Indian J. Applied & Pure Bio. Vol. 37(2), 555-563 (2022). **ISSN: 0970-2091**
A web of Science Journal

Multiple protocorm like body (MPLB) formation and plant regeneration from the PLB culture of a rare orchid *Aerides crispum* Lindl

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Abstract

An efficient protocol for micropropagation of *Aerides crispum* using axenic protocorm like bodies (PLBs) derived from in vitro seed germination was developed. These PLBs were used to induce multiple protocorm like bodies (MPLBs) on different PGRs through the formation of primary and secondary PLBs. The effect of α-Naphthalene acetic acid (NAA), 6- Benzyl amino purine (BAP) and Thidiazuron (TDZ) singly and in combinations were investigated. The formation of primary and secondary PLBs leading to the formation of MPLBs was assessed. The MPLB formation was optimum in ½ MS medium supplemented with TDZ (0.5 mg/l) where 70.3% MPLBs were induced with an average number of 38.5 MPLBs/explants was recorded. To increase the frequency of MPLB formation various combinations of PGRs were also tested. The MPLB formation was greater from the PLB explants cultured on ½ MS medium containing TDZ with NAA, followed by BAP with NAA. The medium fortified with TDZ (1.0 mg/l) + NAA (0.5 mg/l) the highest frequency of MPLBs (91.8%) were recorded with an average number of (68.0) MPLBs/explant. Medium containing BAP (1.0 mg/l) + NAA (0.5 mg/l) stimulated the formation of MPLBs (84.3%) and an average number of MPLBs/explant (51.9) was recorded. On subculturing the MPLBs on to the same media further developed into plantlets. A survival rate of more than 89% under green house conditions was achieved.

The orchidaceae represents approximately 10% of angiosperms. It is one of the largest, most diverse and most important categories of botanically significant flowering plants with 35000 species[9]. These ornamental plants are widely distributed, cultivated for their beautiful flowers and are of economic importance. Due to ruthless collection by

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increasing orchid lovers, over exploitation for medicinal and ornamental purposes, deforestation, killing of pollinators and unauthorized trade has led to reduction in natural populations of orchids. Meanwhile many orchid species have become extinct and many others are on the verge of becoming rare and endangered. At present most of the wild orchids are listed in the International Union for Conservation of Nature and Natural resources (IUCN) Red data book. This family is also included in the Appendix-II of the Convention of International Trade in Endangered Species of Wild Flora and Fauna (CITES).

Aerides crispum is commonly called as curled *Aerides* is an epiphytic rare orchid found in South-western India at elevations of 800-1200 m. The inflorescence is 40-50 cm long, erect to arching, branched 20-25 flowered cluster, carrying fragrant flowers and lasts for more than a month. When it produces beautiful fragrant flowers with long inflorescences with long vase life, thus giving this species great potential as a novel alternative for the floriculture industry. *A. crispum* is now becoming rare because of the destruction of its habitat, the heavy exploitation of its wild resources, its low propagation rate and its slow growth. Therefore, to meet the growing demand of the horticulture and floriculture industries artificial cultivation is beginning to be investigated and attempted. *A. crispum* is usually propagated sexually by seeds. However, the conventional methods of propagation is very inefficient and time consuming as seeds lack endosperm and require suitable mycorrhizal association for germination in nature. Now an effective strategy becomes essential to conserve and multiply this orchid species. An efficient propagation system followed by primary, secondary PLBs formation, MPLB induction, plantlet regeneration and acclimatization has been demonstrated. Such a protocol would allow for large scale propagation to meet commercial demand and conserve this rare orchid species by reducing wild collection.

