh et al. 2009; Roy et al. 2012). In the in vitro cultures of orchids, 2,4-D has been shown to play a significant role in callus induction, seed germination, rhizome induction, and somatic embryogenesis (Novak et al. 2014). The number of shoots produced per rhizome segment (0.8–3.0) was very few (Ueda and Torikata 1969; Shimasaki and Uemoto 1991). Therefore, an efficient in vitro propagation method is needed for the mass propagation of *C. goeringii*. The aim of this study was to develop an efficient method for in vitro propagation of *C. goeringii* using rhizome explants.

## Materials and methods

Mature seed (green-capsules) collected at 180–210 days after self-pollination from greenhouse-grown plants of *C. goeringii* were surface disinfected with 70% (v/v) ethanol solution for 60 s, washing three times with sterile distilled water (sDH<sub>2</sub>O), followed by 1.0% (v/v) sodium hypochlorite solution for 10 min, and then washing four times with sDH<sub>2</sub>O. The seeds obtained from the capsules were placed in culture bottle containing Murashige and Skoog (1962, MS) medium with 0.2% (w/v) activated charcoal (AC), 0.2% (v/v) coconut water, 3% (w/v) sucrose, and 0.8% (w/v) plant agar (Duchefa Biochemie,

The Netherlands). The pH of the medium was adjusted to 5.6, 120 mL of the MS medium was dispensed in 500-mL culture bottles, and autoclaved. The culture bottles were kept at  $22 \pm 2$  °C under a 16-h photoperiod ( $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Seeds germinated within 8–12 weeks to develop PLBs (green) were transferred to the same fresh medium (MS) for rhizome development. After 8 weeks of culturing, the rhizomes (0.5 cm) were transferred to MS medium incorporated with 0.2% (w/v) AC, 3% (w/v) sucrose, and 4  $\mu\text{M}$  NAA and cultured for 4–6 months.

Rhizome (2.0–3.0-cm long) explants were placed in culture bottles containing 120 mL of MS medium incorporated with 0.2% (w/v) AC; 3% (w/v) sucrose; 5, 10, 20, or 40  $\mu\text{M}$  2,4-D; and 1, 2, 4, or 8  $\mu\text{M}$  BA or TDZ with or without 20  $\mu\text{M}$  2,4-D. The MS medium devoid of PGR served as control. The experiment was conducted in triplicate with 25 rhizomes for each treatment. The cultures were kept at  $22 \pm 2$  °C for a 16-h photoperiod ( $45 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). The number of rhizomes and shoots were recorded after 12 weeks of culturing.

The in vitro-produced shoot buds were cultured on  $\frac{1}{2}$  MS medium incorporated with 0.2% (w/v) AC; 3% (w/v) sucrose; and 0, 1, 2, or 4  $\mu\text{M}$  NAA for root induction. The experiment was conducted in triplicate with 50 shoot buds for each treatment. The frequency of root induction and the number of roots were recorded after 12 weeks of culturing. The rooted shoots were subcultured onto the same medium (fresh). After 12 weeks, well-developed plantlets (5–6-cm tall) were transplanted into 72-cell plug trays containing sphagnum moss, irrigated at 2-day intervals, and maintained in the greenhouse at  $22 \pm 2$  °C for a 16-h photoperiod ( $45 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) under a relative humidity of 70–80%. After 7 days, plants were fertigated with Hyponex (N–P–K; 20–20–20) solution, and the survival was recorded after 6 weeks. Data were subjected to Duncan's multiple range test and analysis of variance (ANOVA). The percent values were transformed using arcsine square root ( $\sqrt{P}$ ) to normalize the error distribution before ANOVA (Compton 1994).

