An improved method for rapid propagation of *Phalaenopsis* hybrids *via* culture of longitudinally bisected shoot tips.

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Abstract

A new procedure for *in vitro* propagation of orchids belonging to the genus *Phalaenopsis* was developed.In contrast to the commonly employed micropropagation methodsthat make use of dormant flower stalk buds or intact shoot tips, we have used longitudinally bisected shoot tips derived from 3 – 3.5 month old axenic plantlets of *Phalaenopsis* hybrids for protocorm-like-bodies (PLB) induction. This procedure in combination with the use of New Dogashima Medium for PLB proliferation resulted in large-scale production of plantlets. Culturing the bisected shoot tips in half strength solid Murashige and Skoogmedium supplemented with Thidiazuron (TDZ) (4.54µM–9.08 µM) and 10% coconut water resulted in early PLB induction in 20-25 days and callus-free formation of 13.6 – 15.2 shoot buds in 8 weeks. Intact shoot tips took 6-8 weeks to PLB induction and proliferated to a mean of 5.46 - 7 shoot buds per shoot tip in 12 - 16 weeks in different TDZ concentrations. Continuous multiplication of PLBs and shoot buds was maintained in New Dogashima Medium devoid of hormones by bimonthly subcultivation.About 50,000 hardened plantlets could be produced from a single flowerstalkwith 10 dormant buds, within 2 years from initiation of axenic shoottip cultures.This protocol can be used to produce genetically stable *Phalaenopsis orchids* on a commercial scale.

Keywords: Longitudinally bisected, Micropropagation, *Phalaenopsis*, Physiological age, Protocorm-like-body, Shoot tip

Introduction

In vitro propagation makes it possible to clonally mass propagate *Phalaenopsis* hybrids of commercial value and conserved species. Although seed-raised plants can be used for mass propagation, there is a great deal of variability in plant growth, flowering time and flower characteristics. Micropropagation through the induction and proliferation ofdirect axillary shoots without undesirable callus formation is an important strategy in obtaining genetically stable plants and shortens the time needed for regeneration. However, *in vitro* culture technologies via direct multiple shoots have not been commercially feasible in Phalaenopsis hybrids due to low rate of axillary shoot multiplication. The need for mass propagation in *Phalaenopsis* hybrids has led to the development of *in vitro*methods of propagation through protocorm-like-bodies (PLBs)using shoot tips and flower stalk buds(Mondal et al., 2013). Plantlets regenerated through the direct formation of PLBs did not produce any detectable somaclonal variation (Samarfard et al., 2014). However most of these methods are not adequate enough to meet the needs for commercial propagation of *Phalaenopsis* because the survival rates, percentage of PLB forming explants, time and rate of PLB induction and proliferation have been low.

The present work was done to develop a rapid micropropagation technique in *Phalaenopsis*

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hybrids so as to make commercial ventures profitable.

Materials and Methods

Two-month-old to eight-month-old axenic shoots regenerated from the dormant buds of the flower stalks served as the explant source. These were regenerated from dormant flower stalk buds of six *Phalaenopsis* hybrid mother plants maintained at Rice Research Station,Vyttila. Shoot tip explants of 3-5 mm. size were excised from axenic plantlets and longitudinally bisected using a sharp scalpel before inoculatingin medium. The cut surfaces were placed in contact with the medium inorder to study the effect of vertical cutting on PLB induction percentage, earliness of PLB induction and number of PLBs produced. Intact shoot tips served as control in the experiment.

New Dogashima Medium (NDM) (Tokuhara and Mii, 1993) and solidhalf strength Murashige and Skoog (½ MS) Medium (Murashigeand Skoog,1962)were tried for culture initiation. These media were supplemented with various concentrations and combinations of plant growth regulators (PGRs): Naphthalene Acetic Acid (NAA) 0.54 -10.7 µM, Benzyl Amino Purine (BAP) 4.44-44.4 µM andThidiazuron (TDZ) 4.54 - 9.08µM with and without 0.2% peptone, 1% and 2% sucrose and 10%, 15% and 20% coconut water (CW) to assess the effect on PLB and shoot bud induction. Due to shortage of planting material only the most promising treatments identified from these experiments were used for replicated trials. Observations on effect of age of explant source on parameters like percentage of PLB forming explants, necrosis percentage, time to PLB induction and proliferation and size of PLB mass were recorded.

