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Double phase culture system mediated enhanced protocol for shoot proliferation of *Vanilla andamanica* Rolfe. - an endemic wild relative of commercial Vanilla from the Andaman and Nicobar Islands

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Abstract

A liquid overlay culture system in micropropagation improved shoot proliferation in many species. In the present investigation, we have developed an enhanced shoot proliferation protocol using the double phase culture system (DPS) for *Vanilla andamanica*, an endemic species of the Andaman and Nicobar Islands recognized under the vulnerable category by the IUCN. *In vitro* generated nodal explants were used for shoot proliferation experiments and were tested in Murashige and Skoog medium augmented with various cytokinins (BAP, KIN, 2iP) and auxins (IBA, NAA, IAA) to produce a maximum of 3.24 ± 0.08 shoots per explant in 5 µM BAP and 5.23±0.67 roots per shoot in basal MS medium. A remarkable increase in shoot regeneration was observed when the nodal explant was cultured on DPS system with optimum cytokinin (5 µM BAP). On evaluation of the influence of DPS and conventional single-phase systems (SPS), (solid medium) exhibits an improved multiplication rate on a DPS with optimal BAP (5µM) with an average of 8.66 ± 0.17 shoots per explant, which represents a 2-fold increase over the rate of SPS + 5 µM BAP. The plantlets were rooted at 100% frequency in half-strength MS medium devoid of auxins and acclimatized with 100% success. A double-phase mediated enhanced shoot proliferating procedure could be employed for large scale multiplication and commercial breeding trials for better results with regard to *V. andamanica*.

Keywords

Vanilla andamanica; endemic; double phase culture; liquid medium; micropropagation; shoot proliferation

Introduction

The genus *Vanilla* Plum. ex-Mill., belonging to the family Orchidaceae and comprising more than 110 species, is distributed throughout the tropics (1, 2). Interestingly, *Vanilla* is the only commercial orchid taxon found to be an expensive flavouring material across the world after saffron (3). All the *vanilla* species except commercial *vanilla* are under the threat of serious habitat destruction and massive exploitation, highlighting the relevance of their conservation (3). The commercial crop improvement programmes are apparently meagre owing to limited clonal sources and the availability of a wide range of genetic variability. It highlights the necessity of *ex-situ* conservation in different geographical habitats of the taxon.

V. andamanica is a climbing, hemiepiphytic shrub whose occurrence

in the Andaman and Nicobar Islands was reported by Rolfe in 1918. The genetic distance of *V. andamanica* from other wild relative species of *vanilla* showed that it had a different origin, which may have arisen during past historical introduction events in the Asian sub-continent along with India (3). This evidently emphasises the importance and rarity of *V. andamanica*.

According to Lubinsky (4), the primary gene pool of *vanilla* species is evidently threatened, and therefore the secondary gene pool contains wild relatives of commercial *vanilla* that serves as a source of many desirable characteristics which can be used for crop improvement programmes (5). Disease resistance was considered one of the most important crop breeding traits. The foot rot and wilt diseases commonly found in cultivated species of *vanilla* by *Fusarium oxysporum* and *Phytophthora meadii*, which are rather least infected with *V. andamanica,* are a hallmark of this wild endemic taxon in the breeding programmes among existing germplasm of *vanilla*. Successful isolation followed by protoplast fusion of *V. planifolia* and *V. andamanica* was also reported in order to achieve gene transfer of desirable traits and hybrid production (6).

Habitat destruction coupled with frequently occurring natural calamities in the Andaman and Nicobar group of islands severely threatens many endemic and rare species, highlighting the immediate requirement of *ex-situ* conservation of plant species outside the islands. Micropropagation facilitates enhanced rapid multiplication of plant species from the limited plant material and is the best propagation methodology for the rare species concerned. In the present investigation, an evaluation of a double phase culture system over a conventional system of propagation was employed. A successful protocol for enhanced shoot multiplication for the conservation of *V. andamanica* through a double phase culture system was employed for the first time.