## Results

The rhizome segments did not develop shoots on MS medium incorporated with 2,4-D after 12-weeks of culturing. However, rhizomes were formed from the explants within 3-week of incubation. The percent of rhizome induction improved as the level of 2,4-D in the culture medium increased from 0 to 20  $\mu\text{M}$  and then declined with further increase of 2,4-D level (40  $\mu\text{M}$ ). The highest percent of rhizome formation (100%) was achieved on MS medium incorporated with 20  $\mu\text{M}$  of 2,4-D. The morphology and number of rhizomes varied with the level of

2,4-D in the MS medium (Table 1). In 5 or 10  $\mu\text{M}$  concentration of 2,4-D, the developed rhizomes demonstrated chlorophyll synthesis and several trichomes on their surfaces (Fig. 1a). The culture medium containing 20–40  $\mu\text{M}$  of 2,4-D produced a creamy white rhizome without trichomes (Fig. 1b). Differences were noted in the number of induced rhizomes on MS medium incorporated with various levels of 2,4-D (Fig. 2a, b). The maximum number of rhizomes ( $16.6 \pm 2.6$ ) was produced on MS medium incorporated with 5  $\mu\text{M}$  of 2,4-D. The induced rhizomes were subcultured on PGR-free MS medium for further growth. The shoots developed from the tip of the rhizomes after 6 months of incubation (Fig. 2c, data not shown).

When the medium was incorporated with BA or TDZ, the rhizome explants developed both direct adventitious shoot buds and rhizomes within 4 weeks of culturing. However, the culture medium containing BA in low concentration (1 or 2  $\mu\text{M}$ ) did not develop shoot buds, but the explants produced rhizomes. Whereas, the MS medium containing BA in high concentration produced both adventitious shoot buds and rhizomes (Table 2). Differences were noted in the number of shoot buds per rhizome on the culture medium supplemented with 1–8  $\mu\text{M}$  of TDZ (Table 2). The maximum of  $7.2 \pm 1.2$  shoot buds per rhizome was obtained on MS medium incorporated with 2  $\mu\text{M}$  of TDZ. However, the mean number of shoot buds significantly decreased with increasing level of TDZ (4.0 or 8.0  $\mu\text{M}$ ) in MS medium. The supplementation of auxin (2,4-D) to the MS medium containing cytokinin (BA, TDZ) increased the shoot buds number per rhizome segment. The combination of BA and 2,4-D maximized the shoot bud number as compared to BA alone. A large number of shoot buds ( $8.9 \pm 1.7$ ) was produced per rhizome segment on MS medium incorporated with 20  $\mu\text{M}$  of 2,4-D and 8  $\mu\text{M}$  of BA (Table 2). Similarly, the presence of 2,4-D and TDZ increased the yield of shoot buds (Fig. 3a–c). The highest mean of  $21.8 \pm 1.8$  shoots per rhizome was achieved on MS medium fortified with 2  $\mu\text{M}$  of TDZ and 20  $\mu\text{M}$  of 2,4-D (Table 2).

The regenerated shoot buds (obtained from MS + 8  $\mu\text{M}$  BA + 20  $\mu\text{M}$  2,4-D) developed roots in  $\frac{1}{2}$  MS medium incorporated with 0–4  $\mu\text{M}$  of NAA within 4 weeks of

culturing. However, significant differences were noted in the percent of root induction and the number of roots among the treatments (Table 3). The percent of root induction increased with increasing level of NAA in the MS medium. The greatest percent of root induction (100%) and the mean of  $5.3 \pm 1.1$  roots per shoot were obtained on  $\frac{1}{2}$  MS medium incorporated with 2  $\mu\text{M}$  of NAA (Fig. 3d, Table 3). The inclusion of 4  $\mu\text{M}$  NAA into the  $\frac{1}{2}$  MS medium did not affect the percent of root induction; however, it significantly decreased the formation of roots ( $3.4 \pm 1.3$  per shoot). About 97% of the in vitro-developed plantlets acclimatized in the greenhouse (Fig. 3e, f).

