ORIGINAL ARTICLE

Establishment of in Vitro Phalaenopsis Violacea Plant Cultures from Flower-stalk Cuttings

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ABSTRACT

Phalaenopsis species are much sought after in the orchid flower industry due to their long-flowering periods and their large-sized flowers. There is a need to hasten the micropropagation process and to improve the quality of the Phalaenopsis plantlets through optimization of medium components. Three experiments were carried out in this research to test the following parameters: direct shoot regeneration from nodal explants of Phalaenopsis violacea flower stalks on half-strength MS medium added with different cytokinins (1-benzylaminopurine or BAP, thidiazuron or TDZ, and zeatin) under various concentrations; the effects of different cytokinin concentrations on protocorm-like body (PLB) induction from leaf segments of the orchid; and the effects of different cytokinin concentrations on the orchid’s PLB proliferation rates. Among the three cytokinins tested, BAP produced the highest percentages of induced shoots from the nodal explants of the flower stalks, after 8 weeks of culture. Both 0.8 µM and 1.0 µM BAP resulted in the conversion of 100% nodal explants to shoots, with each corresponding to the formation of 3.7 and 2.9 shoots per nodal explant respectively. Half-strength semi-solid MS media containing 0.6 µM, 0.8 µM and 1.0 µM BAP induced PLBs in 60%, 70% and 50% of the leaf segments respectively, with 0.6 µM also producing the highest PLB proliferation rate at 75% after 12 weeks of culture. Among the TDZ concentration series, 0.6 µM induced 60% of the explants into shoot formation, producing 2.6 shoots per explant, with both 0.6 µM and 0.8 µM producing PLBs from 40% of the leaf segments. Zeatin produced low percentages of nodal explant conversions and shoot formation with the lowest PLB induction and proliferation rates.

Key words: Phalaenopsis violacea, protocorm-like body (PLB), benzylaminopurine (BAP), thidiazuron (TDZ), zeatin.

Introduction

The Phalaenopsis (Orchidaceae), also known as the moth orchids, are mostly found in the Southeast Asian region, with a few exceptions in Taiwan, Sikkhim to Australia and the Pacific (Teob, 1989). Highly profitable in flower markets around the globe (Chen and Chang, 2006), the Phalaenopsis is regarded as the most popular orchid genus in the horticultural industry due to the aesthetic value and durability of the flowers, as well as the adaptability of the plants to room environment. Phalaenopsis violacea have greenish white flowers with purple pigment around the sepals and the lip. P. violacea are native to the peninsular Malaysia and are closely

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related to *P. bellina* of the Borneo. *P. violacea* are important parent’s varieties to produce novel *Phalaenopsis* hybrids with special fragrance (Fig. 1). Due to their epiphytic nature, they are recalcitrant to vegetative propagation (Košir et al., 2004), with most plantlets produced from seeds cultured *in vitro* (Tokuhara and Mii, 1993). They are amenable to both interspecific and intergeneric hybridizations that are capable of producing many beautiful flowers, through common cross breeding techniques (Shrestha et al., 2007). Among the problems encountered from the culture of *Phalaenopsis* and their related hybrids are the low PLB production from various explants such as shoot tips (Intuwon and Sagawa, 1974); flower-stalk cuttings (Reisinger et al., 1976), root tips (Tanaka et al., 1976) and leaf segments (Kano, 1971; Park et al., 2002a); the long period required for PLB multiplication and growth, and somaclonal variation of the plants (Chen et al., 1998; Park et al., 2002b). Hence, there is an urgent need to develop a micropropagation method for *Phalaenopsis* which will enable demands for the orchid to be fulfilled rapidly.

Tokuhara and Mii (1993) stated that the main factors affecting *Phalaenopsis* micropropagation on a commercial scale are the composition of both macronutrients and micronutrients in the culture medium, coupled with the appropriate combinations and concentrations of α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP). Thus, this study aims to determine the following parameters: direct shoot regeneration from nodal explants of *Phalaenopsis violacea* flower stalks on half-strength Murashige and Skoog (MS, 1962) medium with different growth regulators; the effect of different cytokinin concentrations on PLB induction from leaf segments of *Phalaenopsis violacea*; and the effect of different cytokinin concentrations on PLB proliferation rate (%) of *Phalaenopsis violacea*.

