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# Highly Efficient Proliferation and Regeneration of Protocorm-like Bodies (PLBS) of the Threatened Orchid, *Phalaenopsis bellina*

(Proliferasi dan Penjanaan Semula yang Cekap ke atas Jasad seperti Protokorm (PLBS) Orkid Terancam, *Phalaenopsis bellina*)

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ABSTRACT

Phalaenopsis bellina *is an important indigenous fragrant orchid threatened with extinction. In this study, we evaluated the effect of medium strength, sucrose, nitrogen ($NH_4NO_3$) and potato extract on proliferation of* P. bellina *protocorm-like bodies (PLBs) to improve micropropagation in this species. Optimal treatment for PLBs proliferation rate with an average fresh weight (FW) of 0.97±0.16 g was obtained through culturing on half strength (½) MS medium containing 20 g/L sucrose, 15 mM $NH_4NO_3$ and 20% w/v potato extract supplemented with 0.8 µM 2,4 dichlorophenoxyacetic acid (2,4-D). The optimal treatment produced large, healthy and greenish PLBs with reduction in the occurrence of culture browning. In contrast, treatments with high potato extract (>20% w/v) or $NH_4NO_3$ (>30 mM) concentrations tend to have inhibitory effect and resulted in low PLBs proliferation rate, with an average FW of 0.77±0.15 g and 0.69±0.15 g, respectively. Plant regeneration of PLBs was achieved on plant growth regulator (PGR)-free ½ MS medium. In total, 60 healthy PLBs from the optimal treatment were successfully regenerated, acclimatized with 100% survival percentage and grew well in a mixture of soil, sand and vermicompost (8:4:2 (w/w/w)). With the optimal treatment, PLBs proliferation rate was enhanced by 27.63%. Our findings offer an improved micropropagation protocol of the endangered* P. bellina *for conservation and commercial production.*

*Keywords: Indigenous orchid;* Phalaenopsis bellina*; proliferation; protocorm-like bodies; regeneration*

ABSTRAK

Phalaenopsis bellina *merupakan orkid harum asli yang diancam kepupusan. Menerusi kajian ini, kami menilai kesan sukrosa, nitrogen ($NH_4NO_3$) dan ekstrak kentang terhadap proliferasi jasad seperti protocorm (PLBs)* P. bellina *untuk meningkatkan perambatan spesies ini. Rawatan optimum untuk kadar proliferasi PLBs dengan purata berat segar 0.97±0.16 g diperoleh dengan pengkulturan pada separuh kekuatan (½) media MS yang mengandungi 20 g/L sukrosa, 15 mM $NH_4NO_3$ dan 20% w/v ekstrak kentang ditambah dengan 0.8 µM 2,4 asid diklorofenoksiasetik (2,4-D). Rawatan optimum menghasilkan PLBs yang besar, sihat dan hijau dengan pengurangan keperangan kultur. Sebaliknya, rawatan menggunakan kepekatan ekstrak kentang (>20% w/v) atau $NH_4NO_3$ (>30 mM) yang tinggi cenderung memberikan kesan rencatan dan menyebabkan kadar proliferasi PLBs yang rendah dengan purata berat segar masing-masing 0.77±0.15 g dan 0.69±0.15 g. Penjanaan semula tumbuhan daripada PLBs dihasilkan dengan ½ media MS tanpa zat pengatur tumbuhan (ZPT). Secara keseluruhan, 60 PLBs yang sihat berjaya dijana semula dan PLBs dapat menyesuaikan diri dengan 100% kelangsungan hidup dan tumbuh dengan baik di dalam campuran tanah, pasir dan vermikompos (8:4:2 (w/w/w)). Kadar proliferasi PLBs meningkat 27.63% dengan rawatan optimum. Penemuan kami mengusul satu penambahbaikan protokol pembiakan* P. bellina *yang terancam untuk tujuan pemeliharaan dan penghasilan komersial.*

*Kata kunci: Jasad seperti protocorm; orkid asli; penjanaan semula;* Phalaenopsis bellina*; proliferasi*

## INTRODUCTION

The orchid family (Orchidaceae) is one of the largest families of flowering plants consisting of 30,000-35,000 species in 850 genera (Hossain et al. 2013). Orchids are grown as ornamental plants for its high commercial value in horticulture industry. It has become an important export commodity in countries such as Malaysia, Taiwan, Thailand and Singapore.