## Discussion

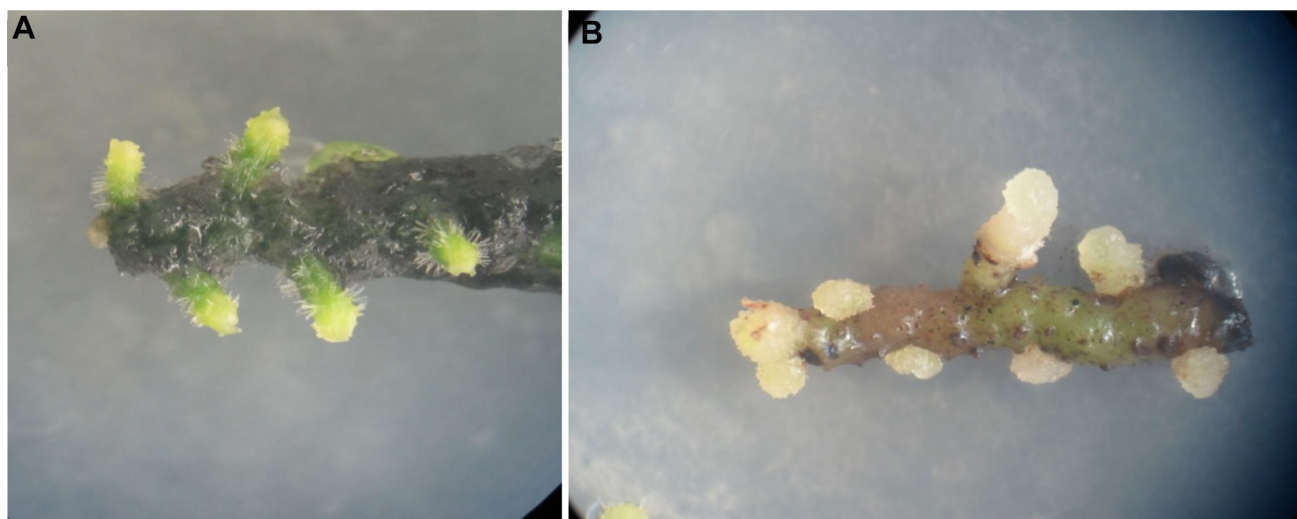
Rhizome formation is an important means of in vitro propagation of terrestrial *Cymbidium* (Chang and Chang 2000a). In hybrid *Cymbidium*, the inclusion of 1 or 2  $\text{mg L}^{-1}$  of 2,4-D into the medium resulted in the induction of embryogenic callus from PLBs (Teixeira da Silva 2014). In contrast, rhizome explants of *C. goeringii* grown on MS medium incorporated with 2,4-D developed only rhizomes (Table 1), which can be attributed to the difference in type and source of explant, genotype, and culture environment. Several studies have demonstrated that exogenous application of auxins is necessary for rhizome induction (Novak et al. 2014). The stimulating effect of auxins in rhizome development has been reported in *C. aloifolium* (Nayak et al. 1998), *C. forrestii* (Paek and Yeung 1991), and *C. kanran* (Shimasaki and Uemoto 1990). These studies have shown that auxin addition inhibits shoot regeneration. In this study, shoots developed from the tip of regenerated rhizomes on the PGR-free medium after 6 months of culturing. The conversion of rhizome meristems into shoots mainly depends on the endogenous levels of PGRs.

In vitro propagation through direct adventitious shoot regeneration is a prerequisite for the extensive commercial propagation and genetic transformation of plants. Exogenous application of cytokinins is necessary for shoot formation from rhizome segments of *Cymbidium* species. In

