KERALA STATE COUNCIL FOR SCIENCE, TECHNOLOGY AND ENVIRONMENT

BACK TO LAB PROGRAMME

FINAL TECHNICAL REPORT

MERICLONING OF *PHALAENOPSIS* AND *VANDA* HYBRIDS

Project Reference No: 008/WSC-BLS/2011/CSTE

Principal Investigator : Preeta Liz Korah

Scientist Mentor : Dr.Shylaraj K.S.

Rice Research Station

Vyttila, Kochi -19

Kerala, India

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INTRODUCTION

Orchids, the most beautiful flowers in God's creation, comprise a unique group of plants.Taxonomically,they represent the most highly evolved family among monocotyledons with 600-800 genera and 25,000-35,000 species. Of late, South East Asia has developed into a major supplier of orchid hybrids. In India, orchids form 9% of our flora and are the largest botanical family of higher plants . About 1300 orchid species have been reported from India scattered all over North eastern Himalayas(600 species), North Western Himalayas(300 species), Maharashtra(130 species), Andaman and Nicobar Islands(70 species) and Western Ghats(200 species). Many species of Dendrobium,*Phalaenopsis* and Vanda are renowned for their very showy and attractive flowers and their great ornamental value. The most important hybrid in trade internationally is *Phalaenopsis Blume* (over 22,000 man-made hybrids). *Phalaenopsis* orchids have high economic value in the floriculture industry.

In vitro propagation makes it possible to clonally mass propagate hybrids of commercial value and conserved species. However, *in vitro* culture technologies are still a challenge because of the slow growth of plantlets, low multiplication rate, poor rooting and somaclonal variation. Although seed-raised plants can be used for conservation and breeding for the selection of superior features, genetic characteristics including seasonality, inflorescence, flower color and type are not uniform. In this regard, micropropagation through mericloning is an important strategy in obtaining genetically stable plants and improvement of quality. However, not all genotypes of *Phalaenopsis* respond to the same protocol under the same culture conditions.

Many species of orchids viz., *Phalaenopsis* and *Vanda* are renowned for their attractive flowers. These species are becoming prominent in the homesteads of Kochi metro both for ornamental and cut flower purpose. The non-availability of good quality/true to type planting material at reasonable price is the most important limiting factor for its spread and cultivation at a large scale. At present these hybrids are imported from Singapore,Malaysia,Thailand etc. by middle men and grown in private nurseries and distributed to consumers at an exhorbitant price. The import cost and the profit realized by the middle men enhances the sale price of these hybrids. This limits its fast spread and its large scale cultivation is made unaffordable for the common man due to high initial investment which hinders the development of orchid industry in Kerala. The present project has been formulated as a solution to this problem.

BRIEF TECHNICAL PROGRAMME

Objectives

1.Collection of the most demanded hybrids of *Phalaenopsis* and *Vanda* already selected through homestead survey of orchid growers.

2. Protocol development for mass multiplication of two each of the selected *Phalaenopsis* and *Vanda* hybrids through mericloning.

Methodology

A preliminary survey among the homesteads of Ernakulam revealed that the *Phalaenopsis/Doritaenopsis* hybrids and *Vanda* hybrids listed below have high consumer demand due to their adaptability to the climatic conditions,free flowering nature and blooms kwith aesthetic appeal. Although *Doritaenopsis* is now a defunct generic name and is botanically categorized as *Phalaenopsis,*the name *Doritaenopsis* is still commonly used horticulturally. Based on the availability in required numbers, two each Phalaenopsis hybrids and two each Vanda hybrids will be mericloned for mass production.Trial experiments will be done for the rearing of micropropagated plants in the nurseries of selected orchid growers in Ernakulam District with the trials to be done in the implementing institute so that plants can be sold directly from lab to the growers without rearing. Germplasm of these varieties is maintained at RRS,Vyttila but not in enough numbers for research purpose. Hence the hybrid materials need to be purchased from KAU,TBGRI or registered private nurseries.

The protocol for mericloning will be standardised which involves

- Standardisation of choice of explant
- Protocol development for adequate surface sterilization of explants particularly against fungus which is most serious
- Standardisation of initiation medium
- Standardisation of multiplication medium
- Standardisation of rooting medium
- Standardisation of hardening technique
- As future line of work, mass production of these hybrids will continued at RRS,Vyttila under Kerala Agricultural University.