*Phalaenopsis* or commonly known as 'Butterfly orchid', is one of the most popular orchids in the trade (Huang et al. 2015). The *Phalaenopsis* genus comprises approximately 66 species that can be divided into five subgenera, namely *Proboscidioides*, *Aphyllae*, *Parishianae*, *Polychilos* and *Phalaenopsis* (Yu et al. 2006). These species are widely distributed in the Asia (India, Malaysia and Taiwan), Australia and the larger islands of the Pacific Ocean (Huang et al. 2015). The plant is an epiphyte with fleshy, copious and adventitious roots arising from the base and lower nodes of stem. The stem is short and leaftly and it does not form pseudobulb. All

*Phalaenopsis* species have uniform chromosome number (2n = 38), but varied in karyotypes and genome sizes (Jing et al. 2015). The *Phalaenopsis* species and hybrids are of high commercial value in floriculture because of their elegance appearance, long-lasting, versatile colour and orderly arranged flowers.

*Phalaenopsis bellina*, classified in subgenus *Polychilos*, is a fragrant orchid endemic to Borneo and Malaysia. The unique features of *P. bellina* include a purplish inner base of the lateral sepals, strong sweet floral fragrance and flowering all year round (Christenson & Whitten 1995). Due to its strong fragrant feature, *P. bellina* is often used as a donor plant to produce novel *Phalaenopsis* varieties with new fragrance (Shrestha et al. 2007). Over the years, high market demand, over collection, habitat destruction and slow multiplication rate caused the species seriously threatened with extinction. Moreover, the International Union for Conservation of Nature (IUCN) has listed *P. bellina* as an endangered species (Chadburn 2013). Thus, conservation of this exotic species is of great importance before the population reaches the verge of extinction.

In general, monopodial orchids like *P. bellina* is difficult and recalcitrant to vegetative propagation. Therefore, *in vitro* micropropagation through tissue culture technique offers a good alternative to conserve and mass propagate this species. Several tissue culture techniques have been reported for *Phalaenopsis* micropropagation using explants such as shoot tips (Ken & Mashiro 1993), buds (Chen & Piluek 1995), rhizome (Park et al. 2002) and protocorm-like bodies (PLBs) (Maziah & Chew 2008). The success of *in vitro* micropopagation is greatly influenced by plant genotypes, type of explant and medium composition. PLBs consisting actively dividing meristematic tissues are often the favourable choice of explant for orchid micropropagation due to its high regeneration efficiency. Besides, proper manipulation of medium composition with appropriate medium strength, plant growth regulators (PGRs), sucrose, nitrogen and organic additive concentrations are essential to ensure the success of mircropropagation. To date, most of the developed micropropagation protocols for *Phalaenopsis* were based on *P. amabilis* and the micropropagation technique for *P. bellina* remains largely untapped.

Hence, following our previous report on micropropagation of *P. bellina* through PGRs manipulation (Maziah & Chew 2008), this study was undertaken to improve the micropropagation protocol by evaluating the effect of medium strength, $NH_4NO_3$, sucrose and potato extract on PLBs proliferation of *P. bellina*. This study was also extended to ascertain whether the manipulation of these parameters have influence on the PLBs morphology, survival percentage and plantlet regeneration efficiency. Ultimately, it is our goal to establish a more efficient micropropagation technique to conserve and mass propagate the endangered *P. bellina*.