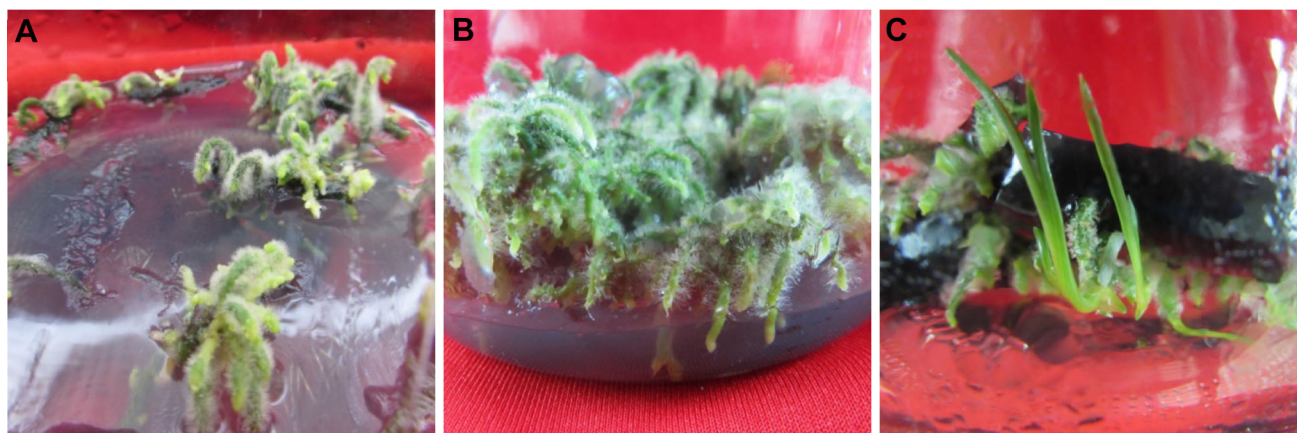
**Table 1** Effect of 2,4-D on rhizome and shoot induction from rhizome explants of *C. goeringii*

2,4-D ( $\mu\text{M}$ )	Rhizome induction (%)	No. of rhizomes/explant	No. of shoot buds/explant
0	$68.1 \pm 3.6\text{c}$	$4.4 \pm 1.7\text{d}$	$0.0 \pm 0.0$
5	$85.6 \pm 3.5\text{b}$	$16.6 \pm 2.6\text{a}$	$0.0 \pm 0.0$
10	$98.3 \pm 1.6\text{a}$	$12.5 \pm 3.1\text{b}$	$0.0 \pm 0.0$
20	$100 \pm 0.0\text{a}$	$8.8 \pm 1.2\text{c}$	$0.0 \pm 0.0$
40	$54.6 \pm 5.3\text{d}$	$7.7 \pm 1.3\text{c}$	$0.0 \pm 0.0$

Value (mean  $\pm$  standard error) within a column followed by the same letters are not significantly different at  $p \leq 0.05$



**Fig. 1** Rhizome induction from seed-derived rhizome explants of *C. goeringii*. **a** Induction of rhizome on MS medium containing 5  $\mu\text{M}$  2,4-D after 8 weeks; **b** induction of rhizome on MS medium containing 20  $\mu\text{M}$  2,4-D after 8 weeks



**Fig. 2** Influence of 2,4-D on rhizome formation *C. goeringii*. **a** Induction of rhizome on MS medium after 4 months; **b** induction of rhizome on MS medium containing 5  $\mu\text{M}$  2,4-D after 4 months; **c** shoots developed from the tip of the rhizomes in hormone-free MS medium after 6 months

this work, direct adventitious shoot formation was achieved on MS medium incorporated with BA or TDZ (Table 2). The use of BA has been reported to promote adventitious shoot formation in rhizome explants of *C. aloifolium* (Nayak et al. 1998), *C. faberi* (Hasegawa et al. 1985), *C. forrestii* (Paek and Yeung 1991), and *C. kanran* (Lee et al. 1986; Shimasaki and Uemoto 1990). In this work, direct adventitious shoot and rhizome induction were observed when the rhizome segments of *C. goeringii* were grown on MS medium incorporated with greater concentration of BA. Similarly, the presence of 20  $\mu\text{M}$  BA induced upright shoot formation from rhizomes of *Cymbidium ensifolium* (Lu et al. 2001). In contrast, a maximum number of shoots (8.3) were obtained when the shoot tip of *Cymbidium iridioides* was grown on MS medium incorporated with a lower concentration (0.5 mg L<sup>-1</sup>) of BA (Pant and Swar 2011). The number of shoots per rhizome segment was

