Plant Tissue Cult. & Biotech. **28**(1): 91-98, 2018 (June)

PTC&B

Efficient Plant Regeneration Through Protocorm-like Bodies Derived from Shoot Tip Cultures of *Vanilla planifolia* Andr.

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Key words: Efficient plant regeneration, Protocorm-like bodies, Shoot tip culture, Vanilla planifolia

Abstract

A protocol for obtaining a ready source of protocorm-like bodies (PLBs) was developed in *Vanilla planifolia*. MS containing BA 1 mg/l, IBA 0.5 mg/l, tryptone/peptone 2 g/l and 2% sucrose was used to induce PLBs from axillary bud explants. PLB formation from the shoot/root tips involved the direct conversion of the apices without an intermediate callus stage. PLBs either directly developed into plantlet or produced secondary protocorm like bodies. Thin cell layer culture of protocorm like bodies can be used as a commercial application of tissue culture technology for mass micro-propagation. The details of production of PLBs and their transformations in culture are also discussed. The plantlets derived from PLB were rooted in culture and established in the field after hardening.

Introduction

The genus *Vanilla* Mill. (Plum. Ex. L) belonging to *Orchidaceae* comprises more than 100 species, distributed in tropical parts of the world (Dressler 1993). *Vanilla planifolia* Andr. is native to the humid tropical rain forests of South-eastern Mexico, Central America, the West Indies and northern part of South America.

Vanilla is one of the world's most labor-intensive agricultural crops, this is why it is so expensive. Even though vanilla flowers are bisexual in nature, because of the presence of a special structure inside the flower, called "rostellum", hand pollination must be used to get an economic crop. Natural seed

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germination is possible only in association with mycorrhiza. In vanilla, the aseptic method ensures nearly cent per cent germination of seeds and has largely been used for breeding fusarium root rot resistant varieties (Knudson 1950, Withner 1955).

Traditionally vanilla is propagated from cuttings of mature vines. This method is not economical since the collection of stem cuttings leads to arrest of growth and developments of the mother plant (Ayyappan 1990). Vanilla, being a monopodial orchid, yields only a small number of planting material (Tessy 1995). More over, the market demand for the propagules is hardly met with such cuttings. As the growers are looking for alternate sources, micropropagated plantlets serve the purpose and are popularly used (Geetha and Shetty 2000).

Protocols on micropropagation of vanilla have been reported using nodal explants (Kononowicz and Janick 1984, George and Revishankar 1997), and aerial root tips (Philip and Nainar 1986) and through the culture of callus masses (Gu et al. 1987, Davidonis and Knorr 1991). In orchids, PLBs, which appear on the vegetative bud in culture, are adventive embryos analogous to the gametic embryos. There are morphological and physiological similarities between the PLBs and the protocorms of orchids (Huang et al. 1990). Orchid PLBs, which originated from vegetative tissues, were considered to be true somatic embryos by Moral (1974).

The present work outlines a method of efficient *in vitro* propagation of vanilla *via* mass production and regeneration of 'protocorm like bodies'.

Materials and Methods

The nodal segments obtained from two-year-old plants of *V. planifolia* were used as explants for inducing protocorm like bodies (PLBs).

Nodal segments obtained from field grown plants were washed in tap water and surface sterilized with 0.1% mercuric chloride for 3 min washed thrice with sterile distilled water and aseptically transferred to MS supplemented with 2% sucrose. The pH was adjusted to 5.8 before adding 0.8% agar. They were maintained in 25 ± 2°C under 12 hrs photoperiod.

The axillary buds regenerated from the nodal explants were transferred to different experimental media combinations (Table 1) for PLB induction, with sufficient replicates. The pH was adjusted to 5.8 before adding 0.8% agar.

The PLB obtained by axillary bud culture were cut into small bits and cultured separately to allow multiplication and growth. For this, thin cell layers (TCL) of explants were excised transversly from PLBs and cultured in MS containing BA 1 mg/l, IBA 0.5 mg/l, tryptone 2 g/l and 2% sucrose. The culture

medium was adjusted to pH 5.8 before autoclaving and solidified with 0.7% agar. The cultures were incubated at 25 ± 1ºC with a light intensity of 2000 lux and 14 hrs light/10 hrs dark photoperiod.

Table 1. The intensity of PLB development in the axillary bud explants to different media supplements.