## Materials and Methods

### Induction and establishment of PLBs

Three month old *in vitro* seedlings of *P. belina* used in this study were from the nursery. Leaf segment (1.0 × 1.0 $cm^2$) excised from the young and healthy seedlings was used to induce PLBs according to protocol described in Maziah and Chew (2008). The PLBs were maintained on Murashige and Skoog (MS) medium (Murashige & Skoog 1962) supplemented with 0.8 µM 2,4 dichlorophenoxyacetic acid (2,4-D), 30 g/L sucrose and 3 g/L gelrite. The pH of the medium was adjusted to 5.8. All cultures were maintained as described previously (Lai et al. 2014, 2011) with temperature at 24±2°C and 16 h photoperiod of 25 µmol $m^{-2}$ $s^{-1}$ white fluorescent light.

### Influence of MS medium, sucrose, nitrogen and potato extract on proliferation of PLBs

Effect on PLBs proliferation was evaluated by using different MS strength (¼, ½, 1 and 2X) with various concentrations of sucrose (10, 20, 30 and 40 g/L), nitrogen ($NH_4NO_3$) (7.5, 15, 30 and 45 mM) and potato extract (5, 10, 20 and 30% w/v) supplemented with 0.8 µM 2,4-D. These parameters were tested independently. Each parameter consisted of 10 replicates with six PLBs per replicate (one PLB weight was 0.15 g). The average fresh weight (FW), morphology, percentage of culture browning and survival of proliferated PLBs were recorded at eight weeks old culture.

### PLBs regeneration and acclimatization

The eight weeks old of proliferated PLBs were transferred into plant growth regulator (PGR)-free ½X MS medium for plant regeneration. Rooted plants with 2-4 leaves were washed thoroughly in distilled water to remove any adhering medium. The plantlets were transplanted into plastic pot containing garden soil, sand and vermicompost in a ratio 8:4:2 (w/w/w). The plantlets were watered twice weekly and placed under a control conditions (25±2°C, 16-h photoperiod of 40 µmol $m^{-2}$ $s^{-1}$ white fluorescent light). After two weeks of acclimatization, the plantlets were grown in greenhouse and the percentage of PLBs plantlet regeneration was recorded.

## Statistical Analysis

All the experiments were conducted as completely randomized design. The average FW was calculated as the mean number of proliferated PLBs from 10 replicates. The data was analyzed by one-way ANOVA and mean values were compared by Tukey's multiple range test at 5% ($p$=0.05) significance level using software SPSS version 24.0 (SPSS Inc. USA).

## Results and Discussion

*In vitro* micropropagation through tissue culture technique offers a viable route for high quality plant material for conversation and commercial production. Proper management of explant source, genotype, medium strength and medium composition are prerequisite to ensure the success of *in vitro* micropropagation. A number of complex organic additives such as banana pulp, corn extract, peptone, slap honey and potato extract have also been shown to enhance and improved plant growth (Rosmah et al. 2010). These extracts are crucial in providing nutrients and growth factors for plant development (Nambiar et al. 2012).

In our laboratory, *in vitro* micropopagation protocol for *P. bellina* PLBs through the supplementation of 0.8 µM 2,4-D is used (Maziah & Chew 2008). Despite being the commonly used medium, there is a need to revise the existing formulation for more efficient micropropagation of *P. bellina* PLBs. Moreover, the effect of medium strength, medium composition and addition of organic additive such as potato extract on micropropagation of *P. bellina* PLBs had not been reported previously. Hence, we sought to refine the medium formulation by evaluating the effect of different medium strength, medium composition and as well as addition of potato extract on proliferation rate of *P. bellina* PLBs. Based on the results obtained (Table 1), half strength MS medium supplemented with 30 g/L sucrose and 0.8 µM 2,4-D was found to be the best medium strength for PLBs proliferation with an average FW of 0.76 ± 0.15 g followed by full (FW: 0.67 ± 0.15 g), quarter (FW: 0.53 ± 0.18 g) and double (FW: 0.31 ± 0.11 g) strengths of MS medium. In general, the MS medium is classified as high salt medium with high level of nitrogen, potassium and some micronutrients such as boron and manganese (Cohen 1995). Although it is commonly used in plant tissue culture, this MS medium is not necessarily always optimal for growth of all plant types. In fact, study showed that halving the strength of the MS medium resulted in significant increase of growth and proliferation of *Dendrobium* PLBs (Budi & Teixeira Da Silva 2015). The beneficial effect of reducing the strength of the MS medium could be associated with particular components of the medium. Minor changes in the medium components could greatly affect the plant organogenesis *in vitro* (Dani et al. 2010). Hence, half strength of MS medium being the best medium strength obtained in this study is in accordance with the results reported from other studies.