**Fig. 1:** The appearance of *Phalaenopsis violacea* orchid flower. The bar in the bottom image represents 1.0 cm.

**Materials and Methods**

The *in vitro* culture of nodal segments of young shoots derived from flower-stalk cuttings were used for clonal propagation of *Phalaenopsis violacea*. Excised flower-stalks were wiped 3 times with 70% (v/v) ethanol, and then cut into nodal cuttings. The cuttings were immersed in 70% (v/v) ethanol for 50 sec, and then rinsed 4 times in sterile water. Thereafter, the cuttings were immersed in the solution which contained several antimicrobials for 30 min in the dark. The cultures were maintained in the dark for first 10 days and then placed under light at 25°C. *Phalaenopsis violacea* PLBs induction experiment was performed with young *in vitro* leaf segments of approximately 1x1cm², excised from aseptically raised 4 months old *in vitro* seedlings derived from the first part of the above mentioned experiment. To study the effect of different BAP, TDZ and Zeatin concentrations on shoot regeneration from the nodal segments, PLBs induction and propagation, a range of cytokinin concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 µM) were added to half-strength Murashige and Skoog (1962) medium supplemented with 5% of Mas banana (AA) extract. Cultures were incubated on tissue culture room at 25°C under 16 hours photoperiod with light intensity of 40 µmolm⁻²s⁻¹ supplied by white fluorescent tubes. All analyses were performed at a significance level of 5% with the mean differences contrasted with the Duncan’s multiple range test using SPSS 10.0 (SPSS Inc., USA).

**Results and Discussion**

**Shoot regeneration:**

The induction of shoots from the nodal explants of *P. violacea* essentially does not require growth regulators as 30% of the nodal explants successfully generated an average of 1.8 shoots per explant, in media
PLB induction:

Somatic cells of cultured orchid tissues can transform into protocorm-like bodies (PLBs) that will later grow into individual plantlets (Arditti and Ernst, 1993).

BAP proved to be the better phytohormone in inducing PLBs from leaf segments of Phalaenopsis violacea. Half-strength semi-solid MS media containing 0.6 \( \mu M \), 0.8 \( \mu M \) and 1.0 \( \mu M \) BAP induced PLBs in 60%, 70% and 50% of the leaf segments respectively (Fig. 2). However, no significant differences were found between the three concentrations. Benzyladenopurine has been proven to be effective in stimulating PLB growth in various orchids such as Phalaenopsis spp. (Tanaka, 1992; Park et al., 2002a) and Dendrobium nobile (Nayak et al., 2008) discovered that the incorporation of BAP ranging from 0.1 to 5.0 mg l\(^{-1}\) enhanced both PLB induction from the explants and the number of PLBs from the stem segments of Dendrobium densiflorum. The best result was obtained from medium supplemented with 5.0 mg l\(^{-1}\) BAP as a mean of 15 PLBs were successfully induced on 72% of the explants within six weeks of culture.

The best TDZ concentrations were 0.6 \( \mu M \) and 0.8 \( \mu M \), with both inducing PLB production from 40% of the leaf segments. PLBs were induced in 20% of the leaf segments when TDZ was used in 0.2 \( \mu M \) and 1.0 \( \mu M \) concentrations. Chen and Chang (2006) reported that the somatic embryos of Phalaenopsis amabilis were formed directly from leaf explant surfaces after 20 days of culture in the dark in media containing TDZ. The embryos subsequently enlarged and formed more embryos after a further 10 days of culture in the same media. They further reported that when the cultures were transferred into light, the embryos turned green and matured into protocorms after two weeks. Chen et al. (2002) reported that 40% of flower stalk explants of Epidendrum radicans produced PLBs when cultured on half-strength MS medium containing 0.45 \( \mu M \) TDZ. Zeatin gave the lowest rate of PLB induction, with 0.4 \( \mu M \), 0.6 \( \mu M \) and 0.8 \( \mu M \) concentrations inducing PLBs in only 10% of the leaf explants. No PLBs were formed in media containing 0.2 \( \mu M \) and 1.0 \( \mu M \) zeatin.

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**Table 1:** Direct shoot regeneration from nodal explants of Phalaenopsis violacea flower stalks on half-strength MS medium with different growth regulators. All results were scored after 8 weeks of culture. The results indicate the mean standard error (+ SE) of 3 independent experiments with 10 replicates for each treatment concentration, with the experiment repeated thrice.