MATERIALS AND METHODS

1. Procurement of Mother Plants and After Care

From preliminary survey among the homesteads of Ernakulam *Phalaenopsis/Doritaenopsis* hybrids and *Vanda* hybrids having high consumer demand due to their adaptability to the climatic conditions,free flowering nature and blooms with aesthetic appeal had already been identified. In the present study, 190 plants belonging to 30 hybrids of *Phalaenopsis* and 15 hybrids of *Vanda* including the identified hybrids were procurement from registered private nurseries. The mother plants purchased were *V*. Dr.Anek, *V*. Pat Delight *V*. Robert's Delight , *P*..Joy Angel Voice , *P*. Leopard Prince , *P*. Everspring Angel , *P*. Sogo Lit Angel and other promising hybrids. Greenhouse grown plants of 16 *Phalaenopsis* hybrids and 4 *Vanda* hybrids listed in **Table 1** were used as explant source.

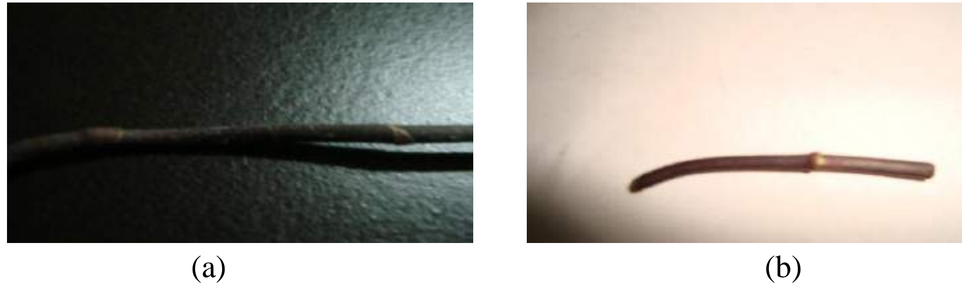
Phalaenopsis and *Vanda* hybrids purchased from registered private nurseries were repotted, labeled and maintained in the green house. The plants were daily watered and sprayed with fertilizers NPK 30:10:10 and NPK 13:27:27 alternately at weekly intervals. Fu Foliar spray with fungicide bavistin 0.05% was done at fortnightly intervals. Protection from snail infestation was done by placing metaldehyde pellets in the pots at regular intervals. These mother plants were used as the source for explants in various experiments.

2. Isolation of Explant and Surface Sterilisation

In *Phalaenopsis* hybrids, explants tested for *in vitro culture* were

1. Nodal segments (0.5-1cm.) bearing vegetative buds excised from both tender flower. stalks (only 2-3 opened flowers) and fully flowered flower stalks **(Fig.1a & b)**.
2. Shoot tips of flower stalk buds, 0.1-0.3cm wide and 0.5mm high.
3. Thin internodal sections (0.3 – 0.5cm.) of flower stalks.
4. Young undifferentiated inflorescence buds.
5. Shoot tips (0.3-0.5cm.) from axenic plantlets or axillary shoots.
6. Fresh root tips (0.8-1cm.) excised from axenic plantlets or shoots
7. Leaf bases (0.8-1.5cm.) excised from axenic plantlets or shoots

Motherplants for explant excision maintained in the greenhouse



(a) (b)

Fig.1(a) *Phalaenopsis* flower stalk with dormant buds
(b) Nodal segment with dormant bud after removing bract

The flower stalks were sprayed with bavistin (0.1%) on the day prior to removal from plant. The excised flower stalks were rinsed well with tap water and detergent and wiped with 95% alcohol. Flower stalks were cut into 1.5-2cm. single node cuttings with parts of internodes, each node holding one dormant lateral bud, treated with bavistin (0.05%) for 3 min and washed thrice in sterile water and taken to the Laminar Air Flow chamber. Inside the Laminar Air Flow a comparison among 4 modes of surface sterilization was done :

(i) 20% (v/v) branded sodium hypochlorite solution

(ii) 20% (v/v) house hold bleach

(iii) 15 % (v/v) house hold bleach after adjusting the pH to 7± 0.02 with HCl

(iv) 20% (v/v) house hold bleach after adjusting the pH to 7± 0.02 with HCl

(v) 15 % (v/v) house hold bleach after adjusting the pH to 9 ± 0.02 with HCl

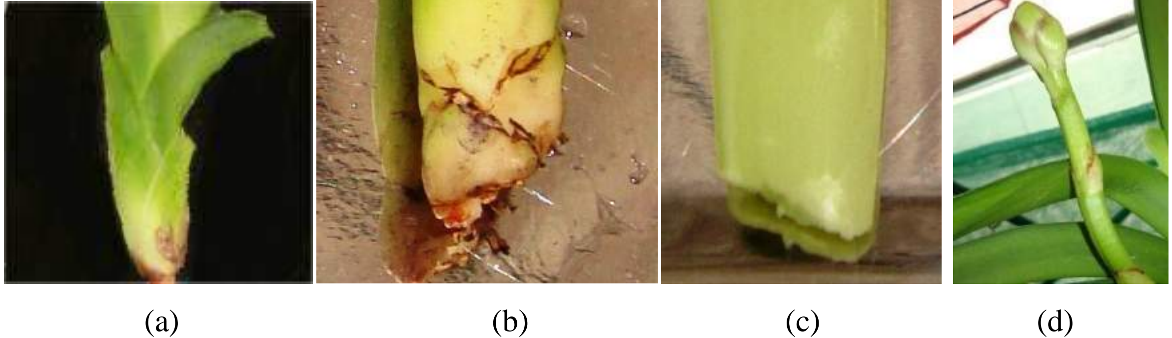
(vi) 20% (v/v) house hold bleach after adjusting the pH to 9 ± 0.02 with HCl

