A simple and efficient protocol for the mass propagation of *Cymbidium mastersii*: an ornamental orchid of Northeast India

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**Abstract**

**Background and aims**

*Cymbidium mastersii* is an epiphytic orchid distributed mainly in Northeast India. Owing to its high commercial value in the floricultural industry, natural populations are under threat from over-exploitation. Mass propagation provides an alternative means of satisfying the demand. Unfortunately, conventional propagation is slow and difficult, suggesting *in vitro* methods for mass multiplication may be more appropriate. The objective of this study was to develop an efficient protocol.

**Methodology and principal results**

Four nutrient media were evaluated for seed germination and early protocorm development: Murashige and Skoog (MS), half-strength MS, Knudson 'C' (KC), and Vacin and Went (VW). In addition, the effects of plant growth regulators 6-benzylaminopurine (BAP), kinetin (KN), α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) were studied alone and in combination. The maximum percentage seed germination (93.58 ± 0.56) was obtained in MS basal medium after 8–9 weeks of culture. Secondary protocorms (protocorm-like bodies) were developed from primary protocorms on MS medium fortified with different concentrations and combinations of cytokinins (BAP and KN) and auxins (NAA and IBA). The highest numbers of secondary protocorms (20.55 ± 0.62)/primary protocorms were obtained in MS medium supplemented with 5.0 μM BAP and 2.5 μM NAA. The most effective auxin source promoting root production (7.46 ± 0.09 per shoot) was 10.0 μM IBA. The plants were acclimatized effectively (survival percentage 88 %) in a greenhouse using a rooting medium of crushed sterile brick and charcoal (1 : 1 v/v) and vermicompost (leaf litter + cow dung, 1 : 1 v/v).

**Conclusions**

An efficient protocol was established for *in vitro* propagation of *C. mastersii* using seed as the starting material. The percentage seed germination varied with the composition of the nutrient media and was highest in full-strength MS basal medium. The number of secondary protocorms that developed from primary protocorms was increased by the addition of 5.0 μM BAP and 2.5 μM NAA. *In vitro* raised plantlets acclimatized in a greenhouse were closely similar to the mother plants in morphology.

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Introduction
The Orchidaceae represents a peak in the evolution of monocots and is one of the most successful families of flowering plants, as is clear from its wide distribution and numerous species (20,000–30,000) (Chowdhery 2001). Increased globalization of the floriculture trade has made orchids the most popular cut flower and potted floriculture crop with a wholesale value estimated at $126 million (United States Department of Agriculture 2004). Cymbidium comprises more than 70 natural species and hundreds of manmade hybrids. This rich variety has contributed significantly to the development of the international trade in orchid cut flowers (Vij et al. 2004). Cymbidium mastersii is an epiphytic orchid found principally in the Sikkim-Himalayas and the Khasi Hills of Meghalaya, India, at an altitude of 1700–2300 m (Bose and Bhattacharjee 1980). Its beautiful sweet-scented white flowers explain its high commercial value. Currently, ruthless collection of this species from its natural habitat, linked to unauthorized trading, fulfils market demand. As a consequence, it is becoming increasingly rare in the wild (Hedge 1999).

An alternative source of these plants is clearly needed and mass propagation offers a solution (Stanberg and Kane 1998).

The regeneration and multiplication of orchids through seeds in nature are limited due to suppressed endosperm and requirements of fungal partners (Sungkumlong and Deb 2008). In vitro asymbiotic seed germination of orchids has been frequently used for the production of commercially important orchids and shown to be an efficient tool for the production of orchids for conservation and rehabilitation (Kauth et al. 2006). The in vitro asymbiotic seed germination of orchids by Knudson (1922) and shoot tip culture in Cymbidium by Morel (1964) laid the milestone of orchid tissue culture. Mass propagation protocols of different Cymbidium spp. using different explants such as mature seeds (Chung et al. 1985; Hassain et al. 2010), immature seeds (Shimasaki and Uemoto 1990; Das et al. 2007), flower stalks (Wang 1988), pseudobulbs (Shimasaki and Uemoto 1990), shoot segments (Nayak et al. 1997), flower buds (Shimasaki and Uemoto 1991), protocorms (Corrie and Tandon 1993), protocorm-like bodies (PLBs) (Begum et al. 1994), thin cell layers (TCLs) of PLBs (Malabadi et al. 2008) and artificial seeds based on PLBs (Nhut et al. 2005) have been standardized. In vitro seed germination of orchids is greatly influenced by several factors like seed age, nutrient media and organic carbon source, etc. The development of protocorms from germinated seeds and the subsequent induction of secondary protocorms or PLBs, from different tissues as explants has become a reliable method for breeding orchids. Propagation by the formation of PLBs is a preferred option because a large number of PLBs can be obtained in a short period of time. Protocorm-like bodies can proliferate and regenerate rapidly into complete plantlets (Liu et al. 2003). 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 appropriate nutrient medium (Ng and Saleh 2011).