Media was solidified with 0.24% w/v gellan gum and pH was adjusted to 5.5 using 1N NaOH or HCl prior to autoclaving at 121^0 C for 15 min. Medium (30 ml.) was dispensed into culture bottles (10.5 cm. height x 5.5 cm inner diameter) and closed with polypropylene autoclavable lids. The cultures wereincubated at 25 ± 2^o C with 16 h photoperiod of 3000 lux light intensity.

Two months after initiation of shoot tip cultures, shoot bud masses (5 mm. - 1 cm.) were cut into smaller pieces (3-4 mm.) and subcultured in NDM without hormone and in NDM fortified with varying concentrations and combinations of NAA (0.54 µM -10.75 µM) and BAP (4.44 µM – 22.2 µM) with and without 0.2% peptone, 1% and 2% sugar and 0.75% activated charcoal. Effect of all the treatments on formation of secondary PLBs, shoot buds, axillary shoots and shoot morphology was recorded. Subsequent subcultivation on the same media was done and data recorded.

Table1.Comparison of effect of TDZ on PLB and shoot bud induction in intact andlongitudinally bisected axenic shoot tips.

PGRTDZ (µM)	Nature of shoot tip*	PLB forming explants (%)	Time to PLB induction (days)	Approx.size of shoot bud mass (mm.)	No. of shoot buds per shoot tip	Time to PLB&shoot bud proliferation (days)	Colour of shoot buds
0(Control)	intact	0	-	-	-	-	-
	bisected	0	-	-	-	-	-
4.54	intact	13.3 [b]	63 [b]	1.8 [b]	5.46 [b]	126 [b]	Yellowish green
	bisected	66.67 [a]	22.3 [a]	7.5 [a]	15.2 [a]	60 [a]	Green
6.81	intact	6.7 [b]	70 [b]	2 [b]	7 [b]	97 [b]	Green
	bisected	66.67 [a]	21.3 [a]	5.4 [a]	13.6 [a]	67 [a]	Green
9.08	intact	13.3 [b]	60 [b]	1.9 [b]	5.73 [b]	121 [b]	Green
	bisected	60 [a]	24.7 [a]	6.7 [a]	14.4 [a]	66 [a]	Green

Means followed by different letters (a & b) are significantly different ($p < 0.05$) based on WASP 2.0 Analysis (ICAR, Goa).