Materials and Methods

Explant source and culture condition

Plant materials for *in vitro* culture were originally collected from South Andaman Island and were well acclimatised in the greenhouse of the Department of Botany, University of Kerala, India (Fig. 1.A).They served as a source of explant. Young top shoots having two to three nodes from healthy plants grown in the greenhouse were defoliated, excised into nodal segments, and washed in tap water for 10-20 min, followed by treatment in the 10% Labolene solution (Galaxo India Ltd., Mumbai) for 10-15 min with constant shaking. Nodal segments were then subjected to washing in running tap water for 30-45 min, were further rinsed thrice using double-distilled water, and were surface sterilised using 0.1% $HgCl_2$ (SRL, Pvt. Ltd., India) for 5-6 min also. The surface-sterilized explants were further washed with sterile double-distilled water three to four times and inoculated into culture medium after the excision of nodal explants into appropriate sizes (1-1.5 cm). Surface sterilisation and inoculation were carried out under an aseptically maintained laminar airflow hood (Klenzaids, India).

For initiating cultures, MS medium (7) containing salts and vitamins augmented with 3% sucrose (w/v) (Himedia, India) and gelled with 0.8% agar (w/v) (Himedia, India) was used, and the pH was adjusted to 5.8 ± 0.2 using 1N NaOH or 1N HCl. Sterilization of a culture bottle (13 x 6 cm) was carried out in an autoclave (Nat Steel, Mumbai) at 120°C for 18 minutes under 15 lbs. pressure. The inoculated cultures were carefully maintained in a culture room provided with 25 ± 2 °C under a 16/8 h photoperiod with a light intensity of 2500 lux ($\mu mol^{-2} S^{-1}$) provided with white LED tubes (Crompton Ltd., Mumbai) and 55- 60 % relative humidity.

Culture initiation and *in vitro* multiplication

The surface-sterilised nodal segment was inoculated into MS medium without plant growth regulators. Nodes containing a petiole attached to a portion of an internode (nodal segments) of appropriate size were excised from proliferated shoots in cultures and subcultures into fresh hormone-free medium for further proliferation. Nodal segments obtained from subcultures served as explants for further *in vitro* experiments. *In vitro*-developed nodal explants were cultured on MS medium supplemented with various concentrations of different cytokinin individually, viz. 6-Benzyl Amino Purine (BAP 1-5 µM), kinetin (KIN 1-5 µM), and 2-isopentenyl adenine (2 iP 1-5 µM) was tested for shoot multiplication and medium devoid of plant growth regulators was used as a control.

Shoot proliferation in Double Phase Culture System

To evaluate the effect of double phase culture systems and their duration, MS medium supplemented with optimum cytokinin (5 µM BAP) for shoot multiplication was selected. A nodal segment derived from stock cultures with one axillary bud was excised and inoculated into the double phase medium (DPS). The explants inoculated on 60 mL of solid medium and 15 mL of liquid medium with optimum BAP (5µM) were added after 30 days of culture without removing the solid medium. For solid phase medium (SPS), the shoots were retained on the same medium itself. The parameters were evaluated at 30, 60, and 90 days after the culture inoculation. The total shoot number, shoot length and percentage of response were recorded.

In vitro rooting

Healthy shoots of about four to five leaves were transferred to half strength-MS medium with different concentrations (0.5-2 µM) of auxins: (IBA (Indole -3- butyric acid), IAA (Indole-3- acetic acid) and NAA (1- Naphthalene acetic acid) to produce *in vitro* roots.

Acclimatization and field establishment of rooted plantlets

In vitro-rooted cultures were taken from the culture room, kept at room temperature for one week, and deflasked and washed using tap water to remove the traces of agar. Rooted plantlets were transferred to plastic pots (6 x 7.5 cm) filled with a potting mixture of garden soil and river sand (1:1) and maintained humidity by covering the

planted pots with a transparent polythene bag. The polythene bags were removed from the pots after 30 days, and the pots were transferred to greenhouse conditions. Hardened plants were planted in a potting mixture (garden soil, sand, and cow dung in a 1:1:1 ratio) containing plastic pots (17 x 15 cm) and then in the field.