Although C. mastersii faces rapid destruction due to exploitation and also urbanization and habitat loss, there is, to date, no published information on how it may be effectively mass propagated in vitro. We have addressed this shortcoming by developing an efficient protocol for rapid propagation of C. mastersii starting with in vitro asymbiotic seed germination, leading to PLB induction followed by plantlet development and successful ex vitro acclimation. Our present study constitutes the first report of the large-scale production of C. mastersii.

Materials and methods
Seed source and sterilization
Seven-month-old fruit capsules of C. mastersii were collected from plants grown in the greenhouse of the Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, India. Capsules were washed with 2–3 drops of Tween 20 and surface-disinfected in 0.1 % mercuric chloride solution for 2 min followed by 70 % ethanol for 30 s and surface flaming. Finally, the capsules were rinsed five times with sterile distilled water and dried. All subsequent work was carried out aseptically in laminar flow cabinets. The capsules were then dissected longitudinally with a surgical blade and extracted seeds were spread thinly over the surface of liquid culture medium contained in 25 × 150 mm glass test tubes (20 test tubes per capsule) each containing 10 ml of medium.

Media and culture conditions
Four nutrient media were compared for their suitability as germination media and for protocorm development: Murashige and Skoog (MS), half-strength MS, Knudson ‘C’ (KC), and Vacin and Went (VW) (Murashige and Skoog 1962; Knudson 1946; Vacin and Went 1949). All media were supplemented with 3 % sucrose (w/v) and solidified with 0.8 % agar (‘Hi media’, India). The pH was adjusted to 5.8 prior to autoclaving at 121 °C,
15 p.s.i. for 15 min. The cultures were maintained at 25 ± 2 °C and 75 % relative humidity under cool fluorescent light at 50 μmol m⁻² s⁻¹ (Philips, India) with a 14-h photoperiod. Seed germination data were recorded 60 days after inoculation of seeds, whereas different developmental stages of protocorms (i.e. percentage of protocorms with vegetative apex, plantlets with 2–3 leaves and 1–2 roots) were recorded every 30 days.

**Growth regulators**

The most effective medium for seed germination and protocorm development was supplemented with different concentrations of 6-benzylaminopurine (BAP), kinetin (KN), α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) (2.5, 5.0, 10.0 μM) alone and in combination to study their effectiveness in promoting proliferation of protocorms and subsequent plantlet development. Sixty-day-old primary protocorms (one protocorm per test tube) were used for the experiment. Data were recorded after 60 days in culture. Means of 20 test tubes were taken for each treatment and all experiments were repeated three times.

**Rooting and acclimatization**

For root induction, 6-month-old plantlets without roots obtained from the above experiments were cultured on full-strength MS medium supplemented with different concentrations (5.0, 10.0 and 15.0 μM) of IBA or NAA. Well-rooted plantlets were rinsed thoroughly with sterile water to remove the residual medium, and transplanted to 8-cm-diameter thermocol/plastic pots containing a potting mixture of coarsely crushed sterile brick and charcoal at 1 : 1 (v/v) and vermicompost (leaf litter + cow dung, 1 : 1 v/v) and kept in the greenhouse of the Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, at 25 ± 2 °C and 70–80 % relative humidity. Plantlets were watered on alternate days. The percentage survival of plantlets was calculated after 90 days in the greenhouse.

