

# Protocorm-like body proliferation of *Grammatophyllum speciosum* through asymbiotic seed germination

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**ABSTRACT:** Viability of *Grammatophyllum speciosum* seeds was evaluated by a TTC (2,3,5-triphenyl tetrazolium chloride) test. The seeds were then asymbiotically germinated on different agar media under aseptic conditions. The results showed that keeping the seeds in the dark at 30 °C for 1 day gave 99% of red staining embryos (referred to as viable). After sowing the seeds on half-strength Gamborg's B5 medium supplemented with 0.1% activated charcoal (AC) for 30 days, germination of those seeds was observed. Half-strength MS medium (½ MS) supplemented with 1 mg/l 6-benzylaminopurine in combination with 0.5 mg/l  $\alpha$ -naphthalene acetic acid, 15% coconut water, 0.2% AC, and 0.7% agar gave the highest percentage of protocorm-like body (PLB) induction and proliferation. The PLBs could be regenerated into complete plantlets with vigorous shoot and root formation on ½ MS medium without any plant growth regulators. The survival rate of plantlets was 70% after hardening off in the greenhouse for 1 month.

**KEYWORDS:** in vitro, tiger orchid, micropropagation

## INTRODUCTION

The Orchidaceae, a highly evolved, diverse, and successful families of flowering plants, constitutes about 40% of the monocotyledonous taxa. There is a great demand for potted orchids and cut flowers in international as well as domestic markets<sup>1</sup>. Orchidaceae is the largest and the most specialized family of flowering plants. All the species in this family are listed in the endangered species of wild fauna and flora in Appendix II of the Convention of International Trade<sup>2</sup>.

*Grammatophyllum speciosum* Blume or tiger orchid is a beautiful endangered species which is also the largest orchid plant in the world, in addition to the largest epiphytes in general. Conservation effort has been challenged to propagate and reintroduce species that have special horticultural or educational value<sup>3</sup>.

Tissue culture technique was firstly introduced and applied to *Cymbidium* orchids by Morel<sup>4</sup>. By this technique, culture methods, culture media, and

various explants for orchid tissue culture were introduced and developed by several workers. Shoot tips are the most frequently used for orchid tissue culture. However, protocorms derived from germinating seeds and protocorm-like bodies (PLBs) are also applied<sup>5-7</sup>. Culture medium, namely Vacin and Went (VW)<sup>8</sup> and Murashige and Skoog (MS)<sup>9</sup>, are usually employed for in vitro orchid culture<sup>5,10,11</sup>. Half strength MS medium is also used successfully for orchid culture<sup>12</sup>.

In *G. speciosum*, the first report on an in vitro establishment for micropropagation has been described by Sopalan<sup>13</sup>. In this report, PLBs were induced from shoot tips. The explants were incubated in ½ MS liquid medium containing 2% (w/v) sucrose without any plant growth regulators (PGRs). In this study, effort has been made to develop a protocol for the in vitro propagation or conservation of *G. speciosum*. The goal of this study was to establish an effective propagation system for the large-scale propagation to meet commercial needs and to re-establish this threatened orchid species back into the original habitats.

## MATERIALS AND METHODS

### Plant materials

The capsules or pods of *G. speciosum* orchid were collected from plants with self-pollination for 150 days after pollination (DAP). The capsules were harvested in March 2012 at Suratthani province. The capsules were brought to the laboratory for disinfection and culture.

### Seed morphology and viability studies

For seed viability analysis, freshly isolated seeds from capsules were stained with 1% (v/v) 2, 3, 5-triphenyl tetrazolium chloride (TTC) in darkness overnight under shaking condition at 5 rpm for 30 min and kept under static condition at 30 °C for 24 h. The seeds were then observed under a stereomicroscope. Percentage of seed viability was calculated by the number of red colour staining embryos divided by the total number of seeds examined and multiplied by 100.

### Effects of basal media on seed germination

To determine the effects of basal media on seed germination and subsequent development to protocorms, capsules at 150 DAP were used as seed explants. The capsules were disinfected and cut vertically over a sterile Petri-dish. The seeds were then removed and placed on the filter paper. The seeds were surface sterilized again by dipping into 15% (v/v) sodium hypochlorite (NaOCl) solution containing 0.5% (v/v) Tween-20 for 10 min, rinsed three times with sterile distilled water, and placed on half strength Gamborg's B-5 (½ B5) or New Dogashima (ND) or half strength Murashige and Skoog (½ MS) medium supplemented with 0.1% activated charcoal (AC) and kept at 25 ± 1 °C with a 16/8 h photoperiod. All experiments were performed in three independent replicates with 10 culture Petri dishes per replication. In each Petri dish, one gram of seeds (approximately 815 seeds) was used. The germination percentage of seeds at 30 days was calculated by the number of germinated seeds or protocorms divided by the number of seeds cultured and multiplied by 100. The seed-derived protocorms were used in these experiments related to PLB induction and proliferation.