In all these experiments Tween - 20 (0.1%) was also added to the surface sterilant and shaken for 30 minutes in a rotary shaker. The cuttings were rinsed thrice with sterile water. Bracts were then carefully removed and a final surface sterilization with 5% commercial bleach for 10 minutes was given..After washing off the sterilant remains in four changes of sterile water, the nodal segments were subjected to drying for 5, 7, 9 and 10 min. in different experiments to study the effect of duration of drying under flame on the survival % of explants. Nodal segments which were not exposed to drying served as the control. Approximately 10 – 15 mm. of damaged tissue on both sides of the sterilized segments was cut off . The explants were then placed on 30ml. of solid medium in glass bottles with plastic cap with cut surface in contact with the medium.

In *Vanda* , the explants tested to establish *in vitro* culture were

1. Leaf sections (0.5cm.x1cm.) excised from well expanded leaves measuring 8-10 cm. 2 months after their emergence in the mother plants.

2. Shoot tip along with leaf base of both mature plants and keikis (0.9 -1.5 cm.) **(Fig.2a & b)**
3. Leaf base of both mature plants and keikis (2 -3 cm.) **(Fig.2c)**
4. Shoot tips of axenic plantlets (0.3-0.5 cm.)
5. Fresh root tips of mature field plants and axenic plantlets (0.2- 0.5cm.)
6. Young inflorescence stalk tip of 5cm. length before bud differentiation**(Fig.2d)**



(a) (b) (c) (d)

Fig. 3 (a) Vanda keiki used as explant source for shoot tips **(b)** Shoot tip with leaf base **(c)** Leaf base **(d)** Young inflorescence stalk tip of 5cm. length before bud differentiation

A comparison between the following two protocols was done for surface sterilization of *Vanda* explants.

Surface Sterilisation Method I in *Vanda*

Mother plants were sprayed with bavistin (0.1%) on the day prior to explant excision Expalnt source (keiki, leaf or inflorescence stalks with unopened budsk) were excised and cut to 2.5-3.5 cm, washed with tapwater and detergent, wiped with 95% alcohol, treated with bavistin (0.05%) for 3 min., took into LAF under blue flame, washed with sterile water thrice, treated with 20% commercial bleach + 1% Tween 20 with constant shaking for 30 min., washed with sterile water thrice, treated with 20% v/v hydrogen peroxide for 30 min., washed with sterile water thrice, leaves separated and cut ends trimmed to get 2– 3 cm. explants and inoculated in media.

Surface Sterilisation Method II in *Vanda* [Protocol standardized by Seeni and Latha (2000)]

Mother plants were sprayed with bavistin (0.1%) on the day prior to explant excision. Leaves, inflorescence stalks with unopened buds, top shoot cuttings with 4 to 5 leaves from mature plants and keikis (1-3 months old) were rinsed well with tap water and detergent. These were surface decontaminated by successive immersion in 1% (v/v) Tween-20 for 30 min., 70% ethanol (30 sec.) and 0.1% $HgCl_2$ solution (3 min.) and washed thrice with sterile distilled water.

3. Media and Culture Conditions

Both solid and liquid media with different nutrient composition and hormonal combinations were tested for explant response. pH of the media was adjusted to 5.5 and autoclaved at 121°C for 15 min. In all experiments, media with no hormones served as controls.

Experiments to standardize more cost effective protocols was done by substituting

i) Sucrose (Rs.650/kg.) by ordinary sugar (Rs.40/kg.) during media preparation.

ii) High grade agar (Rs.4050/kg.) by industrial agar (Rs.1100/kg.) for solidification of media.

iii) Branded sodium hypochlorite solution @ Rs.40/ l. by household commercial bleach @ Rs.15/ l. for explant sterilization.

All cultures were incubated at 25 ± 2°C with a 16 h. photoperiod of 3000 lux light intensity provided by cool-white fluorescent lights.