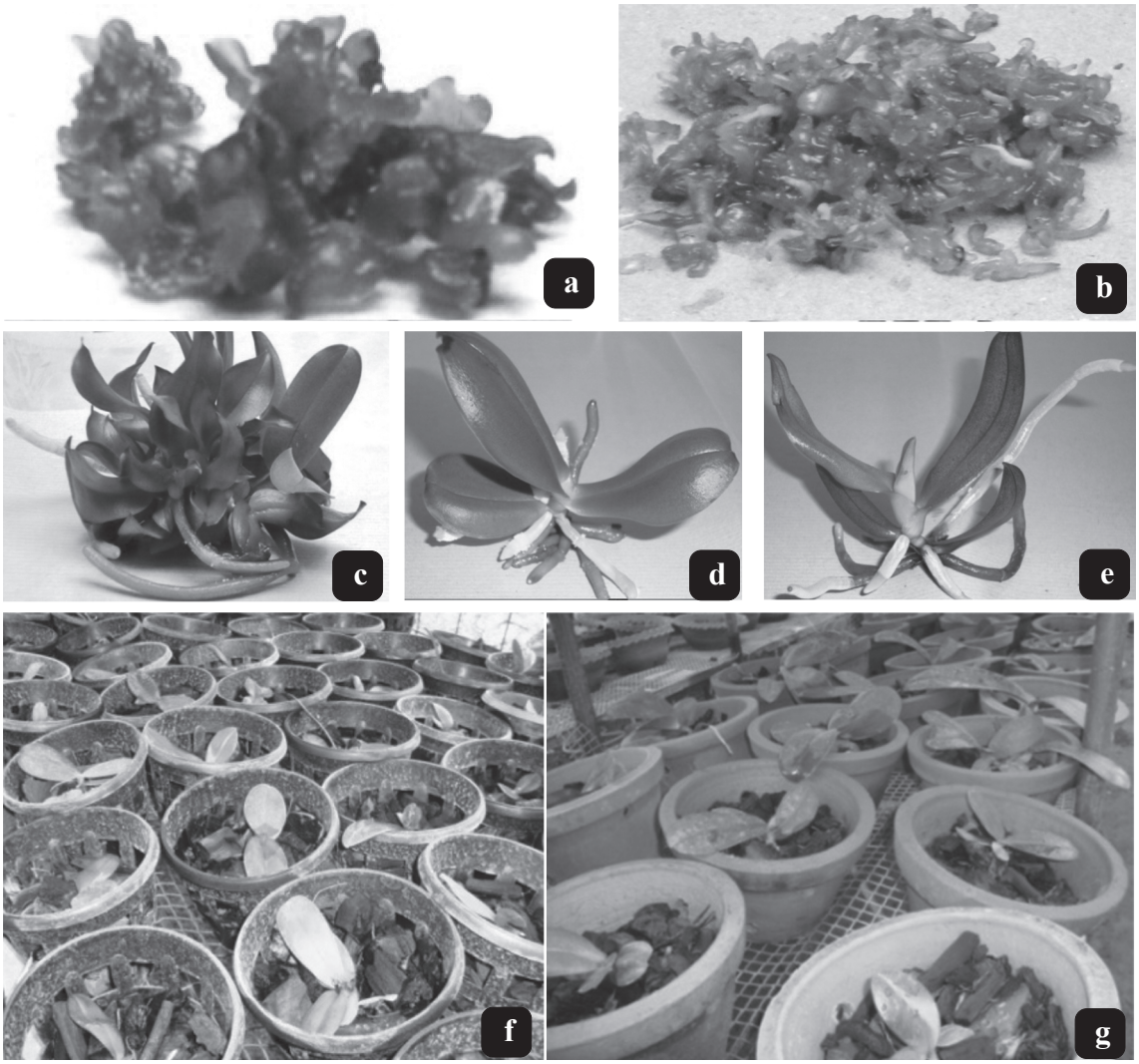

Figure 1 Micropropagation in *Phalaenopsis* via PLB and shoot bud formation from axenic shoot tips of *Phalaenopsis*hybrids **a.** PLB & shoot bud induction from shoot tip in TDZ fortified medium. **b.** PLB & shoot bud proliferation in no-hormone NDM. **c.** Multiple shoot and plantlet formation in NAA:BAP fortified medium **d** & **e**. *In vitro* hardened rooted plantlets ready for transplanting to field **f.** Primary hardening in green house **g**. Secondary hardening 6 months after transplanting to field.

Shoot clumps with an average of 3 shoots per clump were subcultured in ½ MS medium fortified with PGRs (NAA 2.69 µM and BAP 11.1 µM), 0.2% peptone, 2% sugar and 0.075% charcoal and solidified with 0.6% industrial agar.

Experiments were performed in Completely Randomised Design and repeated twice. Each treatment had 15 replicates and each replicate consisted of 5 explants per culture bottle. Morphogenetic response (primary PLB and direct shoot bud formation) from shoot tips was evaluated after 8 weeks of culture and response from subculturing PLB and shoot bud sections was evaluated after 12 weeks of culture. Morphogenetic response was expressed as percentage of explants producing PLBs and shoot buds in relation to number of surviving explants. Average number and diameter of PLBs and shoot buds per explant and time taken to induction and proliferation of PLBs

and shoot buds were also recorded.The data was statistically analyzed using Web Based Agricultural Statistical Software Package (WASP 2.0) designed and developed by Central Coastal Agricultural Research Institute (ICAR), Ela Goa (www.ccari.res.in/waspnew.html).