**Data analysis**

All results were analysed by one-way analysis of variance. Comparisons of the mean and standard error were determined by Duncan’s multiple range test at P ≤ 0.05. Statistical tests used the SPSS statistical package version 15.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**Asymbiotic seed germination**

Percentage seed germination was highest in MS (93.058 ± 0.56) followed by half-strength MS (85.72 ± 0.66), KC (73.55 ± 0.58) and VW (67.13 ± 0.64) (Table 1). In C. mastersii, an undifferentiated embryo occupies the centre of the seed covered by a transparent integument or testa (Fig. 1A). Germination was marked by swelling and emergence of the embryo from the testa. Seed germination was first evident by swelling and within 5–9 weeks the undifferentiated embryos formed an irregular shaped cell mass—the spherules. After 3–4 weeks, these spherules turned green and formed round structures—the protocorms. After 12 weeks (Fig. 1B), protocorms became visible microscopically at the vegetative apex while rhizoids appeared below (Fig. 1C). This was followed by the development of 2–3 leaf primordia and 1–2 roots (after 14 weeks) when MS basal medium or half-strength MS medium was used. In KC and VW medium, development of protocorms reached the promeristem stage after 12 weeks of culture. Murashige and Skoog basal medium was demonstrably the most effective of the media tested (Table 1). Protocorms cultured in VW medium did not develop beyond the vegetative apex stage, while half-strength MS and KC supported only poor growth of protocorms in comparison with MS medium (Table 1).

**Production of secondary protocorms from primary protocorms**

Sixty-day-old protocorms obtained from MS basal medium were subcultured on fresh MS medium supplemented with different concentrations of BAP (2.5, 5.0, 10.0 μM), KN (2.5, 5.0, 10.0 μM), NAA (2.5, 5.0, 10.0 μM) and IBA (2.5, 5.0, 10.0 μM) alone and in combination. This resulted in the generation of secondary protocorms (PLBs) instead of shoot formation directly (Fig. 2A and B). Protocorm-like body development initiated after 30 days of culture (Fig. 2A). The maximum number of PLBs (20.55 ± 0.62) per protocorm was observed after 60 days of culture in MS medium containing BAP (5.0 μM) and NAA (2.5 μM), followed by BAP (5.0 μM) alone (Table 2). Almost all the PLBs were converted into plantlets (Fig. 2C and D) in the following 20–30 days of the time period, giving rise to multiple numbers of shoots on the same media.

**Rooting and acclimatization**

In attempts to increase the number of roots and their length, plantlets were supplied with different concentration of auxins (Table 3). Murashige and Skoog medium supplemented with 10 μM IBA induced the most roots (7.46 ± 0.09) per shoot (Fig. 2E), followed by 15 μM IBA. In comparison, NAA was much less effective at inducing new root formation and promoting root extension. The plants were acclimatized in a greenhouse after potting up in a mixture of sterile brick and charcoal.
pieces mixed with vermicompost. The percentage survival was 88% after 12 weeks of hardening (Fig. 2F).

Discussion

We show that an in vitro asymbiotic seed germination technique is an effective tool for the mass propagation of *C. mastersii*. If adopted commercially this will reduce the demand for wild-grown material and thus should benefit conservation of the surviving natural populations.

Nutrient requirements for orchid seed germination are thought to be species specific (Arditti and Ernst 1984; Kauth et al. 2008). It was, therefore, not surprising that the various asymbiotic media used in this present study, with their different compositions and concentrations of mineral salts, organic supplements and vitamins, varied in their suitability for in vitro germination. The full-strength MS medium tested contained higher concentrations of macro and micro elements than the half-strength MS, KC and VW media with which we compared it. The nitrogen source plays a major role in effecting orchid seed germination (Van Waes and Debergh 1986; Anderson 1996; Stewart and Kane 2006). This may explain the superior seed germination on MS. It was reported by Kramer and Kozlowaski (1979) that nitrogen present in MS medium greatly influences the growth and differentiation of cells. According to Dohling et al. (2008), the presence of nitrate in the form of ammonium nitrate in MS medium is the most suitable source for seed germination and plantlet development of *Dendrobium longicornu* and *Dendrobium formosum*. Also, pyridoxine, thiamine and nicotinic acid, as vitamins, are absent from KC and VW media but present in the MS media. This, too, may have promoted germination in *C. mastersii*. Our findings parallel those obtained for *Cattleya* spp. by Mead and Bulard (1979). These additional factors included in full-strength MS may also have aided protocorm development.