Design of experiment and data collection

A completely randomized experimental design was employed for conducting experiments with three replications and three explants per flask. Experimental data were recorded after 30, 60, and 90 days in the case of the double phase culture system experiment. After 60 days, data were recorded for *in vitro* rooting and shooting experiments using PGRs and subjected to ANOVA, and the data means were compared by Tukey's test ($p \leq 0.05$) (Minitab ver. 20.4.0., Minitab, LLC., Pennsylvania, US).

Result and Discussion

Influence of PGRs on shoot multiplication

The effect of plant growth regulators supplemented with MS medium on nodal explant response for shoot multiplication after 60 days of culture is represented in Table 1. *In vitro* derived nodal segments were inoculated with MS medium supplemented with various concentrations of cytokinins like BAP, KIN, and 2iP to induce multiple shoots. Among the cytokinins used, the maximum multiple shoot induction (3.24 ± 0.08) and shoot length (2.52 ± 0.10cm) were obtained on 5µM BAP augmented MS medium after 60 days with a 79.20 ± 2.77 percentage of response (Table 1) (Fig. 1.B). A reduced number of shoots per explant was given by other cytokinins tested (KIN and 2iP), which also did not contribute much shoot length when compared to BAP.

The present investigation reveals that the MS medium augmented with 5µM BAP showed a better regeneration response in *V. andamanica* when compared to other tested cytokinins. BAP induced multiple shoot regeneration was also reported in *V. planifolia* by Abede *et al*. (8) and de Oliveira *et al*. (9). However, Diwakaran *et al*. (10) report a combinational medium (4.44 µM BAP and 2.4 µM IBA) for enhanced shoot production in *vanilla* species, which was completely absent in the present study.

Influence of double phase culture system on shoot multiplication

In order to enhance multiple shoot induction, the explants were inoculated with double phase medium containing optimum cytokinin (5µM BAP) supplemented with agar-gelled solid MS medium and a layer of liquid MS medium. The results obtained on experiments with SPS and DPS with different culture durations are shown in Table 2. It was observed that the use of DPS with 5µM BAP induces enhanced shoot multiplication (8.66 ± 0.17) and significantly improves the rate of shoot multiplication and shoot elongation (2.14 ± 0.09) (Fig. 1.C) compared to other systems (SPS, SPS + BAP, DPS) with a higher percentage of response (84.70 ± 0.25) after 90 days of culture. The DPS culture system improves the shoot multiplication efficiency at least two times higher than that of a conventional system of culture with a culture duration of 90 days. Rapid development of explants as well as the healthy development of multiple shoots was enabled by the DPS system.

Several plant species, such as *Malus sylvestris* (11), pineapple (12, 13), *Cymbidium giganteum* (14), *Pyrus calleryana* (15), potato (16), and *Gynerium sagitatum* (17), provide evidence for the use of liquid medium for improved shoot regeneration. Usually, liquid medium based culture systems use a support such as a bridge or paper raft. In the present DPS system, the solid phase basement acts as an anchor, whereas the liquid phase replenishes plant growth regulators and nutrients, which

Table 1. The effect of various cytokinins & augmented - with MS medium on shoot multiplication from *in vitro* generated nodal explants of *Vanilla andamanica*.