4. Optimisation of Protocol for Initiation Medium

Preliminary experiments for initiation of hybids were tried in different basal media,viz.,

1.Murashige and Skooge medium (MS)

2.Half-strength Murashige and Skooge medium (1/2 MS)

3.Vacin and Went medium (V&W)

4.Knudson C(modified) medium

5.Mitra medium (M) and

6.New Dogashima medium (NDM) (Tokuhara and Mii.,1993)

In *Phalaenopsis*, the different hormonal combinations tested for regeneration of shoot or plantlet from dormant bud of flower stalk were NAA (0,0.1,0.5 mgl^{-1}) and BAP (0,1, 2, 2.5, 3, 3.5, 4 mgl^{-1}) with peptone 2 gl^{-1}.

In *Vanda* hybrids the hormones tried for direct and indirect (via PLBs) shoot induction were NAA (0, 0.1, 0.2, 0.5, 1, 2 & 2.5mgl^{-1}) ,BAP (0, 0.5, 1, 2, 2.5, 3, 4.5, 5, 10, 20 mgl^{-1}) and TDZ (1,1.5 & 2mgl^{-1}) alone or in combinations along with or without additives like coconut water (10% ,15% & 20%) and peptone 2 gl^{-1} **(Table 1)**. Initiation was not obtained in liquid medium.

In *Phalaenopsis*, explants excised from the regenerated *in vitro* plantlets or axillary shoots induced in the initiation media viz., shoot tips, leaf bases and root tips were inoculated in ½ MS and NDM fortified with hormones NAA (0, 0.1, 0.2, 0.5, 1, 2 & 2.5mgl^{-1}) ,BAP (0, 0.5,

1, 2, 2.5, 3, 4.5, 5, 10, 20 mgl^{-1}) and TDZ (1,1.5 & 2mgl^{-1}) alone or in combinations along with or without additives like coconut water (10% ,15% & 20%) and peptone 2 gl^{-1} with the aim of producing direct or indirect (via PLBs) multiple shoot induction (**Table 1,2&3**).

Table 1. List of culture media tested for tissue culture of orchid cultivars used in the experiment

Hybrid	No. of expla-nts	Culture Media	Hormonal conc. tried (mgl^{-1})			Additives used	
			BA	**NAA**	**TDZ**	**Coconut water (%)**	**Peptone (%)**
			***PHALAENOPSIS* HYBRIDS**				
MO	16	MS,KC,VW	1-5	0.1-2.5	-	-	0.2
FGL	8	1/2MS,M,NDM	1-20	0.1-1	1-2	10-20	0.2
BJ	20	1/2MS,VW,M	1-4.5	0.1-1		10-20	0.2
JAV	92	KC,VW, 1/2MS,NDM	1-20	0.1-1	1-2	10-20	0.2
LP	18	1/2MS,NDM, M	1-4.5	0.1-1	-	-	0.2
LHD	35	1/2MS,NDM	1-20	0.1-1	-	-	0.2
ML	22	1/2MS,NDM	1-20	0.5-1	-	10-20	0.2
SS	12	1/2MS,NDM	2	0.5	-	-	0.2
TSZ	17	1/2MS,NDM	1-20	0.1-1	1-2	10-20	0.2
PY	13	1/2MS,NDM	1-20	0.5-1	-	10-20	0.2
SY	15	1/2MS,NDM	1-10	0.1-1	-	-	0.2
PR x BJ	23	NDM	1	0.1	-	-	0.2
CXM	7	1/2MS	1	0.1	-	-	0.2
SB	15	1/2MS	0.5-2	0.5	-	-	0.2
JS	23	1/2 MS,NDM	0.5-10	0.1-2	1-2	10-20	0.2
FC	11	1/2 MS,NDM	0.5-10	0.1-2	1-2	10-20	0.2

VANDA **HYBRIDS**							
DA	23	VW,KC,M, 1/2MS,NDM	1-20	0.1-1	-	10-20	0.2
KD	7	VW,KC,M, 1/2MS,MS	0.5-20	0.1-2	-	10-20	0.2
PD	6	M	1-20	0.1-1	1-2	10-20	0.2
RD	4	NDM,KC	1-2	0.1,0.2	1-2	10-20	0.2

*** *Phalaenopsis* hybrids**

MO – Mini Orange ; FGL-Fusheng's GladLip ; HBJ- Hsin Black Jak ; JAV- Joy Angel Voice ; LP – Leopard Prince ; LHD – Lainher Happy Dancer ; ML – Mona Lisa ; SS – Sunset; TSZ- Tying Shin Zebra ; PY – Phal Yellow ; SY – Sogo Yukidian ; SB – Shulong Butterfly; PR x BJ – Plum Rose x Black Jak ; CXM – Chian Xen Magpie; JS - Jinseng Sun; FC – Fuller's Cheese