According to Teixeira da Silva et al. (2006), the development of plantlets from orchid seed can either be direct or indirect through secondary protocorms/PLBs.

### Table 1 Comparative effect of four culture media on asymbiotic germination of seeds and protocorm development of *C. mastersii*.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time required (weeks)</th>
<th>Spherule formation</th>
<th>Protocorm formation</th>
<th>% seed germination (mean ± SE)</th>
<th>Protocorm development (% response)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Stage 1</td>
</tr>
<tr>
<td>MS</td>
<td>5–6</td>
<td>8–9</td>
<td></td>
<td>93.58 ± 0.56a</td>
<td>71.72 ± 1.85</td>
</tr>
<tr>
<td>Half MS</td>
<td>5–6</td>
<td>7–8</td>
<td></td>
<td>85.72 ± 0.66b</td>
<td>61.11 ± 0.45</td>
</tr>
<tr>
<td>KC</td>
<td>7–8</td>
<td>9–10</td>
<td></td>
<td>73.55 ± 0.58c</td>
<td>53.03 ± 0.83</td>
</tr>
<tr>
<td>VW</td>
<td>8–9</td>
<td>11–12</td>
<td></td>
<td>67.13 ± 0.64d</td>
<td>38.44 ± 0.26</td>
</tr>
</tbody>
</table>


Mean values within a column followed by the same letters are not significantly different at P < 0.05 according to Duncan’s multiple range test; n = 20 per treatment conducted in triplicate.
These PLBs are the outcome of continuous division of the outer tissues of protocorms (Huan and Tanaka 2004a, b) or pseudobulbs, rhizomes, roots and shoot segments (Chang and Chang 1998). Protocorm origin holds advantages because a large number of plantlets can be obtained from a single protocorm. Protocorms were shown by Griesbach (1983) to be the most effective explants for PLB regeneration in several orchid species. This may be ascribed to the presence of highly meristematic tissues. In the case of C. mastersii, we observed that protocorms cultured on the MS basal medium alone did not give rise to PLBs. This was perhaps because endogenous auxins and cytokinins are insufficient for PLB development since the addition of auxins and cytokinins resulted in PLB proliferation. Our results revealed optimal concentrations of auxins (NAA or IBA) for PLB generation with yield decreasing at higher levels. The yield of PLBs tended to increase with the cytokinin concentration (BAP and KN), which agrees with findings by Kim and Kako (1982). Primary protocorms responded significantly to a combination of 5.0 μM BAP and 2.5 μM NAA, yielding the highest numbers of PLBs, suggesting a synergistic effect which was also observed in the case of Cymbidium nativity and C. lapine dancer (Fujii et al. 1999). Kusumoto (1978) suggested that the negative effect of NAA on PLB proliferation in the case of cymbidiums was overcome by BA. This was paralleled in our results. In Cymbidium, PLBs were found to regenerate complete plantlets that were closely similar to those arising from somatic embryos (Teixeira da Silva et al. 2006). This was also observed in C. mastersii. Indole butyric acid was found to be the superior auxin for promoting rooting. The maximum number (7.46 ± 0.09) of roots per shoot was recorded in the medium supplemented with 10.0 μM IBA. Giridhar et al. (2001) and Nongdam et al. (2006) have also reported the effectiveness of IBA in the case of the medicinal orchids Vanilla planifolia and Cymbidium pendulum.

Conclusions and forward look

Cymbidium mastersii is an endangered epiphytic orchid of Northeast India of high commercial value. An alternative source of material is needed to satisfy commercial demand.
demands if wild populations are to be saved. In the present study, a successful attempt was made to develop an in vitro system for the mass propagation of *C. mastersii*. This was based on the germination of seeds from a small number of fruit capsules and manipulating their development through primary protocorm production. Each protocorm in turn produced numerous well-rooted plantlets that could be successfully weaned in greenhouse conditions to generate independently growing plants that were indistinguishable from the original parent. The protocol is uncomplicated and will enable commercial production of *C. mastersii*, thereby relieving pressure on the surviving natural population. This study is an initial attempt to mass propagate *C. mastersii* and the method we describe could be rendered commercially successful with the application of bioreactor technology.

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### Contributions by the authors

All the authors contributed to a similar extent overall and have seen and agreed to the submitted manuscript.

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### Conflict of interest statement

None declared.
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