Sl. No.	Media combinations	Intensity of PLB development
1	MS + BA 0.5 mg/l	0
2	MS + BA 1 mg/l + 2,4 D 0.5 mg/l	0
3	MS + BA 1 mg/l + NAA 0.5 mg/l	0
4	MS + BA 1 mg/l + IBA 0.5 mg/l	++
5	MS + BA 1 mg/l + IBA 0.5 mg/l + peptone 2g/l	+++
6	MS + BA 1 mg/l + IBA 0.5 mg/l + tryptone 2g/l	+++

0, nil; ++, moderate, +++, high. *Data scored at the end of four weeks based on visual observations of culture (10 replicates per treatment).

Subculturing of the plantlets derived from PLBs was carried in MS supplemented with 1 mg/l BA, 0.5 mg/l IBA and 3% sucrose to allow rooting. The rooted plants (10 - 15 cm long) were washed under running water to remove traces of agar and transferred to plastic cups containing sand. The cups were kept covered with polythene bags and kept in a shade house. The polythene cover was removed after one month and the plants were transplanted to polythene bags containing sterile potting mixture.

Results and Discussion

In vitro propagation is normally based on the stimulation of multiple-shoot growth from cultured shoot-tip and nodal explants (Murashige 1974, Brown and Thorpe 1995). It has not always been possible to adapt this approach to certain plant taxa like orchids where the proliferation rate is too low (Litz and Gray 1992). Therefore, the *in vitro* technology for culture and propagation of orchids *via* PLBs is an improved efficient method for development and regeneration of the plantlets.

For PLB induction BA in combination with different auxins was tried (Table 1). The combination of BA with 2,4-D produced callus but failed to survive. Among the three auxins tried, only the combination of BA with IBA could induce PLB development; and shiny white elongated protocorms were obtained (Fig. 1a). When this medium was supplemented with tryptone/peptone, the intensity of PLB development was found to be enhanced. The multiplying protocorms

appeared as thick stout clumps in these combinations (Fig. 1b). The PLB proliferated via the TCL procedure in MS containing BA 1 mg/l, IBA 0.5 mg/l, tryptone 2 g/l and 2% sucrose (Fig. 1c). PLBs presented two basic patterns of development; one of them represented by direct plant formation (Fig. 1d) and the other by a secondary PLB regeneration giving rise as a consequence, to clusters of new PLBs (Fig. 1c). The types of responses included root tip/shoot apex conversion into PLB and further proliferation and multiplication of PLB (Fig. 1e, 1f). The plantlets derived from PLB rooted well during subculturing and the establishment rate after hardening (Fig. 1g, 1h) was more than 50 per cent.

In the orchid family, the direct conversion of root tip into PLBs *in vitro* was described in *Catasetum* (Kerbauy 1984a) and *Cyrtopodium* (Sanchez 1988). Techniques for the rapid mass propagation of orchids *via* PLB is reported in orchids like *Geodorum densiflorum* (Biswajit and Datta 2000), *Dendrobium* (Yu et al. 2001), *Vanda* (Munu et al. 2000), *Oncidium* (Chen and Chang 2000), *Cattleya intermedia* (Mello e Silva et al. 2000), *Ipsea malabarica* (Gangaprasad 1999), *Pongonia japonica* (Takahashi and Kondo 1998), *Catasetum fibriatum* (Kerbauy and Colli 1997), *Cymbidium* (Kirdmanee et al. 1992).

PLB formation may occur from yellow white embryonic calli in *Oncidium* (Chen and Chang 2000), friable calli in *Phalaenopsis*, *Doritaenopsis* and *Neofinetia* (Islam and Ichihashi 1999), root apex conversion to PLB as in *Catasetum fimbriatum* (Kerbauy and Colli 1997), rhizome derived PLB (Takahashi and Kondo 1998), or from nodal explants (Vij et al. 1994).

PLB induction is promoted by various media additives like peptone (Chen et al. 2000, Biswajit et al. 2000), casein acid hydrolysate (Gangaprasad and Decruse 1999), maltose and sorbitol (Islam and Ichihashi 1999), potato juice (Kimura and Kurihara 1991), p-coumaric acid (Colli and Kerbauy 1993), peptone and tryptone (Amaki and Higuchi 1989). Kerbauy (1993a) studied the effect of nitrogen sources, auxins and cytokinins on PLB induction and suggested that NAA and ammonium ions were the most effective substances to overcome the intrinsically low rate of regeneration of the root tip protocorm like bodies. Relatively low concentrations of sucrose and agar favored the formation of PLB (Kerbauy 1993b). Park et al. (1996) investigated the multiplication of PLB of *Phalaenopsis* in liquid culture and concluded that Vacin and Went (VW) liquid medium was the most suitable for PLB multiplication.