*** *Vanda* hybrids**

DA – Dr.Anek ; KD – Kasem's Delight ; PD – Pat Delight ; RD – Robert's Delight

Table 2. Leaf base explant cultures tested in different initiation media

Hybrid	No.of leaf base cultures	S1	S1p	S1w1	S1w2	S1w3	S1pw1	S1pw2	S1pw3
JS	13	1	3	1	1	1	6	-	-
FGL	9	1	1	1	1	1	1	1	2
FC	18	4	2	3	3	2	4	-	-
TSZ	12	1	-	-	5	1	2	1	2
PY	28	7	5	2	5	2	4	2	1

S1 - ½ MS (0.1mgl^{-1} NAA ;20 mgl^{-1} BAP); CW-coconut water

w1 - 10% CW; w2 - 15% CW; w3 - 20% CW; p - 2gl^{-1} peptone

FGL-Fusheng's GladLip ; JS – Jinseng Sun ; ML – Mona Lisa ;

TSZ- Tying Shin Zebra ; FC – Fuller's Cheese

Table.3 Root tip explant cultures tested in different initiation media

Hybrid	**No.of root tip cultures**	**N1**	**N1p**	**N1w1**	**N1w2**	**N1w3**
JS	8	2	2	1	1	2
FGL	5	1	1	1	1	1
FC	13	3	2	2	1	5
TSZ	6	2	1	1	1	1
PY	19	4	4	5	1	5

N1- NDM (0.1mgl^{-1} NAA ; 1mgl^{-1} BAP;);

w1 - 10% CW; w2 - 15% CW; w3 - 20% CW; p - 2gl^{-1} peptone

FGL-Fusheng's GladLip ; LP – Leopard Prince ; ML – Mona Lisa ;

TSZ- Tying Shin Zebra ; PY – Phal. Yellow

4.1 Optimisation of Vertical (Longitudinal) Sectioning of Axenic Shoottip for PLB Induction in *Phalaenopsis*

Shoot tips used as explants for PLB induction were excised from the axenic shoots regenerated from the dormant buds of the flower stalk. These shoot tips (0.3-0.5cm.) were vertically cut using a sharp scalpel and inoculated in MS medium containing half- strength major elements (1/2 MS) fortified with varying concentrations of the hormone Thidiazuron/TDZ (1, 1.5 & 2 mgl^{-1}), 10% coconut water and 2% sucrose with the cut surface in contact with the medium inorder to study the effect of vertical cutting on PLB mediated and direct shootbuds induction percentage, earliness of PLB induction and number of PLBs produced. Intact shoot tips served as control in the experiment.

4.2 Optimisation of Age of axenic plantlets on PLB or Shoot bud Induction in *Phalaenopsis*

Since experiments to optimize micropropagation in *Phalaenopsis* via direct or indirect regeneration of axillary shoots from the above explants ,viz., in vitro regenerated axillary shoots after excising leaves, leaf bases and roottips did not give good results, we tried to induce multiple shooting via PLB induction by culturing shoot tips of axenic plantlets. Shoot

tips of size 0.3 – 0.5cm. were excised from two-month-old to eight-month-old *in vitro* regenerated plantlets to study the effect of age of explants source on PLB or shoot bud induction.

5. Optimisation of Protocol for Multiplication Medium

In both *Vanda* and *Phalaenopsis* hybrids, MS medium containing half- strength major elements (1/2 MS) and New Dogashima Medium(Tokuhara and Mii.,1993) were supplemented with various concentrations and combinations of NAA (0.1, 0.2, 0.5, 0.8, 1 & 2 mgl^{-1}), BAP (1,2, 2.5, 3, 3.5, 5, 8 & 10 mgl^{-1}) with and without 0.2% peptone, 1% and 2% sucrose and 10%, 15% and 20% coconut water to study effect in multiple shoot induction, PLB and shoot bud proliferation.

Two sets of experiments were done to optimise multiplication media

i) To standardise a multiplication medium for direct axillary shoot multiplication

In *Phalaenopsis,* experiments to effect direct axillary shoot multiplication were done in six-month-old to one-year-old *in vitro* regenerated plantlets or axillary shoots subcultured after excising leaves (**Table 4**).

ii) To standardise a multiplication medium for PLB proliferation

Two months after initiation of shoot tip cultures in both *Phalaenopsis* and *Vanda*, each PLB or shoot bud mass (0.5 cm. - 1cm.)was cut into smaller pieces (0.3-0.4cm.) and subcultured. Effect of all the treatments on formation of secondary PLBs, shoot buds, axillary shoots and shoot morphology was recorded. Subsequent subcultivation on the same and different media was done and data recorded. Due to shortage of planting material, only the most promising treatments identified from these experiments were used for replicated trials.