### PLB induction, proliferation, and plantlet regeneration

For PLB induction, seed-derived protocorms at 3 months of culture (approximately 5 mm in length) from previous step were transferred to ½ MS medium supplemented with 1 mg/l of 6-benzylaminopurine

(BA) alone or in combination with 0.5 mg/l of  $\alpha$ -naphthalene acetic acid (NAA), with or without 0.2% AC, 15% coconut water (CW) and 0.7% agar. Ten explants were put in each culture bottle and six culture bottles were used for each treatment. The percentage of PLB formation and the number of PLBs per explant were recorded after 8 weeks of culture. The proliferation rate was also calculated after 10 weeks of culture.

For plantlets developed from PLBs, the cluster of PLBs (3–5 PLBs) were cultured on ½ MS medium without any plant growth regulators or supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA, 15% CW, 0.2% AC, and 0.7% agar. The number of shoots was recorded after 3 months of culture.

All cultures were maintained at 25 ± 2 °C under a 16 h photoperiod with light at an intensity of 10  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density. The experiments were established in a completely randomized design. ANOVA and Duncan's multiple range test (DMRT) were used for comparison among the treatment means.

### Acclimatization

Six-month-old-vitro-grown complete plantlets were rinsed under running water to remove agar, soaked in 0.2% Bavistin for 1 h, transferred to 1.5 inch plastic pots containing sterilized coconut peat and tree fern (1:1), and placed in trays. Those plantlets were grown in the greenhouse under 60% shading and control relative humidity at 80%. One hundred plantlets were used for 5 replications, 20 plantlets per replication. After 1 month of acclimatization, the percentage of survival plantlets was recorded.

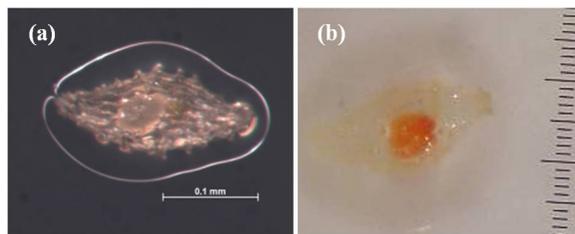
## RESULTS AND DISCUSSION

### Seed morphology and viability

The seed viability test, as stained by TTC, showed the presence of non-staining small embryos in their seeds (Fig. 1a) and red staining (Fig. 1b). The viability was recorded to be 99.02%. Those embryos could germinate in an appropriate culture medium and environment. In addition, embryos or seeds could germinate because they were completely developed<sup>14</sup>.

### Effects of basal media on seed germination

The orchid seeds cultured on ½ B5 medium supplemented with 0.1% AC for 30 days gave the highest germination percentage at 97%; whereas, ½ MS medium and ND medium gave only 76% and 67%, respectively (Table 1, Fig. 2). During sowing seeds in this culture medium, seed germination occurred after the imbibition, which resulted in swelling of the



**Fig. 1** Viable seeds of *G. speciosum* evaluated by TTC staining; (a) a non-red stained embryo; (b) a red stained embryo.