To further refine the medium composition, we also investigated the effect of different sucrose concentrations on PLBs proliferation. Our results showed that ½ MS medium with 20 g/L sucrose and 0.8 µM 2,4-D gave the highest PLBs proliferation rate with an average FW of 0.81±0.17 g (Table 1). In contrast, medium supplemented with 10 g/L sucrose gave the lowest PLBs proliferation rate with an average FW of 0.57±0.10 g (Table 1). Sucrose acts as carbon source for plant growth and development *in vitro*. It functions in signaling of plant life cycle (Chandran et al. 2006) and acts as osmotic agents to facilitate the uptake of the constituents from medium (Nowak et al. 2004). It is also for proper maintenance of the structure and semi-permeability of the plasma membrane (Chandran et al. 2006). Since sucrose plays an important role in plant tissue culture, adequate supply of sucrose is essential for optimal plant growth. It has been reported that high amount of sucrose may have inhibitory effect on nutrient uptake by inducing osmotic stress (Shohael et al. 2006) and lowering water potential of the medium (Johnson et al. 2011; Shim et al. 2003). Increasing sucrose concentration in the medium significantly affects the development of *Bletia purpurea* orchid. Therefore, 20 g/L sucrose is appropriate for *P. bellina* PLBs as this gave a better proliferation rate.

Nitrogen is among the essential nutrients known to have the greatest impact on plant growth and tends to be species specific (Marschner 1995). Numerous studies have shown that the yield of horticultural crops can be greatly affected by the ratio of nitrogen (Toor et al. 2006). Despite this, study on the effect of nitrogen ratio on orchids, particularly *Phanaelopsis* is lacking. Hence, we examined the effect of different $NH_4NO_3$ concentrations on *P. bellina* PLBs proliferation. Addition of 15 mM $NH_4NO_3$ to the ½ MS medium supplemented with 20 g/L sucrose and 0.8 µM 2,4-D gave an optimal PLBs proliferation with an average FW of 0.88 ± 0.20 g (Table 1). Previously, orchid has been known for its slow growth, mainly attributed by its sluggish N metabolism (Poddubnaya-Arnold 1967). Hence, addition of optimum nitrogen concentration can enhance the orchid growth as shown in this study. Consistent with our result, it has been reported that addition of nitrogen in medium increased the growth of *Cymbidium* PLBs (Teixeira Da Silva 2013). However, at higher $NH_4NO_3$ concentration (>30 mM), the proliferation rate of *P. bellina* PLBs was greatly reduced (Table 1). This inhibitory effect could be clearly seen on the increase of browning occurrence (16.6%) with smaller size of PLBs cultured on medium fortified with 45 mM $NH_4NO_3$ (Table 2; Figure 1(c), 1(g), 1(h)). Moreover, these PLBs also recorded a lower survival percentage of 83.4% (Table 2). Similarly, high level of nitrogen inhibiting the growth of *Cymbidium kanran* shoots was noted, but moderate level of nitrogen promoted shoot growth (Shimasaki & Uemoto 1990). Therefore, proper manipulation of nitrogen ratio is of great importance to achieve an optimal PLBs growth.