Table 4. *Phalaenopsis* axenic plantlets subcultured after excision of leaves in different multiplication media

Hybrid	No. of cultures	S	Sp	Sw1	Sw2	Sw3	Spw1	Spw2	Spw3
JS	4	-	-	1	-	1	1	1	-
FGL	5	3	-	1	1	1	1	-	-
FC	10	-	-	2	2	2	2	-	2
TSZ	8	-	1	3	2	1	-	-	1
PY	24	4	4	2	3	4	3	2	2

S - ½ MS (1mgl^{-1} NAA ;20 mgl^{-1} BAP); CW – coconut water
w1 - 10% CW; w2 - 15% CW; w3 - 20% CW; p - 2gl^{-1} peptone
FGL-Fusheng's GladLip ; JS – Jinseng Sun ; ML – Mona Lisa ;
TSZ- Tying Shin Zebra ; FC – Fuller's Cheese

6.Optimisation of Protocol for Rooting Medium

Half-strength Murashige and Skooge medium with different hormonal combinations which were identified as good for rooting in multiplication media itself were compared to standardize rooting medium in *Phalaenopsis and Vanda* . The different hormonal combinations tested were (NAA 0.5 mgl $^{-1}$ & BAP 2.5 mgl $^{-1}$), (NAA 0.5mgl $^{-1}$ & BAP 4.5mgl $^{-}$1), (NAA 1mgl $^{-1}$ & BAP 5mgl $^{-1}$) and (NAA 2mgl $^{-1}$ & BAP 5mgl $^{-1}$) with and without sugar (2% & 3%) and coconut water (10% & 20%) .

The inoculation density in rooting medium for *Vanda* was five plantlets or shoots per bottle. For *Phalaenopsis* it was three plantlets or shoots per bottle since it requires more space for adequate growth to robust plantlets compared to *Vanda*.

7.Optimisation of Protocol for Hardening Medium

The regenerated plantlets were hardened *in-vitro* on a PGR(plant growth regulator) free medium with and without sugar (2% & 3%) for 1 month before transplanting under field conditions. The regenerated rooted plantlets of *Phalaenopsis* and *Vanda* hybrids were further subjected to *ex-vitro* hardening. The plantlets were taken out of the culture bottles and washed thoroughly under running tap water to remove traces of adhering agar. These were then taken to the green house, treated with 0.1% bavistin solution for 5 min.and again washed with water.

In *Phalaenopsis*, three different primary hardening methods were tried to study the effect of hardening medium on survival percentage of the plants. The rooted plantlets were transferred to

1. Plastic pots 8cm. diameter, containing small brick pieces, coir and charcoal in the ratio 1:1:1 as single plantlets per pot.
2. Clay pots 20cm. diameter, containing small brick pieces, coir and charcoal in the ratio 1:1:1 as 4 plantlets per pot.
3. Coconut husk 15-18cm. long, soaked in water containing 0.1% bavistin for 24 hours on the day prior to planting as 2 plantlets at either ends of the husk.

After 4 months plants were transferred individually to clay pots for secondary hardening. Husks were cut in the middle and each plantlet with a piece of the husk was placed in the center of claypots filled with brick pieces, coir and charcoal in the ratio 1:1:1.

In *Vanda*, the plantlets were planted in plastic pots (4cm diameter) filled with brick pieces, coir and charcoal in the ratio 1:1:1. The pots were hung from frames inside the green house. Inorder to maintain humidity, water filled trays were kept under the hanging pots. After 6 months were transferred to plastic pots of diameter 8cm. The primary hardened plants were transferred individually to claypots for secondary hardening after 4 months.

Plantlets were covered with plastic bags initially to maintain humidity. After one week, the plastic cover was removed, *Vanda* plantlets were watered twice daily and *Phalaenopsis* plantlets were watered twice daily. The plantlets were sprayed with 0.1% fertilizer NPK 30:10:10 at weekly intervals and bavistin 0.1% at monthly intervals. Plants became acclimated to a reduced relative humidity and finally hardened to green house conditions.

RESULTS AND DISCUSSION

1. Isolation of explant and surface sterilisation

Based upon observations on floral attributes, adaptability to climatic conditions and response of explants to *in vitro culture* in preliminary experiments, 8 hybrids were selected to be carried forward for explant isolation and further mericloning experiments **(Table 5).**

Table 5. Observation on Floral Attributes (Mean of observations from 5 plants of each hybrid)

Hybrid	Mean no. of inflores-cence per plant	Mean no.of flow-ers	Flower colour	Lip colour	*Flower size	Flower longe-vity	Spike length	Spike colour
***PHALAENOPSIS* HYBRIDS**								
Tying Shin Zebra	3.33	17.33	Pinkish offwhite with purple striations	Purple	Medium	30-45	Long 46.6	Dark green
Hsin Black Jack	1	5.33	White with pink dots	Pinkish yellow with pink dots	Medium	15-30	Short 16.6	Light green
Joy Angel Voice	1.67	10.67	White with purple dots clustered towards the centre	Dark purple with yellow along periphery	Medium	30-45	Medium 28.2	Green