PGRs (µM)			Means no. of shoots/explant (cm) ±SE	Mean shoot length in (cm) ±SE	Percentage of Response
BAP	KIN	2ip			
0	0	0	1.00±0.00^{f}	1.53±0.01^{ef}	42.85±0.00^{i}
1			0.90±0.08^{gh}	1.65±0.04^{de}	61.27±1.09^{e}
2			1.62±0.08^{de}	1.99±0.03^{bc}	66.23±0.25^{d}
3			2.14±0.14^{bc}	2.13±0.06^{b}	70.94±0.82^{c}
4			2.42±0.14^{b}	2.10±0.04^{b}	75.96±0.05^{b}
5			3.24±0.08^{a}	2.52±0.10^{a}	79.20±2.77^{a}
	1		1.00±0.14^{gh}	1.55±0.02^{ef}	47.20±0.20^{h}
	2		1.14±0.14^{fgh}	1.38±0.07^{c}	52.33±0.45^{g}
	3		1.38±0.08^{ef}	1.66±0.05^{de}	56.70±0.60^{f}
	4		1.61±0.16^{de}	1.79±0.09^{cd}	70.80±0.72^{c}
	5		1.85±0.14^{cd}	1.84±0.03^{cd}	66.04±0.14^{d}
		1	0.80±0.08^{h}	1.35±0.24^{c}	42.85±0.00^{i}
		2	1.19±0.08^{fg}	1.33±0.03^{a}	52.42±0.42^{g}
		3	1.42±0.14^{ef}	1.63±0.02^{de}	60.43±2.12^{e}
		4	1.38±0.08^{ef}	1.74±0.01^{de}	60.80±0.73^{e}
		5	1.66±0.08^{de}	1.85±0.02^{cd}	57.14±0.00^{f}
Treatment Df(n-1)=15			94.50***	32.32***	364.76***
Cytokinin(T) Df(n-1)=2			4.59*	6.67**	15.51***
Cytokinin con.(C) Df(n-1)=4			481.41***	130***	157.71***
TxCDf (n-1)=14			62.09***	3.84*	2.27^{NS}

Means followed by same letter within a column are not significantly different ($p < 0.05$) as determined by Tukey's test

NS – Non significant, *** F value is highly significant at $p < 0.001$, ** significant at $p < 0.01$ and *significant at $p < 0.05$

Table 2. The effect of various *in vitro* culture systems (SPS - solid phase system and DPS - double phase system) with 5 µM BAP on shoot proliferation from *in vitro* generated nodal explants of *Vanilla andamanica*.

Culture System	Culture Duration (No. of days)	Mean Shoot Number Per explant ±SE	Mean Shoot Length ±SE	Percentage of Response
	30	1.00±0.00^{f}	1.25±0.02^{f}	40.63±1.19^{f}
	60	1.00±0.00^{f}	1.40±0.01^{e}	41.90±1.65^{f}
	90	1.00±0.00^{f}	1.47±0.01^{e}	46.66±1.65^{ef}
	30	2.19±0.08^{d}	1.76±0.04^{cd}	73.73±4.03^{c}
SPS+BAP	60	3.28±0.14^{c}	1.86±0.03^{c}	79.63±1.48^{abc}
	90	4.29±0.15^{b}	2.03±0.01^{b}	81.43±1.25^{ab}
	30	1.14±0.14^{f}	1.49±0.02^{e}	50.10±0.26^{e}
DPS	60	1.52±0.08^{e}	1.71±0.01^{d}	51.67±2.89^{de}
	90	1.48±0.08^{e}	2.01±0.01^{c}	56.53±0.92^{d}
	30	2.14±0.15^{d}	1.47±0.02^{e}	77.77±3.78^{bc}
DPS+BAP	60	4.14±0.14^{b}	1.74±0.03^{d}	81.57±1.40^{ab}
	90	8.66±0.17^{a}	2.14±0.09^{a}	84.70±0.25^{a}
Treatment Df(n-1)=11		1182.8***	197.04***	206.41***
Culture system(S) Df(n-1)=3		17.05***	40.74***	95.95***
Culture Duration(D) Df(n-1)=2		233.75***	656.07***	61.68***
DxSDf (n-1)=11		105.06***	42.35***	0.17^{NS}

Means followed by same letter within a column are not significantly different ($p < 0.05$) as determined by Tukey's test

NS – Non significant, *** F value is highly significant at $p < 0.001$, ** significant at $p < 0.01$ and *significant at $p < 0.05$

Table 3. The effect of various auxins augmented - with half strength MS medium on rooting from *in vitro* generated shoots of *Vanilla andamanica*.