**Table 1** Percentage of seed germination after 30 days of culture.

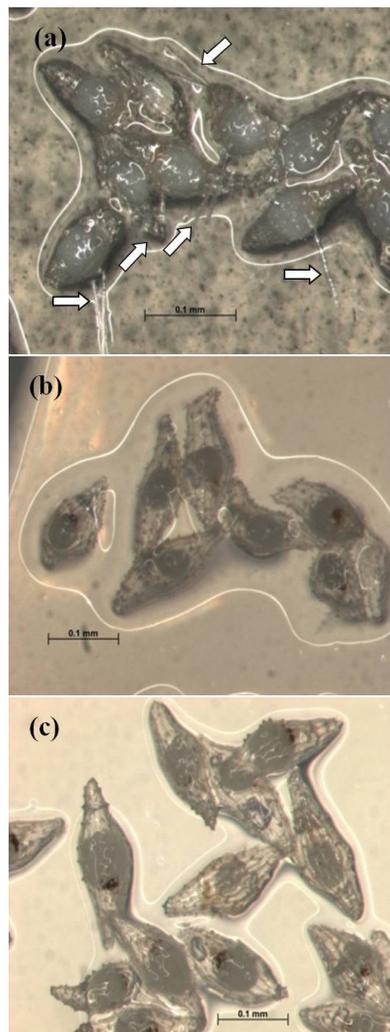
Medium	Germination (%)
½ B5	96.9 <sup>a</sup>
½ MS	76.5 <sup>b</sup>
ND	66.7 <sup>c</sup>
F-test	$p \leq 0.01$
C.V. (%)	4.3

Means followed by different letters within a column are significantly different by DMRT.

embryos, and then the seed coats were ruptured by the emerging of germinated embryos, so the embryo enlargement and the papillae appeared on the one end of protocorms (Fig. 2a). Since seed viability was high, the effects of ½ MS and ND media on lower seed germination were confirmed. Hence ½ B5 medium is recommended for asymbiotic germination of *G. speciosum*. Moreover, asymbiotic germination technique is much easier than symbiotic germination.

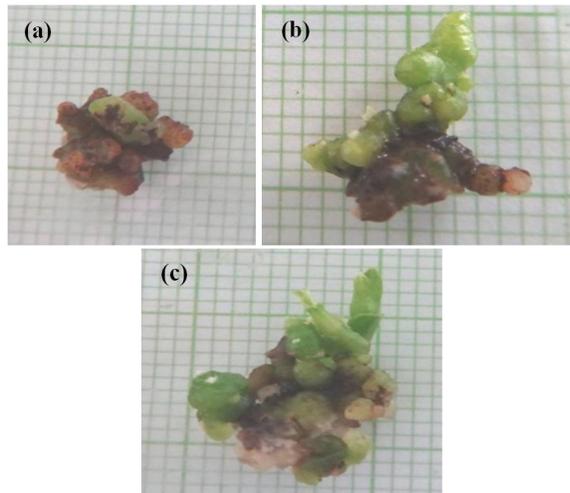
Asymbiotic seed germination of orchid seeds is an efficient propagation method for large-scale production of orchid plants. However, ½ B5 and ND media have never been applied to promote seed germination in *G. speciosum* before. An in-depth review on in vitro orchid seed germination was reported by Kauth et al<sup>15</sup>. Many protocols for in vitro seed germination, in orchids have been described<sup>14,16</sup>. In those reports, seed germination and development were significantly influenced by capsule maturity and pretreatment of seeds, medium compositions, culture conditions, culture methods, as well as other conditions. Some experimental results, such as the germination percentage of seeds of the same species on the same medium, were inconsistent or even contradictory<sup>16</sup>.

For propagation of *G. speciosum* in vitro, Sopalun et al<sup>13</sup> reported the induction of PLBs from shoot tips in ½ MS liquid medium without any plant regulators (PGRs). This study showed that the seeds germinated



**Fig. 2** Germination of *G. speciosum* seeds after 30 days of culture: (a) on ½ B5 medium which showed the enlargement of embryos and papillae (arrow), (b) on ND medium, and (c) on ½ MS medium.

well on ½ B5 solidified medium but the seeds cultured on ½ MS medium resulted in a larger protocorm enlargement after 3 months of culture. Similar results were obtained by Abbas et al<sup>17</sup> who reported successful propagation of *G. scriptum* by seed germination in vitro. The best medium for germination of *G. scriptum* was Knudson C medium supplemented with 30% CW and the best medium for seedling development was ½ MS medium supplemented with 40% CW. Moreover, Zhang et al<sup>18</sup> reported that full strength of MS medium contains high ionic concentration of nutrient salts; therefore, ½ MS could adequately support rapid production of protocorms in orchids. Thus the ½ MS medium was suitable for PLB induction in this study.



**Fig. 3** Development of new PLBs from culturing a protocorm (a) on  $\frac{1}{2}$  MS medium without PGRs, (b) on  $\frac{1}{2}$  MS medium containing 1 mg/l BA and 0.5 mg/l NAA without AC, and (c) with 0.2% AC.