Addition of complex organic additives is a common practice to promote orchid growth. Besides being a natural carbon source, complex organic additives contain fiber, hormones, vitamins, phenols, proteins and minerals needed for orchid development (Namibiar et al. 2012). Consistent with its essential role in plant growth and development, medium supplemented with 20% w/v of potato extract gave the highest PLBs proliferation rate with an average FW of 0.97 ± 0.16 g (Table 1). Our results also in agreement with studies on the growth enhancement effect of potato extract in *Cattleya* (Islam et al. 2000), *P. gigantea* (Rosmah et al. 2010) and *Vanda roxburgii* (Islam et al. 2011). Perhaps, the

TABLE 1. Effects of medium strength, sucrose, $NH_4NO_3$ and potato extract on PLBs proliferation

| Treatment | | | | Mean | PLBs |
|---|---|---|---|---|---|
| Medium strength (X) | Sucrose (g/L) | $NH_4NO_3$ (mM) | Potato extract (% w/v) | fresh weigth (g) | size and morphology |
| 1/4 | 30.0 | 0.0 | 0.0 | $0.53 \pm 0.18^{ab}$ | small, greenish |
| 1/2 | 30.0 | 0.0 | 0.0 | $0.76 \pm 0.15^{c}$ | moderate, greenish |
| 1 | 30.0 | 0.0 | 0.0 | $0.67 \pm 0.15^{b}$ | small, greenish |
| 2 | 30.0 | 0.0 | 0.0 | $0.31 \pm 0.11^{a}$ | small, greenish |
| 1/2 | 10.0 | 0.0 | 0.0 | $0.57 \pm 0.10^{b}$ | small, greenish |
| 1/2 | 20.0 | 0.0 | 0.0 | $0.81 \pm 0.17^{c}$ | large, greenish |
| 1/2 | 30.0 | 0.0 | 0.0 | $0.76 \pm 0.15^{c}$ | moderate, greenish |
| 1/2 | 40.0 | 0.0 | 0.0 | $0.62 \pm 0.11^{b}$ | small, greenish |
| 1/2 | 20.0 | 7.5 | 0.0 | $0.83 \pm 0.15^{b}$ | large, greenish |
| 1/2 | 20.0 | 15.0 | 0.0 | $0.88 \pm 0.20^{c}$ | large, greenish |
| 1/2 | 20.0 | 30.0 | 0.0 | $0.75 \pm 0.16^{bc}$ | moderate, slight browning |
| 1/2 | 20.0 | 45.0 | 0.0 | $0.69 \pm 0.15^{a}$ | small, browning |
| 1/2 | 20.0 | 15.0 | 5.0 | $0.86 \pm 0.13^{a}$ | large, greenish |
| 1/2 | 20.0 | 15.0 | 10.0 | $0.90 \pm 0.12^{a}$ | large, greenish |
| 1/2 | 20.0 | 15.0 | 20.0 | $0.97 \pm 0.16^{b}$ | large, greenish |
| 1/2 | 20.0 | 15.0 | 30.0 | $0.77 \pm 0.15^{c}$ | moderate, browning |

All data are the mean ± Standard Deviation of ten replicates after eigth weeks of culture. Different letters within a column indicate a significant difference at $p<0.05$ level

presence of niacin or thiamine in potato extract may have accounted for this positive growth effect. Nevertheless, higher concentration of potato extract (>20% w/v) decreased PLBs proliferation rate with an average FW of 0.77±0.15 g. In addition, occurrence of PLBs browning (13.3%) with reduced survival percentage (86.7%) was also observed on PLBs cultured in medium supplemented with 30% w/v potato extract (Table 2). Taken together, ½ MS medium containing 20 g/L sucrose, 15 mM $NH_4NO_3$ and 20% w/v potato extract supplemented with 0.8 µM 2,4-D is the optimal treatment obtained in this study for rapid *P. bellina* PLBs proliferation. Using the optimal treatment, PLBs proliferation rate was enhanced by 27.63% as compared to the medium reported by Maziah and Chew (2008). Besides, the optimal treatment also produces large, compact and greenish PLBs with 100% survival percentage (Table 2; Figure 1(b), 1(e), 1(f)). Moreover, small young leaves and roots were often seen derived from the PLBs cultured using optimal treatment (Figure 1(e), 1(f)), indicating an actively dividing and rapid cell growth.