Before transplanting to field conditions, the rooted plantlets were subjected to *in vitro* hardening for 2-3 weeks in ½ MS medium devoid of hormones and sugar. The regenerated plantlets (4-8 cm. height) were then transplanted toplastic pots (8 cm. diameter) containing small brick pieces, coir and charcoal in the ratio 1:1:1 and kept in green house for primary hardening. Plantlets were watered daily, sprayed with 0.05% fertilizer NPK 30:10:10 at weekly intervals and 0.1% bavistinat fortnightly intervals. After 16 weeks plants were transferred individually to 20 cm. diameter claypots.

Results and Discussion

Effect of shoot tip bisection on PLB induction

It was observed that 3-5 mm. long shoot tips excised from 3-3.5 month-old axenic plantlets showed early induction of PLBs and shoot buds in all treatments with TDZ (4.54, 6.81 and 9.08 µM) when inoculated after bisecting longitudinally and placing the cut surfaces in contact with medium. Rate of PLB induction and proliferation was higher in bisected shoot tips compared to intact shoot tips. PLBs were induced in 20-25 days resulting in 13.6 – 15.2 shoot buds in 8 weeks. Intact shoot tips took 6-8 weeks to PLB induction and proliferated to a mean of 5.46 - 7 shoot buds per shoot tip in 12 - 16 weeks in different TDZ concentrations (Table 1). The bisected shoot tips derived from axenic plantlets above 3 months old induced PLBs whereas intact shoot tips derived from older axenic plantlets above 5 months old alone induced PLBs.

Mondal et al. (2013) were able to induce an average 7.33 PLBs and 1.47 axillary shoots from shoot tips derived from 6 month old axenic seedlings in *Phalaenopsis*. In comparison, since 3- 3.5 month old axenic shoot tips were used as explants in the present study, time to shoot bud induction was reduced by 2.5 - 3 months and number of shoot buds per explant was two-fold higher (12 – 18) onvertically cut shoot tips cultured in all the three TDZ fortified media. Udomdeeet al.(2012) also observed enhanced multiple shooting from vertically cut axenic shoot tips compared to intact shoot tips in *Paphiopedilum*, a terrestrial orchid. Earliness and induction of more number of PLBs and shoot buds, upon bisection of the shoot tip may be due to the direct contact of the inner cells of the shoot tip with the hormone fortified medium. Histological observations on thin flower stalk intermodal sections (1-1.5 mm.) of *Phalaenopsis* cultured *in vitro*by Lin (1987) revealed that a number of initial cells with large nuclei and dense protoplasm existing in the epidermis, cortex and immature vascular bundles begin to divide 7-11 days after the sections are placed in the culture medium and form PLBs on the cut surfaces and epidermis of the sections 20-45 days after the start of cell division.

Effect of age of axenic plantlet on PLB induction

In a separate experiment to study the effect of age of axenic plantlets used as explant source, on shoot bud and PLB induction, it was observed thatverticallycutshoot tips excised from less than three-month-old *invitro*plantlets showed low shoot bud induction percentage (13.3%). The rate of PLB proliferation and size of PLB mass produced was less.It took 3 months to proliferate to a mass of 3.6 mm. Shoot tips above 3 months of age exhibited an approximately five- fold increase in induction percentage of PLBs or direct shoot buds (66.7 – 73.3%) reaching a size of 9.4 – 9.7 mm. shoot bud mass in 8 weeks. Although *shoot tips* from axenic *plantlets* above 6 months old also exhibited good response to shoot induction, 3- 3.5 month old *in vitro* plantlets were standardised as the explant source in order to reduce the time delay to culture initiation (Table 2).