Begum et al. (1994) made histological studies on the developing protocorm like bodies of *Cymbidium* and traced the PLB producing initials to a group of cells found just below the surface layer of epidermis. The histological details of the development of protocorms and buds as observed in culture are described by Kerbauy and Estelita (1996).

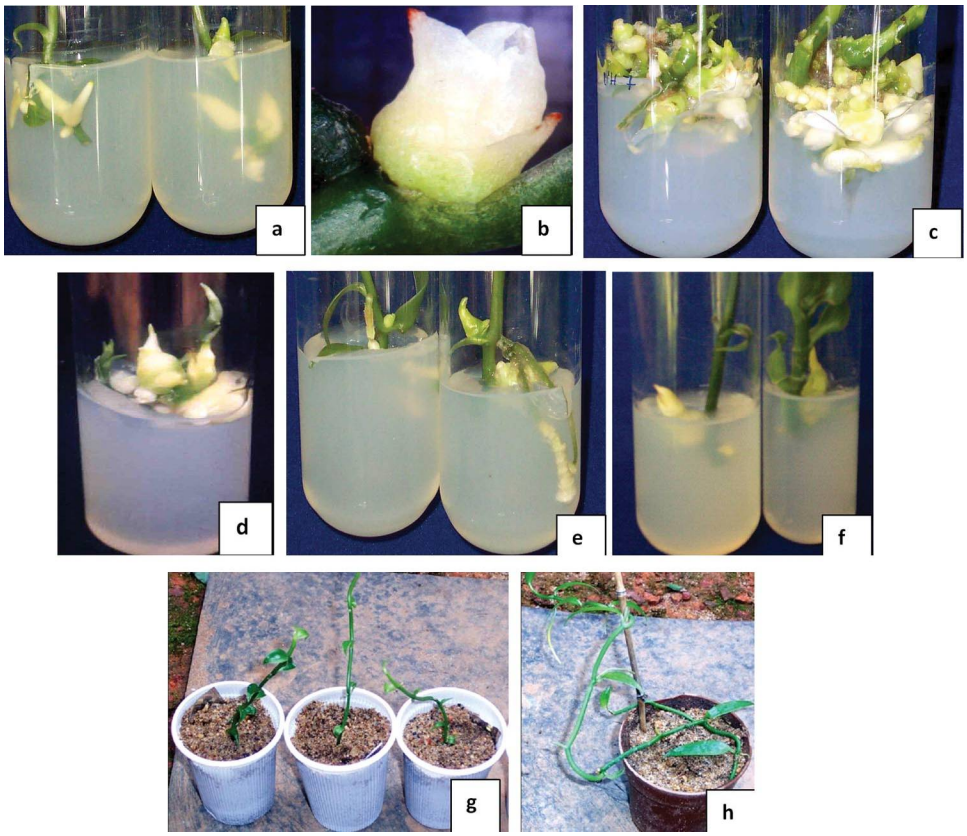

Fig. 1 a-h: (a) Shiny white elongated protocorms, (b) stout protocorm as observed under stereomicroscope (scale 1 cm = 0.1mm), (c) proliferation of PLB via thin cell layer culture, (d) direct plant formation from PLB, (e) root apex conversion to PLB, (f) shoot tip conversion to PLB, (g) hardening of plant after rooting, (h) established plant.

The young protocorms formed during seed germination of *V. planifolia* is generally elongated and its tip often curved; it is much less thick than the PLBs (Veyret 1974) and fails to develop chlorophyll. The fact that PLBs and protocorms of the cultivated vanilla are slow to develop chlorophyll in experimental conditions suggests that they would be obligatory mycorrhizal in natural conditions. Among the vanillas (Bouriquet and Boiteau 1937, Knudson 1950), the formation of the roots in the seed derived protocorms is much later.

The pattern of PLB formation was common to all the media additives. Basically PLB formation in root tips and shoot tips of vanilla involves a direct conversion of the apices, while in most other orchid species studied a callus stage has proved to be an intermediate condition for *in vitro* regeneration (Stewart and Button 1978, Kerbauy 1984b). The behavior of protocorm-like bodies (PLB's) of *Vanilla planifolia* in culture was similar to the results obtained by Kerbauy and Colli (1997) in *Catasetum fimbriatum*.

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