significantly increased when the medium was fortified with TDZ. The explants also developed rhizomes, except in higher concentration of TDZ. Similar results have been observed in *C. sinense* (Chang and Chang 2000a). Multiple PLBs were induced from pseudostem explants of *C. giganteum* grown on MS medium containing a low concentration of TDZ (Roy et al. 2012). In *Cymbidium* hybrid, direct organogenesis was achieved when PLBs were cultured on the medium containing TDZ (Teixeira da Silva 2014). In general, the presence of cytokinin in the medium induced both the shoot and rhizome formation in rhizome explants of *Cymbidium*. Among the two cytokinins, TDZ was found to be better for direct adventitious shoot formation in *C. goeringii*. Similarly, TDZ has been used to maximize shoot formation in various orchids such as *Cypripedium lentiginosum* (Jiang et al. 2017), *Dendrobium crepidatum* (Bhattacharyya et al. 2016), *Dimorphorchis*

**Table 2** Effects of BA, TDZ and 2,4-D on rhizome and shoot induction from rhizome explants of *C. goeringii*

PGRs ( $\mu\text{M}$ )	PGRs ( $\mu\text{M}$ )			No. of rhizomes/explant	No. of shoot buds/explant
	BA	TDZ	2,4-D		
0	0	0	0	4.4 $\pm$ 1.7a	0.0 $\pm$ 0.0h
1	0	0	0	4.1 $\pm$ 1.4a	0.0 $\pm$ 0.0h
2	0	0	0	3.2 $\pm$ 1.0a	0.0 $\pm$ 0.0h
4	0	0	0	3.0 $\pm$ 0.7ab	1.8 $\pm$ 0.9g
8	0	0	0	1.5 $\pm$ 0.5c	2.9 $\pm$ 0.7f
1	0	20	20	1.8 $\pm$ 0.8bc	0.0 $\pm$ 0.0h
2	0	20	20	0.0 $\pm$ 0.0d	4.5 $\pm$ 0.9e
4	0	20	20	0.0 $\pm$ 0.0d	6.5 $\pm$ 1.5d
8	0	20	20	0.0 $\pm$ 0.0d	8.9 $\pm$ 1.7c
0	1	0	0	3.6 $\pm$ 1.2a	3.1 $\pm$ 0.9f
0	2	0	0	2.3 $\pm$ 0.9b	7.2 $\pm$ 1.2d
0	4	0	0	1.9 $\pm$ 0.7bc	4.0 $\pm$ 1.6e
0	8	0	0	0.0 $\pm$ 0.0d	3.6 $\pm$ 1.1ef
0	1	20	20	0.0 $\pm$ 0.0d	10.5 $\pm$ 1.8c
0	2	20	20	0.0 $\pm$ 0.0d	21.8 $\pm$ 1.8a
0	4	20	20	0.0 $\pm$ 0.0d	15.0 $\pm$ 2.0b
0	8	20	20	0.0 $\pm$ 0.0d	9.2 $\pm$ 1.3c

Value (mean  $\pm$  standard error) within a column followed by the same letters are not significantly different at  $p \leq 0.05$