Explant and culture medium :

PLBs derived from in vitro grown seeds of *Aerides crispum* were used as the source of explant. In the present study half-strength MS medium was used to initiate MPLBs. After 4-5 weeks of seed culture, well developed PLBs were used in the second set of experiment to initiate MPLBs through primary and secondary PLBs. To assess the efficacy of different plant growth regulator (PGR) formulations for the initiation of MPLBs and regeneration into complete plantlets, PLBs were inoculated on to ½ MS medium and fortified with varying concentrations of PGRs namely, α-Naphthalene Acetic Acid (NAA), 6-Benzylamino purine (BAP) and Thidiazuron (TDZ) in the concentrations of (0.5, 1.0, 2.0 and 3.0 mg/l) individually and combinations of TDZ + NAA and BAP + NAA. In experiments to select optimal nutrient medium ½ MS medium devoid of any PGR was used as control. Observation on induction of primary, secondary PLBs, average number of MPLBs, frequency of MPLB formation and differentiation of MPLBs into shoot and roots were recorded.

Culture conditions :

MS medium ½ was supplemented with 2% sucrose and gelled by using 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 either with NaOH or HCl prior to

autoclaving at 121°C and 105 kPa for 15 minutes. Cultures were incubated in culture room at 25±2 °C and 65% relative humidity, with a 16/8 h (light/dark) photoperiods supplied by cool white fluorescent light (Philips, India) with light intensity of 40 μ mol $m^{-2}s^{-1}$. For each treatment 12 replicates were taken and the experiments were repeated twice.

Ex-vitro Culture :

Well developed plantlets with a height of at least 3-4 cm were removed from the culture jars and washed thoroughly under tap water to eliminate all media attached to the roots. They were then planted in a autoclaved potting mixture of charcoal, brick pieces, chopped coconut husk and vermiculite in the ratio 1:1:1:1. The plantlets were covered with transparent polythene sheets for a month in order to reduce infection and to maintain high humidity (80-90%), day/night temperature 25/18°C in 12/12 h photoperiod and 30 μ mol $m^{-2}s^{-1}$ light intensity. After one month the polythene bags were removed and humidity was gradually reduced to (50-60%). The hardened plants were watered daily and sprayed with 1/10th liquid MS without sucrose for alternate days. Plant growth was monitored periodically and well developed healthy plantlets were shifted to green house after 30 days and subsequently to small pots. The number of surviving plants was recorded after 12 weeks of transfer.

Data analysis :

The experimental units were set up in a completely randomized block design. Twelve replicates were raised for each treatment and the best treatments were repeated twice. Data on time taken for primary, secondary PLB formation, an average number of MPLBs per explant and time taken for differentiation of MPLBs into shoot and roots were tested applying Tukey's multiple comparison test ($P \leq 0.5$) in one way ANNOVA to separate of significantly different groups. The statistical analysis was performed by using statistical package for social science (SPSS) v. 18.0 software package. The results were expressed as mean ± SD of twelve replicates.

Existing literature shows variation in usage of PGRs in protocorm formation and proliferation in the medium. While in some species germination and protocorm formation has been recorded in the basal media devoid of any PGRs[1,19]. In this study it was observed that addition of cytokinin and auxin (alone or in combinations) in ½ MS medium gave significantly better results of MPLB formation and plantlet development through in vitro culture of PLB explants. Mature seeds of *Aerides crispum* were cultured on ½ MS medium, the germination was marked by the swelling of seeds and developed into PLBs after 5-6 weeks of culture. When these PLBs were subcultured on to the same medium normally differentiated shoot and roots after 10 and 14 weeks of culture respectively. But on the other hand, an interesting result were recorded, when these seed derived PLBs cultured on different PGRs individually and in combinations developed MPLBs through the formation of primary and secondary PLBs.