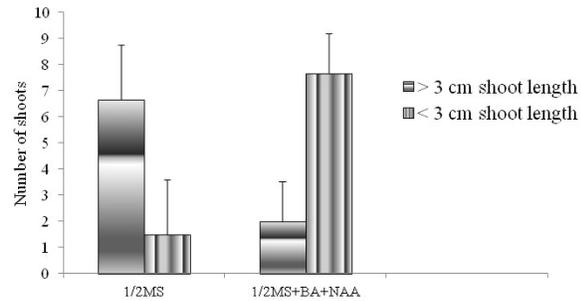
**Table 2** Effects of PGRs and AC on PLB formation and proliferation after 10 weeks of culture.

Medium	AC (%)	PLB formation (%)	No. of PLBs per explant	PLB proliferation (%)
PGR-free $\frac{1}{2}$ MS	0.0	21.7 <sup>b</sup>	2.91 <sup>d</sup>	6.7 <sup>c</sup>
	0.2	28.3 <sup>b</sup>	4.63 <sup>c</sup>	21.7 <sup>b</sup>
$\frac{1}{2}$ MS+BA+ NAA	0.0	46.7 <sup>a</sup>	5.84 <sup>b</sup>	11.7 <sup>bc</sup>
	0.2	63.3 <sup>a</sup>	6.87 <sup>a</sup>	46.7 <sup>a</sup>
F-test		$p \leq 0.01$	$p \leq 0.01$	$p \leq 0.01$
C.V. (%)		36.4	12.0	39.1

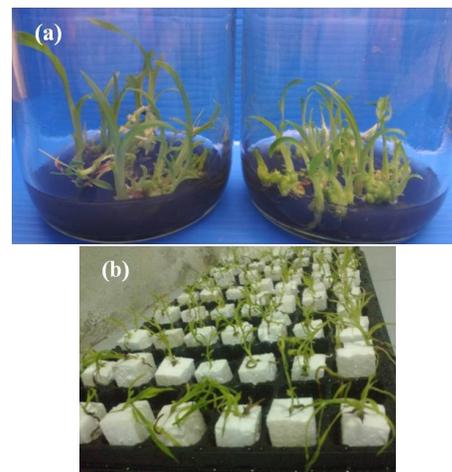
Means followed by different letters within a column are significantly different by DMRT.

### PLB induction, proliferation, and plant regeneration

The highest percentage of PLB formation (63%) was obtained on  $\frac{1}{2}$  MS medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA, 15% CW, 0.2% AC and 0.7% agar. Statistically significant difference was observed among treatments. Culture media supplemented with AC seemed to induce PLB proliferation better than culture medium without AC (Fig. 3). AC adsorbed the inhibitory phenolic and carboxylic compounds produced by the tissues in culture<sup>19</sup>, so the medium without AC showed browning of PLBs (Fig. 3b) as a result of low proliferation rate of PLBs (Table 2). Half-strength MS medium without PGRs was suitable for plantlet regeneration which gave the number of shoots (> 3 cm long) at 6.67 shoots. The medium supplemented with BA promoted the PLB proliferation resulted in an inferior growth



**Fig. 4** Shoot development from PLBs cultured on  $\frac{1}{2}$  MS medium without PGRs and  $\frac{1}{2}$  MS containing 1 mg/l BA, 0.5 mg/l NAA and 0.2% AC after 3 months of culture.



**Fig. 5** (a) Shoot development from PLBs cultured on  $\frac{1}{2}$  MS medium without PGRs (left) and  $\frac{1}{2}$  MS containing 1 mg/l BA, 0.5 mg/l NAA, and 0.2% AC (right) after 3 months of culture and (b) survival of plantlets after 1 month of acclimatization.

of plantlets (Fig. 4). Similar results were obtained by Abbas et al<sup>17</sup> who found that the best culture medium suitable for seedling development of *G. scriptum* was  $\frac{1}{2}$  MS medium supplemented with 15% CW. The PLBs can be regenerated into complete plantlets with vigorous shoot and root formation after 3 months of culture (Fig. 5a).

### Greenhouse acclimatization

After 6 months of culture on  $\frac{1}{2}$  MS medium without PGRs and supplemented with 2% sucrose and 0.2% AC, complete plantlets were formed. Fully developed plantlets with vigorous shoots and roots were successfully acclimatized and grown under the greenhouse with about 60% shading and 80% relative humidity. A total of 100 plantlets were planted. After 1 month

(Fig. 5b), the numbers of survival were 84 plantlets. The survival rate of plantlets was 70%.

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