<table>
<thead>
<tr>
<th>Growth regulators (( \mu M ))</th>
<th>Induced shoots (%)</th>
<th>Number of shoots per explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
<td>1.8( ^a )</td>
</tr>
<tr>
<td>BAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>40</td>
<td>1.3( ^a )</td>
</tr>
<tr>
<td>0.4</td>
<td>50</td>
<td>1.8( ^a )</td>
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<tr>
<td>0.6</td>
<td>80</td>
<td>2.5( ^a )</td>
</tr>
<tr>
<td>0.8</td>
<td>100</td>
<td>3.7( ^a )</td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>2.9( ^a )</td>
</tr>
<tr>
<td>TDZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>30</td>
<td>1.3( ^a )</td>
</tr>
<tr>
<td>0.4</td>
<td>40</td>
<td>1.7( ^a )</td>
</tr>
<tr>
<td>0.6</td>
<td>60</td>
<td>2.6( ^a )</td>
</tr>
<tr>
<td>0.8</td>
<td>30</td>
<td>1.2( ^a )</td>
</tr>
<tr>
<td>1.0</td>
<td>40</td>
<td>0.9( ^a )</td>
</tr>
<tr>
<td>Zeatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>20</td>
<td>1.1( ^a )</td>
</tr>
<tr>
<td>0.4</td>
<td>40</td>
<td>1.7( ^a )</td>
</tr>
<tr>
<td>0.6</td>
<td>20</td>
<td>1.2( ^a )</td>
</tr>
<tr>
<td>0.8</td>
<td>20</td>
<td>0.7( ^a )</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
<td>0.4( ^a )</td>
</tr>
</tbody>
</table>
PLB proliferation rate:

Many orchid genera such as *Phalaenopsis* (Chen and Piluek, 1995), *Oncidium* (Chen and Chang, 2000) *Cypripedium* (Shimura and Koda, 2004), and *Dendrobium* (Martin et al., 2005) had been micropropagated and regenerated through PLB formation. The PLBs of *P. violacea* in this experiment were able to proliferate without the addition of cytokinins to their growth media. This was shown when the PLBs proliferated at a rate of 32% in media devoid of the three cytokinins. This result was not significantly different from most of the cytokinins used at different concentrations in this experiment.

Among all the cytokinins used, zeatin produced the lowest significant proliferation rates, descending from 18% to 8% at concentrations ranging from 0.6 µM to 1.0 µM (Fig. 3). However, Chen et al. (2002) reported that the highest PLB proliferation rate for the orchid *Epidendrum radicans* was produced by 0.28 µM zeatin-riboside, as the fresh mass of the PLBs increased 30.3 times from the original 0.8 g of homogenized tissues. In this experiment, no significant difference was detected in the PLB proliferation rates in media containing 0.6 µM and 0.4 µM TDZ, resulting in 58% and 52% proliferation rates respectively, the best results for that cytokinin. Chen and Chang (2006) reported that 3 mg dm⁻³ TDZ produced the best proliferation rates of both the fresh mass (5.4%) and the mean number of embryos per explant of *Phalaenopsis amabilis* (13.8%), when the embryos were subcultured through the division of the embryo clusters.

![Effect of different cytokinin concentrations on PLBs induction from leaf segments of *Phalaenopsis violacea*. All results were scored after 12 weeks of culture. The results indicate the mean standard error (+ SE) of 3 independent experiments with 10 replicates for each treatment concentration, the experiment was repeated thrice.](image1)

![Effect of different cytokinin concentrations on PLBs proliferation rate (%) of *Phalaenopsis violacea*. All results were scored after 12 weeks of culture. The results indicate the mean standard error (+ SE) of 3 independent experiments with 10 replicates for each treatment concentration, the experiment was repeated thrice.](image2)
The better cytokinin was still BAP, which produced the highest proliferation rate (75%) at 0.6 µM. The efficiency of BAP alone in inducing PLB formation was also observed in Dendrobium sonia hybrids 17 and 28 (Martin and Madassery, 2006) and D. nobile (Nayak et al., 2002). This value, however, did not differ significantly from proliferation percentages produced in media containing 0.8 µM and 1.0 µM BAP, and 0.6 µM TDZ, which resulted in 62%, 48% and 58% proliferation rates respectively.

In conclusion, efficient propagation protocols for the orchid Phalaenopsis violacea has been established which enables the demand for the orchid species to be fulfilled using the optimised selected cytokinin concentration.

References