Fuller's Cheese	1	6	Light violet with striations	Violet with striations	Small	15-25	Short 16.4	Green
Jinseng Sun	1.4	7	White with pink streaks towards centre of petals	Dark pink	Medium	20-30	Medium 27.6	Green
***VANDA* HYBRIDS**								
Pat Delight	1	9	Pink with dots	Dark pink	Large	15-20	Mediu m 27.8	Dark green
Robert's Delight	1	10	Bluish violet	Dark purple	Large	15-18	Mediu m 26.4	Dark green
Dr.Anek	1	8	Dark maroonish pink with dark pink dots	Dark pink	Large	15-20	Mediu m 27	Dark green

*Flower size – Large (Petal – 4.5 to 5.2cm.); Medium - (Petal – 3.5 to 4.2cm.); Small - (Petal – 2.8 to 3.2cm.)

In *Phalaenopsis* hybrids, explants that responded to *in vitro culture* were

1. Nodal segments (0.5-1cm.) bearing vegetative buds excised from both tender flower. stalks (only 2-3 opened flowers) and fully flowered flower stalks
2. Shoot tips of flower stalk buds, 0.1-0.3cm wide and 0.5mm high.
3. Shoot tips (0.3-0.5cm.) from axenic plantlets or axillary shoots.
4. Fresh root tips (0.8-1cm.) excised from axenic plantlets or shoots

5. Leaf bases (0.8-1.5cm.) excised from axenic plantlets or shoots

In *Vanda* , the explants that responded to *in vitro* culture were

1. Shoot tip along with leaf base of both mature plants and keikis (0.9 -1.5 cm.)
2. Shoot tips of axenic plantlets (0.3-0.5 cm.)

Bacterial contamination was found to be the major problem during initiation in *Phalaenopsis*. It was found that explants from tender flower stalks (only 2-3 opened flowers) showed lesser contamination and better initiation percentage. Contamination rate was high when fully flowered inflorescence stalks were used as explant source **(Table 6).** A cost effective protocol was standardized by substituting branded sodium hypochlorite solution (Sigma) (4% a.i.) @ Rs.40/l. by local commercial bleach (5 - 6 % a.i.) @ Rs.12/l. There was no significant difference in explant survival percentage when bleach was used as surface sterilant **(Table.7)**. Good quality commercial bleach (20% v/v) was found equally as effective as sodium hypochlorite (3%) for explant sterilisation . Sodium hypochlorite and Calcium hypochlorite are the commonly used surface sterilants (Tokuhara and Mii, 1993).

An alternate explant sterilization method by treating with sodium dichloro isocyanate (16.6g/l) was tried for nodal segments taken from the fully flowered inflorescences. But there was no significant reduction in contamination. In the case of younger inflorescence stalks (1-3 flowered stage), when the pH of bleach (20%) was adjusted to 7± 0.02 and 9 ± 0.02 contamination decreased by 4% and 6% respectively , but most of the explants did not survive due to loss of chlorophyll and whitening of buds **(Table 7)**. Hence household bleach 20% (v/v) was standardised as the optimum dose for the surface sterilant. The optimum time to keep the explants to dry under blue flame inside laminar hood was found to be 9 minutes as contamination percentage of explants from tender flower stalks was then reduced to 32% **(Table.8)**.

Contrary to the present study, George (1993) suggested that for the most effective disinfection of plant material, hypochlorite solutions should be used at pH 6-7 . He explained that the bactericidal action of hypochlorite solutions (bleach) is due to both hypochlorous acid (HOCl) and the OCl^- ion with the former being more active so that the disinfecting efficiency of chlorine is best in slightly acid hypochlorite solutions. It has been reported earlier that acidified bleach (pH 7) reduced both fungal and bacterial contamination in field grown medicinal explants (Webster *et al.*,2003)

Since older inflorescence stalk sections and leaf sections produced phenolics, charcoal @ 1g/l was added to the medium. Optimal results for shoot initiation was got by culturing nodal explants.

In *Vanda* hybrids, both bacterial and fungal contamination were found to be major problems during initiation. Although many methods for surface sterilization were tried, the following protocol standardized by Seeni and Latha (2000) gave the best results. Mother plants were sprayed with bavistin (0.1%) on the day prior to explant excision. Leaves, inflorescence stalks with unopened buds, top shoot cuttings with 4 to 5 leaves from mature plants and keikis (1-3 months old) were rinsed well with tap water and detergent. These were surface decontaminated by successive immersion in 1% (v/v) Tween-20 for 30 min., 70% ethanol (30 sec.) and 0.1% $HgCl_2$ solution (3 min.) and washed thrice with sterile distilled water.