The initiation and proliferation of MPLBs are largely influenced by the external addition of PGRs. In the present study the

addition of external PGRs in appropriate concentrations promoted MPLB formation from the PLB explants in the ½ MS medium. The 5-6 weeks old seed derived PLBs were then subcultured on fresh ½ MS medium supplemented with different concentrations of NAA, BAP and TDZ alone and in combinations. The medium supplemented with TDZ (0.5 mg/l) stimulated the formation of higher primary PLBs at the base of the PLB explant. Within 2 weeks of culture light green small granules of primary PLBs emerged from the basal surface of the PLB explants and became green after another 1 week of culture. The globular secondary PLBs started to develop on the surface of primary PLBs after 5 weeks of subculture. The primary and secondary PLBs further multiplied and grew faster formed clumps of MPLBs in 7 weeks of subculture. These formed MPLBs were comprised of a relatively compact mass of isodiametric granules on explant surface, where 70.3% of PLB explants induced MPLBs with an average number of 38.5 MPLBs/explant. The induction of MPLB was enhanced at the lower concentration of TDZ in the medium, but it was inhibited the formation of MPLBs when used in higher concentrations (Table-1).

The TDZ involved either directly or indirectly in several morphological and physiological responses in plant tissues[8]. In this study the optimal individual concentration of TDZ (0.5 mg/l) stimulated the formation of MPLBs (70.3%) through the formation of primary and secondary PLBs from the explant. The similar positive effect of TDZ was reported for the induction of both callus and direct PLB formation from cut surface of shoot tip explants of *Dendrobium chrysotoxum*[24]. An observation similar to the present study the TDZ was effective in inducing in vitro morphogenesis in *Xenikophyton smeeanum*[21] and *Anectochilus elatus*[2]. The induced MPLBs were pale green to green in color. Subculturing of these on medium containing the same level of TDZ (0.5 mg/l) resulted in the proliferation into shoots and roots after 5 and 9 weeks of cultures respectively. However, TDZ (0.5 mg/l) was more effective in inducing MPLBs as compared to BAP and NAA in *A. crispum*.

When PLB explants were cultured in ½ MS medium supplemented with BAP singly, formation of MPLBs were also reported. The optimum percentage of MPLBs (46.4%) was induced in the medium supplemented with BAP (1.0 mg/l), with an average number of 28.7 MPLBs/explant. When the concentration of BAP was increased (2.0- 3.0 mg/l), it drastically reduced the formation of MPLBs (Table-1). From the existing literature BAP has proved to be an effective cytokinin in case of orchid micropropagation, but in this report it was found to be comparatively less efficient than TDZ in inducing MPLBs in *A. crispum*. The inhibitory effect of BAP on the conversion of protocorms into plantlets and root formation in *Dendrobium huoshanense*[14] and *Doritis pulcherrima*[20]. In contrast to this the stimulatory effects of BAP on induction of highest mean values of PLBs in *Phalaenopsis* hybrid[13] and stimulated the formation of highest number of shoots in *Dendrobium chryseum*[15], MPLBs formation in *Aerides ringens*[3] and induction of callus, Primary and secondary protocorms in *Dendrobium barbatulum*[4] were also reported.

Table-1. Effect of different PGRs on the induction of MPLBs and Plant regeneration in *A. crispum*

PGRs (mg/l)	Initiation of primary PLB (Wks)	Initiation of secondary PLB (Wks)	Time taken for MPLB formation (Wks)	Frequency of MPLB formation (%)	Average number of MPLB/ explant	Differen-tiation of shoots (Wks)	Differen-tiation of roots (Wks)
½ MS	-	-	-	-	-	-	-
NAA 0.5	4	6	10	8.3±0.8	8.6±0.8	8	12
1.0	4	6	10	112.1±0.7	8.2±0.7	8	12
2.0	6	8	12	8.2±0.8	2.4±1.2	8	13
3.0	-	-	-	-	-	-	-
BAP 0.5	3	5	8	39.6±0.9	24.6±0.7	6	11
1.0	3	5	8	46.4±1.0	28.7±0.5	6	11
2.0	3	5	8	29.4±0.3	12.1±0.6	6	11
3.0	-	-	-	-	-	-	-
TDZ 0.5	3	5	7	70.3±1.2	38.5±0.3	5	9
1.0	3	5	7	61.4±0.8	25.1±0.6	6	11
2.0	3	5	7	36.0±0.9	14.3±0.7	6	11
3.0	4	6	8	33.4±0.7	12.8±0.4	6	11
BAP+NAA							
0.5 + 0.5	3	5	7	80.1±1.1	47.3±0.8	6	12
1.0 + 0.5	3	5	7	84.3±0.9	51.9±1.3	5	11
2.0 + 0.5	3	5	8	69.8±1.2	50.0±1.2	6	11
3.0 + 0.5	-	-	-	-	-	-	-
TDZ+NAA							
0.5 + 0.5	3	5	7	80.0±1.1	50.4±1.2	5	9
1.0 + 0.5	3	5	7	91.8±0.8	68.0±1.0	4	8
2.0 + 0.5	4	6	8	70.0±0.3	37.1±0.3	6	10
3.0 + 0.5	4	6	9	64.1±1.2	37.0±0.4	6	11