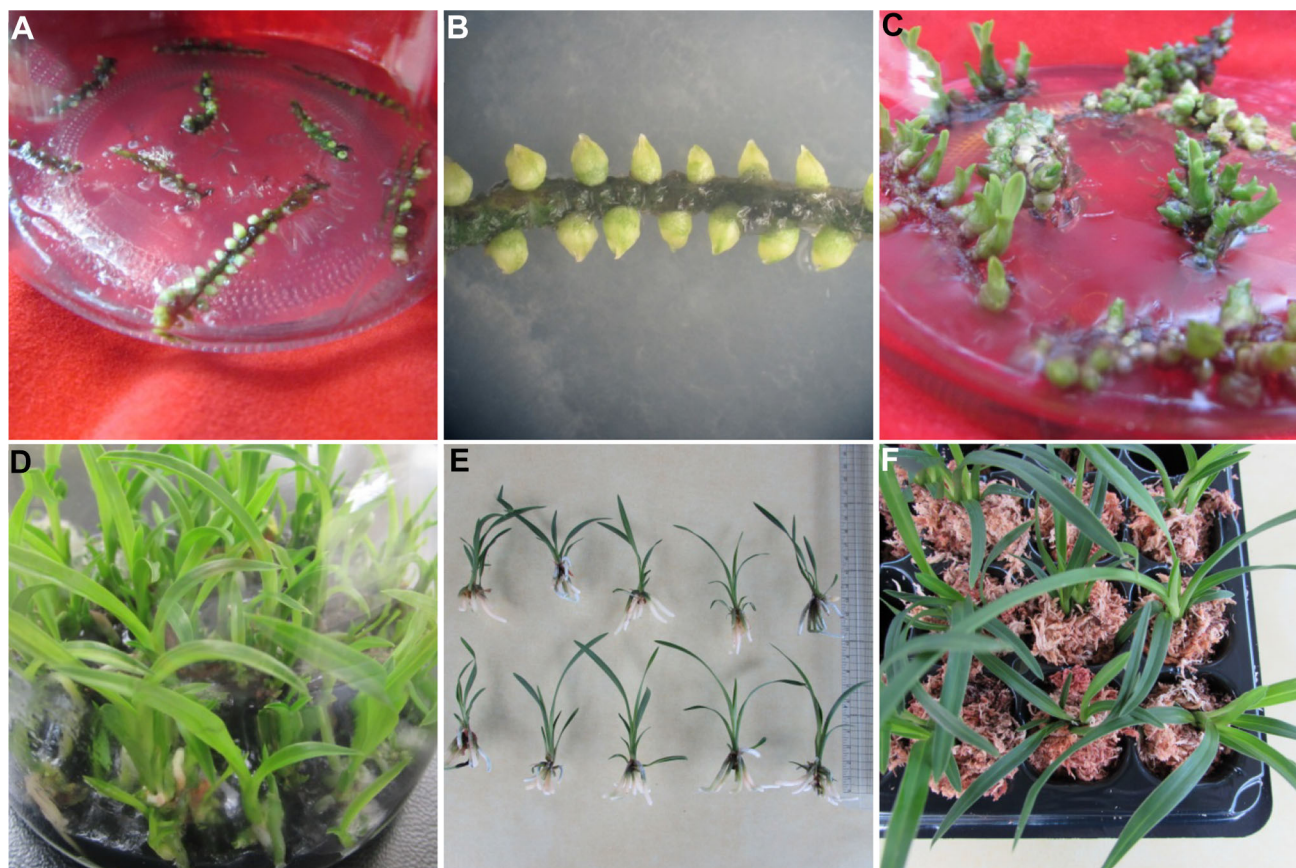
*lowii* (Jainol and Gansau 2017), *Herminium lanceum* (Singh and Babbar 2016) and *Phalaenopsis* ‘Surabaya’ (Balilashaki et al. 2015).

A combination of cytokinin and auxin has often been used to promote shoot regeneration in *Cymbidium* species (Peak and Kozai 1998). Nayak et al. (1998) reported that a combination of BA and NAA maximized adventitious shoot production in *C. aloifolium*. In *C. giganteum*, the maximum number of shoots or PLBs were obtained in medium incorporated with BA and NAA (Hossain et al. 2010). In *Cymbidium* hybrids, the highest frequency of regeneration (62%) with a mean of 3.3 shoots per explant was obtained in SH medium with 1.0 mg L<sup>-1</sup> BA and 0.1 mg L<sup>-1</sup> NAA (Pal et al. 2016). Similarly, the presence of BA and NAA in medium induced multiple shoots from rhizome explants of *C. goeringii* (Shimasaki and Uemoto 1991), *C. forrestii* (Paek and Yeung 1991), *C. kanran* (Shimasaki and Uemoto 1990), and *C. sinense* (Gao et al. 2014). In this study, BA in combination with 2,4-D was more useful for direct adventitious shoot production compared to BA alone. However, more shoot buds per rhizome explant of *C. goeringii* was achieved on MS medium incorporated with TDZ and 2,4-D. The beneficial effect of TDZ in combination with other PGRs has also been reported for other *Cymbidium* species. In *C. faberi*, TDZ combination with NAA was effective for adventitious shoot production from PLB (Tao et al. 2011). The combination of N<sup>6</sup>-(2-isopentenyl) adenine, TDZ,