Earliness and regeneration potential of explants is

*Table 2.*Effect of age of axenic plantlet on PLB and shoot bud induction from longitudinally bisected axenic shoot tips in TDZ fortified media.

Age of axenic plant let	PLB and shoot bud formation(%)	Necrosis (%)	Time to PLB/ shoot bud induction (days)	Time to shoot bud proliferation (days)	Approx. size of proliferated mass (cm.)
< 3 months	13.3[b]	46.6	32[c]	90[c]	0.36[b]
3 -6 months	73.3[a]	26.7	24[b]	53[a]	0.97[a]
>6 months	66.7[a]	20.0	21.3[a]	61[b]	0.94[a]

Means followed by same letters are not significantly different ($p < 0.05$) based on WASP 2.0 Analysis (ICAR, Goa).

markedly influenced by their physiological status. This was supported by Murthy and Pyati (2001) whoreported that the physiological age of an explant is an important factor in regeneration of multiple shoots in orchids.

Effect of TDZ on PLB induction

From preliminary experiments it was found that PLBs were induced in ½ MS medium supplemented with PGRs (NAA 2.69 µM and BAP 19.98 µM), (NAA 5.4 µM and BAP 22.2 µM) and (NAA 10.7 µM and BAP 22.2 µM). But PLB induction rate was less and time consuming. It took 3-5 months to produce a PLB mass of size 2 -5 mm and was induced only in shoot tips from six-month-old axenic plantlets. Hence keeping all the other parameters the same, with a view to induce early and increased shoot bud induction NAA and BAP were replaced with the plant growth regulator Thidiazuron (TDZ). It was observed that explantsshowed early induction of PLBs and shoot buds in all treatments with TDZ (4.54, 6.81 and 9.08 µM), 10% CW and 1% sugar. Since all the three TDZ concentrations did not exhibit significant differences, the hormonal concentration for PLB induction was optimised as TDZ at 4.54 µM.The PGR treatment 4.54 µM TDZ produced the highest number of shoot buds (15.2 per shoot tip) in 8 weeks(Table 1).

Gantait et al. (2012) working with leaf explants of a hybrid between *Aranda*and *Vanda* concluded that the best concentration of TDZ for stimulation of PLBs was 6.81µM. Park et al.,(2002) reported that in leaf explants of *Doritaenopsis* TDZ at $2mgl^{-1}$ gave the best response, much higher than the concentration suggested in this study. Increasing the concentration of TDZ did not seem to increase PLB proliferation. Contrary to this, Ernst (1994) reported that proliferation increased with increased concentrations of TDZ. He also reported that shoot and root development were reduced with increasing concentrations of TDZ. TDZ was highly potential in inducing PLBs or the proliferation of shoot buds from thin shoot tip sections in *Vanda coerulea* (Malabadi et al.,2004). Sujjaritthurakarn and Kanchanpoom (2011) reported that TDZ gave a superior response to BA for inducing PLBs in dwarf *Dendrobium*. These results can be attributed to its chemical properties. TDZ is a di-phenyl-urea derivative with influential cytokinin activity. TDZ has been estimated to be 50-100 times more efficient than regular cytokinin compounds in inducing cytokinin-like-effects (Genkov and Iordanka, 1995). It was observed that the addition of 10% coconut water to culture medium promoted induction of PLBs and axillary shoot buds from shoot tips. However there was no significant difference when higher concentrations (15% and 20%) were used.

*Table 3.*Effect of NAA and BAP in varying concentrations on *in vitro* response of primary PLBs and shoot buds after 3months of culture period.