Values represent mean ± SD; the same letters within a column are not significantly different based on ANOVA followed by Tukey's test Pd"0.5.

The effect of NAA on the induction of MPLBs was also evaluated in *A. crispum*. it is very less efficient in inducing primary PLBs and MPLBs when compared with BAP and TDZ. The NAA in the lower concentrations (0.5- 1.0 mg/l) induced only 18.3% of MPLB formation with an average number of 8.6 MPLBs/explant. In higher concentrations (2.0-3.0 mg/l), it almost inhibited the formation of MPLBs. The effect of NAA in inducing MPLBs through the formation of primary and secondary PLBs followed a similar pattern but to vary lesser degree than TDZ and BAP. These obtained results were conformity with the earlier report of the lowest efficiency/ inhibitory effect of NAA on the induction of callus or somatic embryos in *Dendrobium Sonia Earsakul*[11]. But in contrast to present

study the NAA was able to produce shoots and roots in *Dendrobium aqueum*[22] and *Dendrobium longicornu*[7].

To evaluate the synergistic effect of the PGRs, the PLBs were cultured on ½ MS medium with the combinations of TDZ with NAA and BAP with NAA the concentrations and the ratio between them are critically important. In the present study the explant response intern of primary, secondary PLBs and MPLB induction was highest in the medium supplemented with TDZ (1.0 mg/l) in combination with NAA (0.5 mg/l). The initiation of primary PLBs was observed at the lower portion of the PLB after 3 weeks of culture (Fig. 1A), subsequently the formation of secondary PLBs was also documented on the surface of the primary PLBs in the same medium after 5 weeks of culture. Wherein it was also recorded the highest frequency of MPLBs formation (91.8%) (Fig. 1B) with an average number of 68.0 MPLBS/PLB explant. These formed MPLBs were subcultured on to the same medium the shoot apex was differentiated at the tip regions (Fig. 1C), later these developed leafy shoots after another 4 weeks of subculture (Fig. 1D). These well developed shoots differentiated roots on the same medium after 8 weeks of subculture (Fig. 1E). This corroboration suggests the synergistic effect of auxin in amalgamation with cytokinin promotes MPLB formation and plantlet regeneration. However, with the increase in the concentration of TDZ and NAA in the ½ MS medium reduction in both frequency and average mean number of MPLB formation was recorded (Table-1). These obtained results were conformity with earlier report of TDZ and NAA, were involved either directly or indirectly in several morphological and physical responses in plant tissues and these are reported to promote the formation of callus, PLBs and shoots from various species of orchids including *Oncidium flexuosum*[17], *Xenicophyton smeeanum*[21] and *Anoectochilus elatus*[2]. It was reported that the combination of TDZ and NAA were in MVM medium provided a high mean number of shoots/ explants in *Vanda coerulea*[10]. The similar positive effect of TDZ and NAA was also reported in *Malaxis acuminata*[18] and *Dendrobium thyrsiflorum*[5]. In the present investigation the combination of TDZ with NAA induced maximum frequency of MPLBs formation (91.8%) along with highest average number of (68.0) MPLBs/explant. The MPLB produced in this combinations was healthier and superior than in medium containing the combination of BAP and NAA. All these results clears that the media fortified with TDZ and NAA combination stimulated various morphogenic responses in many orchid taxa. Another combination effect of BAP and NAA on the induction of primary, secondary PLBs and MPLBs were also examined. It was observed that the frequency of MPLB formation differed with the concentrations of BAP and NAA. In *A. crispum* best results were obtained on ½ MS medium fortified with BAP (1.0 mg/l) and NAA (0.5 mg/l). On this combination primary, secondary PLBs and MPLBs were developed after 3, 5 and 7 weeks of culture respectively and optimum frequency of MPLB formation (84.3%) and an average number of MPLBs/explant (51.9%) were obtained. The induced MPLBs were differentiated into shoots and roots on the same