To evaluate the effect of optimal treatment on PLBs plantlet regeneration efficiency, a total of 60 PLBs cultured using optimal treatment were transferred into PGR-free medium. After a week, most of the PLBs developed protrusion in the anterior site and posterior region (Figure 2(a)). These protrusion regions formed sheath leaves (Figure 2(b), 2(c)) that eventually enlarged and differentiated into shoots with well-developed leaves (Figure 2(d)). At maturity, the basal regions of the PLBs contracted and dislodged from the parent PLBs to form rooted plantlet (Figure 2(e)). In short, well developed PLBs plantlets with both shoots and roots were produced after four to five months cultured on PGR-free medium (Figure 2(f)). These plantlets were then planted into plastic pot (Figure 2(g)) and acclimatized for two weeks before transferring to greenhouse. All plantlets were successfully acclimatized with 100% regeneration and survival

TABLE 2. Effects of different treatments on PLBs browning, survival percentage and plantlet regeneration

| Treatment | PLBs browning (%) | PLBs survival (%) | PLBs plantlet regeneration (%) |
|---|---|---|---|
| ½ MS + 15 mM $NH_4NO_3$ + 20% w/v potato extract | $0.0^{a}$ | $100.0^{a}$ | $100.0^{a}$ |
| ½ MS + 45 mM $NH_4NO_3$ + 20% w/v potato extract | $16.6^{b}$ | $83.4^{c}$ | $98.7^{b}$ |
| ½ MS + 15 mM $NH_4NO_3$ + 30% w/v potato extract | $13.3^{c}$ | $86.7^{b}$ | $99.4^{b}$ |

All data are the mean ± Standard Deviation of ten replicates with six PLBs per replicate. Different letters within a column indicate a significant difference at $p<0.05$ level

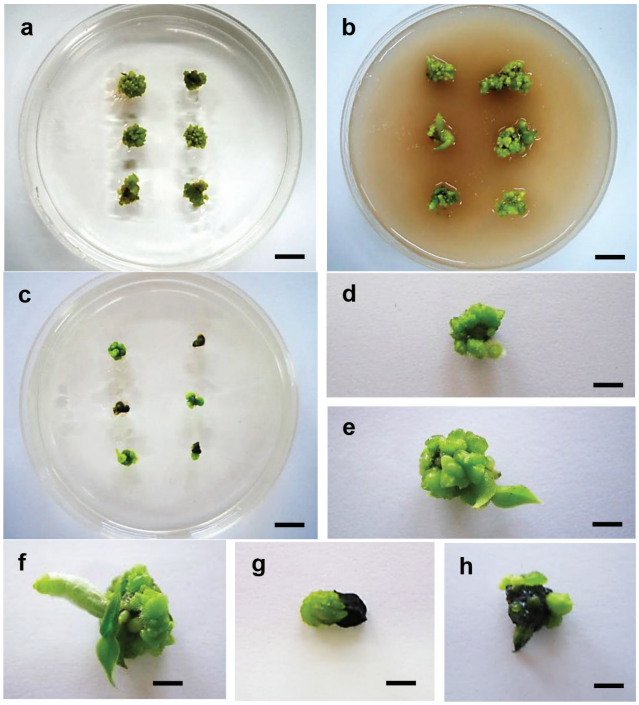


FIGURE 1. Effects of different treatments on PLBs proliferation. The morphology of PLBs after 8 week old culture on ½ MS medium containing (a, d) 20 g/L sucrose + 0.8 µM 2,4-D; (b, e, f) 20 g/L sucrose + 15 mM $NH_4NO_3$ + 20% w/v potato extract + 0.8 µM 2,4-D and (c, g, h) 20 g/L sucrose + 45 mM $NH_4NO_3$ + 0.8 µM 2,4-D. Bar a-c is 1 cm and bar d-h is 0.5 cm