PGRs (µM)			Means no. of Roots/explant ±SE	Mean Root length in (cm) ±SE	Percentage of Response
IBA	IAA	NAA			
0	0	0	5.23±0.67^{a}	1.36±0.11^{cd}	100
0.5			4.61±0.36^{ab}	1.27±0.02^{cde}	100
1			4.57±0.53^{ab}	1.59±0.01^{b}	100
2			4.76±0.16^{ab}	1.92±0.02^{a}	100
	0.5		4.71±0.14^{ab}	1.17±0.06^{cde}	100
	1		4.80±0.16^{ab}	1.15±0.03^{de}	100
	2		4.80±0.08^{ab}	1.26±0.01^{cde}	100
		0.5	4.19±0.08^{ab}	1.13±0.02^{e}	100
		1	4.38±0.08^{b}	1.13±0.06^{e}	100
		2	4.42±0.38^{b}	1.38±0.17^{bc}	100
Treatment Df(n-1)=9			2.95^{NS}	34.94***	-

Means followed by same letter within a column are not significantly different ($p < 0.05$) as determined by Tukey's test

NS – Non significant, *** F value is highly significant at $p < 0.001$, ** significant at $p < 0.01$ and *significant at $p < 0.05$

enhance shoot proliferation (18). Reduction of subculture with maintenance of explants for several months in a culture system had another advantage in double phase culture system, as reported earlier (13). A DPS system devoid of physical barriers increases nutrient availability and facilitates enhanced absorption by the explants, thereby promoting shoot growth. The DPS system ensures a higher quantity of nutrients in the culture system. From the present investigation, the evaluation of the efficiency of DPS in shoot enhancement of *V. andamanica* provides a cost-effective and reliable method for routine micropropagation and thereby ensures *in vitro* conservation strategies for this endangered and endemic species.

Rooting

Generally, influences of auxin quality and concentration showed a significant influence on rooting in the micropropagation technique. The observed results for *in vitro* rooting of micro stems with various concentrations of auxins are recorded in Table 3. Significant improvement in rooting was not observed in the addition of auxins, and the basal half strength MS medium provided the maximum result in an average of 5.23±0.67 roots per explant (Fig. 1.D) with 100% rooting after 60 days of culture. However, IBA showed a maximum root length of 1.92±0.02cm (Fig. 1.E).

Spontaneous rooting in basal MS medium was exhibited by *V. andamanica* micro stems. Endogenous regulators for rooting at the optimum level were present in the nodal explant and are suggested as the reason for the spontaneous rooting of microstems even in the basal MS medium of *Vanilla* species (10). Several herbaceous species produce roots easily in auxin devoid or mediums with low levels of auxin, as already reported (8, 19, 20).

Hardening and acclimatization

In vitro produced rooted plants were taken out, washed thoroughly, and planted in plastic pots with river sand and garden soil (1:1). The potted plants were transferred to a greenhouse for acclimatization. It was observed that the plants raised from both SPS and DPS culture systems exhibited 100% survival rates (Fig. 1.F). A high percentage survival rate of *in vitro* regenerated *Vanilla* species was reported earlier by de Oliveira *et al*. (9).

Fig. 1. **A.** Habitat of *V. andamanica* **B.** *In vitro* produced multiple shoots with SPS after 60 days **C.** *In vitro* produced multiple shoots using DPS system (5µM BAP) after 90 days **D.** Basal MS medium producing roots without any PGRs **E.** IBA induced *in vitro* rooting **F.** Hardened *in vitro* plants of *V andamanica*

Conclusion

The present investigation evaluated the efficiency of a double phase system and revealed that enhanced shoot multiplication of *V. andamanica* could be achieved through this novel technique rather than conventional methods. As regards large-scale propagation of *V. andamanica,* the DPS system can be used as a cost-effective and reproducible method that ensures its conservation, which may be applicable for other orchid members as well. This is the first report of the usage of DPS -mediated shoot proliferation in *V. andamanica,* an endangered endemic species on the road to extinction, from the Andaman and Nicobar Islands in the Bay of Bengal.

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Authors' contributions

SJ carried out the whole experiment and drafted the manuscript. AGP and SPM incorporated corrections to the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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