Fig. 1. The induction of MPLBs through the formation of primary and secondary PLBs in ½ MS fortified with TDZ (1.0 mg/l) and NAA (0.5 mg/l).
A. Initiation of primary PLBs at the base of protocorm explants after 3 weeks of culture, scale bar =0.5mm. B. Formation MPLBs after 7 weeks of culture, scale bar =1 cm. C & D. development of shoot apices and leafy shoots from MPLBs after 4 weeks of subculture, scale bar = 1 cm. E. Well developed plantlets after 8 weeks of subculture, scale bar = 1 cm. F. well acclimatized plant let, scale bar = 1 cm.

medium after 5 and 11 weeks of subculture respectively. A similar synergistic effect of BAP and NAA has been reported in many orchid taxa. It was reported that highest shoot and root length in *Aerides multiflora*[12]. The similar positive effect of BAP and NAA has been reported to be highly effective in protocorm regeneration in *Aerides ringens*[25] and *Aerides multiflora*[6]. The highest number of adventitious shoot formation was also recorded in this combination in *Dendrobium Hybrid Sonia*[16]. It was also reported that the combination of BAP and NAA induced highest number of secondary protocorm from the primary protocorms in *Dendrobium chrysanthum*[23]. These observations are supported by previous findings where BAP and NAA combinations have proven to stimulate morphogenic responses in many taxa.

Earlier studies have opined that proliferative PLBs of orchids fail to root unless they are shifted to rooting medium containing auxin, the need being mandatory for rooting in some orchid species like *Oncidium, Rhynchostylis*[26]. However, in this study the proliferative MPLBs could develop healthy shoot and root system in a single step without the need to transfer to a rooting medium. The completely regenerated plantlets (3-4 cm) were initially acclimatized in a community pot (Fig. 1F) containing the autoclaved potting mixture of charcoal, brick pieces, chopped coconut husk and vermiculite in the ratio of 1:1:1:1, where the survival rate was recorded to be 89%. Initially during the acclimatization period older leaves were observed to die with the emergence of new leaves from the shoots in due course of time[5]. The role of vermiculite has been found to be very important in the successful acclimatization of these in vitro raised plantlets, as it has better moisture retaining capacity as well as constant absorption of oxygen which can be availed by the plants during the acclimatization period.

Plant tissue culture technology is an important prospective method for conservation of several difficult to cultivate plant species. This technique finds a special mention in the conservation and large scale production of many wild orchid species which are routinely harvested in an unscientific manner for commercial purposes. The present report gives a rapid in vitro propagation protocol for a rare orchid *A. crispum*. Individual levels of TDZ and BAP and in combinations of TDZ with NAA and BAP with NAA successfully produced healthy plants through the formation of MPLBs. The most suitable PGR combination is TDZ and NAA where optimum percentage of MPLBs and more healthy plants were produced. The efficient production of MPLBs and subsequent differentiation into plantlets provides a simple and cost effective protocol for mass propagation and conservation of this rare epiphytic orchid in nature.

The author gratefully acknowledges the financial support in the form of Major Research Project F.No. 42-927/2013 (SR) by the University Grants Commission, New-Delhi, India.

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