Culture media	Plant growth regulators(µM)		No. of shoot buds	Response of explants after subsequentsubculturing
	NAA	BAP		
NDM	-	-	46.3[a]	m+s+r
NDM	0.54	4.44	37.3[b]	m+s+r
NDM	1.07	8.88	34.3[c]	m+s+r

Means followed by different letters are significantly different ($p < 0.05$) based on WASP 2.0 Analysis(ICAR, Goa) m– multiplication of shoot buds & PLBs ; s - shoot formation ; r – regeneration to plantlets

Previous studies have proven that the supplementation of coconut water in both liquid and solid medium enhanced the proliferation of PLBs of *Phalaenopsis* and *Vanda* hybrids (Gnasekaran et al., 2010 and Gnasekaran et al., 2012). According to them higher concentrations of coconut water (20% and 30%) tend to be inhibitory to PLB proliferation and was not significant at all except to that of control.

Effect of NDM on secondary proliferation of PLBs and direct shoot buds

Secondary PLBs and shoot buds were formed 2 weeks after subculturing in all the three media of NDM viz., NDM without hormones, NDM supplemented with (NAA 0.54 µM and BAP 4.44 µM) and (NAA 1.07 µM and BAP 8.88 µM). This media composition was selected based on an earlier report on *Phalaenopsis* and *Doritaenopsis* by Tokuhara and Mii (1993).Proliferation of PLBs and shoot buds was observed after 3-4 weeks of subcultivation. At the end of 8 weeks direct axillary shoot buds and shootbuds via PLBs multiplied and covered the medium that it was unable to count and to distinguish between the direct shoot buds and shoot buds from PLBs. Hence the experiment was evaluated at the end of 12 weeks of culture when shoot buds started to regenerate. Mean number of shoot buds ranged from 34.3 – 46.3; the highest mean of 46.3 was observed in the medium NDM without hormones (Table 3).

Continuous multiplication of PLBs, shoot buds, multiple shoots and small plantlets could be maintained in NDM devoid of hormones and supplemented with lower hormonal concentrations. This was supported by the findings of Mondal et al. 2013 in which lower NAA: BAP hormonal combinations proved to be appropriate when it is desired to carry forward PLB multiplication for many generations. Higher hormonal levels were adopted when plantlets for field planting were desired in a short time by easily rooting the large shoots.

Robust plantlets (4-6 cm in height) with optimum shoot elongation (5-8 mm) and thick roots (2-6 cm in length) ready for *ex-vitro* hardening were produced after subculturing for 8 weeks in modified ½ MS medium fortified with PGRs (NAA 2.69 µM and BAP 11.1 µM), 0.2% peptone, 2% sugar and 0.075% charcoal and solidified with 0.6% industrial agar. The plants were gradually acclimatized to greenhouse conditions and percentage survival of plants was 85%. About 50,000 hardened plantlets were produced from a single flower stalk with an average of 10 buds, within 2 years from initiation of axenic shoot tip cultures using this protocol.

The present study describes a commercial scale micropropagation method for multiple shoot proliferation *via* PLB mediated shoot buds and direct shoot buds from longitudinally bisected axenic shoot tips without an intervening callus formation. In the present study axenic plantlets served as explant source.According to Liao et al. 2011, the aseptic nature of mother plants ensures lack of microbial contamination and protects their meristem-derived juvenility status, rendering them responsive to tissue culture regeneration. Age of the axenic plantlets from which the shoot tips were excised markedly influenced PLB induction in *Phalaenopsis*. Explants from three-month-old and above *in vitro* plantlets exhibited optimum PLB induction and less necrosis. It was observed that bisecting shoot tips longitudinally prior to inoculation and substituting NAA and BAP hormonal combination by TDZ resulted in early PLB and direct shoot bud induction. Highest PLB proliferation was observed on subcultivation to New Dogashima Medium devoid of any hormones which reduces further the chances of somaclonal variation due to continued exposure to synthetic hormones and also makes the protocol more cost effective. Using this protocol about 50,000 hardened plantlets can be produced from a single flower stalk with an average of 10 buds, within 2 years from initiation of axenic shoot tip cultures. By repeated bimonthly subculturing of PLB and shoot buds on proliferation medium, it is possible to maintain continuous

production of plantlets which makes the present protocol an efficient means for commercial production of genetically stable *Phalaenopsis* hybrids.

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