6-aminopurine adenine, and NAA was highly effective for shoot bud regeneration from rhizome explants of *C. ensifolium* var. *misericors* (Chang and Chang 2000b).

Rooting of in vitro-regenerated shoots and acclimatization of plantlets are crucial steps in micropropagation. Root induction mainly depends on PGRs that are used to induce shoots and the composition of culture medium. In this study, 37.4  $\pm$  4.2% of the shoots developed roots obtained from MS medium incorporated with 20  $\mu\text{M}$  of 2,4-D and 8  $\mu\text{M}$  of BA when grown on 1/2 MS medium. Whereas, 1/2 MS medium incorporated with NAA showed a significant improvement in the rooting of in vitro-developed shoots of *C. goeringii*. The promoting effect of NAA on root induction has also been reported for *C. aloifolium* (Nayak et al. 1998) and *C. sinense* (Chang and Chang 2000a; Gao et al. 2014). On the contrary, rooting of *C. kanran* was inhibited by the inclusion of auxin to the culture medium (Shimasaki and Uemoto 1990). The acclimatized plants (97%) grew well in the greenhouse.

This is the first report on direct adventitious shoot regeneration of *C. goeringii* using seed-derived rhizome explants. The combination of TDZ and 2,4-D was found to inhibit rhizome induction and promote direct adventitious shoot formation. In vitro-produced shoots rooted the best on 1/2 MS medium incorporated with NAA. The protocol proposed in this study could be useful for germplasm conservation, in genetic transformation studies, and for large-scale commercial propagation of *C. goeringii*.



**Fig. 3** Micropropagation of *C. goeringii*. **a** Induction of direct adventitious shoot buds in MS medium containing 20  $\mu\text{M}$  2,4-D and 2  $\mu\text{M}$  TDZ after 4 weeks; **b** a closer view of shoot buds formation from seed-derived rhizome explant; **c** shoot bud development in MS medium containing 20  $\mu\text{M}$  2,4-D and 2  $\mu\text{M}$  TDZ after 12 weeks;

**d** in vitro-regenerated shoots developed roots in half-strength MS medium fortified with 2  $\mu\text{M}$  of NAA after 12 weeks; **e** rooted shoots after 24 weeks of culture in half-strength MS medium containing 2  $\mu\text{M}$  of NAA; **f** acclimatized plantlets after 6 weeks of cultivation in the greenhouse

**Table 3** Effect NAA on root induction from in vitro-developed shoots of *C. goeringii*

NAA ( $\mu\text{M}$ )	Root induction (%)	Number of roots per shoot
0	37.4 $\pm$ 4.2c	2.4 $\pm$ 0.7c
1	81.3 $\pm$ 2.3b	3.9 $\pm$ 1.0b
2	100 $\pm$ 0.0a	5.3 $\pm$ 1.1a
4	100 $\pm$ 0.0a	3.4 $\pm$ 1.3b

Value (mean  $\pm$  standard error) within a column followed by the same letters are not significantly different at  $p \leq 0.05$

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**Authors contributions** Han Yong Park and Iyyakkannu Sivanesan designed the experiment, analyzed the data and prepared the manuscript. Kyung Won kang and Doo Hwan Kim edited the manuscript.

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