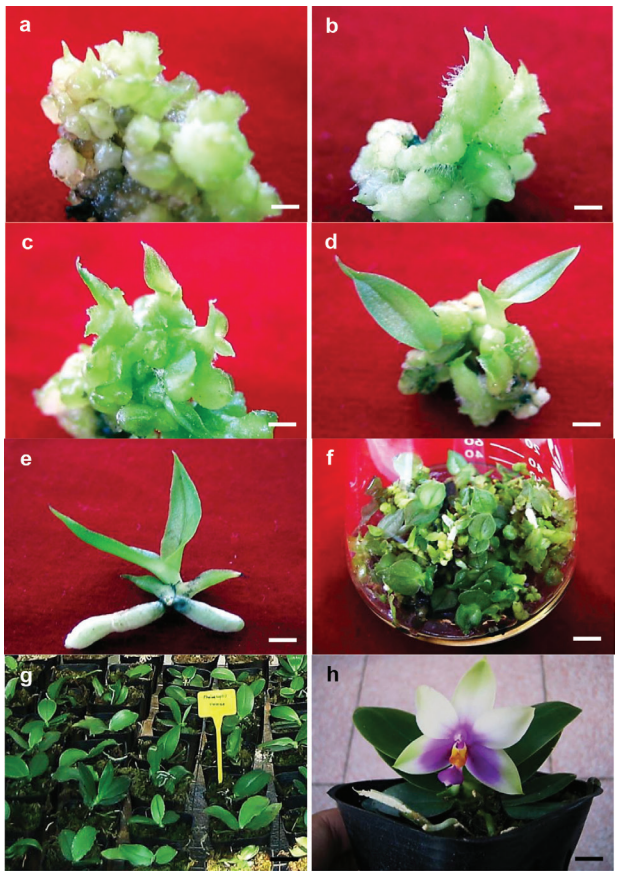


FIGURE 2. *P. bellina* plantlet regeneration from PLBs cultured in PGR-free medium. (a) PLBs with protrusion in anterior region after 1 week culture; (b,c) PLBs with sheath leaves after 1 month culture; (d) PLBs differentiated into shoots with well-developed leaves after 2 month culture; (e) A rooted plantlet after 4 month culture; (f) Clusters of PLBs with well-developed leaves and roots; (g) Plantlets 2 week after acclimatization; (h) Potted plantlet that bear the first flower after 6 month growing in greenhouse. Bar a-e: is 0.2 cm, bar f,h is 2 cm and bar g is 10 cm

percentage in a mixture of soil, sand and vermicompost (8:4:2 (w/w/w)) (Table 2; Figure 2(g)), that eventually gave first flower after 6 months of growing in greenhouse (Figure 2(h)). Unlike PLBs treated with optimal treatment, PLBs cultured on medium with high concentrations of $NH_4NO_3$ (45 mM) or potato extract (30% w/v) recorded slightly reduced in percentage of plantlet regeneration efficiency with 98.7% and 99.4% respectively (Table 2).

## Conclusion

Our study has shown that proper manipulation of medium strength, sucrose, $NH_4NO_3$ and potato extract could significantly enhanced PLBs proliferation of *P. bellina*. Half strength of MS medium containing 20 g/L sucrose, 15 mM $NH_4NO_3$ and 20% w/v potato extract supplemented with 0.8 μM 2,4-D is an optimal treatment for PLBs proliferation. Using the established optimal treatment, the PLBs proliferation rate could increase to 27.63%. Taken together, we have successfully developed an improved micropropagation protocol for efficient PLBs proliferation and plantlet regeneration of *P. bellina*. This protocol allows for sustainable high quality of *P. bellina* PLBs to be produced rapidly for conservation and commercial production.

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