Table 6. Survival percentage and response of dormant buds from old inflorescence of different varieties after 1 month

Cultivar	**Infl. stage**	**No. of of explants**	**No. of of explants contaminated**	**Survival %**	**Nodes remaining green**	**Nodes showing shoot initiation**	**Nodes turned yellow or or albino**
GB	**FF**	19	11	42.1	6		2
HBJ	**5F**	25	10	60	6	3	6
PRxBJ	**5F**	20	6	70	11	2	1
ML(v)	**FF**	18	7	61.1	-	10	1
ML	**FF**	31	19	38.7	4	3	5
TSZ	**FF**	15	6	60	6	-	3
SY	**FF**	14	6	57.1	7	-	1
FC	**FF**	15	9	40	3	-	3
GB	**FF**	13	8	38.5	2	-	3
FGL	**FF**	25	9	64	4	-	12

GB - Golden Beauty ; HBJ - Hsin Black Jack ; PR x BJ - Plum Rose x Black Jack
ML(V)- Mona Lisa(variant); ML – Mona Lisa; FGL – Fuscheng's Glad Lip;
MLxCA – Mount Lip x Ching Ann; TSZ – Tying Shin Zebra; SY- Soga Yukiden;
FC – Fuller's Cheese. FF – Fully flowered ; 5 F- 5 flowers opened stage

Table 7. Comparison between sodium hypochlorite and commercial bleach as surface sterilants on survival % of *Phalaenopsis* explants after 2 months of culture

Surface sterilant	**No. of explants**	**Explants from tender flower stalks (1- 3 flowered stage)**		**Explants from old flower stalks (flowers wilted)**	
		Contamination(%)	**Necrosis (%)**	**Contamination(%)**	**Necrosis (%)**
Sodium hypochlorite solution (branded) (20% v/v)	50	40	6	64	8
Household bleach (20% v/v)	50	32	4	62	6
Household bleach (15% v/v) (pH –7 ± 0.02)	50	38	46	54	46
Household bleach (20% v/v) (pH –7 ± 0.02)	50	28	46	36	48
Household bleach (15% v/v) (pH –9 ± 0.02)	50	46	4	60	46
Household bleach (20% v/v) (pH –9 ± 0.02)	50	26	2	38	44

Table 8. Effect of drying time under flame on survival% of *Phalaenopsis* explants (from tender flower stalks) after 1 month of culture

Drying time	**0 min.**	**5 min.**	**7 min.**	**9min.**	**10min.**
No. of explants	102	15	18	50	15
Contamination %	90.2	46.7	33.4	**32**	33
Necrosis%	-	6.7	11.1	8	27

2. EFFECT OF BASAL MEDIUM

2.1 Effect of Media Composition on Explant Response

In the preliminary experiments, different media like MS, ½ Murashige &Skooge, Vacin & Went, Knudson's C, Mitra, New Dogashima Medium were tested both for direct shoot regeneration and PLB induction. Based on these experiments, two initiation media were optimized, viz.,

i) Half-strength Murashige and Skooge medium (1/2 MS) **(Table 9)**

ii) New Dogashima Medium (NDM) **(Table 10)**

iii) Modified Half-strength Murashige and Skooge medium (Modified ½ MS) – Vitamins of ½ MS medium was replaced with vitamins of NDM **(Table 11)**

For the same hormonal combinations, early shoot regeneration with two to three leaves was obtained from flower stalk nodal segments cultured in ½ MS medium in *Phalaenopsis* and shoot bud induction was obtained from shoot tip explants of both keikis and mature plants in *Vanda* cultured in ½ MS medium . This might be due to the optimum dose of nitrogen provided by the major nutrients in the medium. A combination of $0.95gl^{-1}$ potassium nitrate and $0.825gl^{-1}$ ammonium nitrate was found to be optimum for shoot regeneration and plant let differentiation **(Table 12).** Hence MS medium (Murashige & Skoog,1962) containing half-strength major elements(1/2 MS) was standardized as the basal medium for establishment of *in vitro* culture via shoot regeneration in *Phalaenopsis* and direct multiple shoot induction in *Vanda* hybrids.

Optimal results for initiation of PLB (protocorm-like-bodies) from shoot tips of axenic plantlets in *Phalaenopsis* and *Vanda* was observed in NDM (Table) and satisfactory shoot elongation was observed in Modified ½ MS medium.

Table 9. Components of ½ MS medium used for orchid tissue culture

Macroelements	**Concentration (mgl^{-1})**
Ammonium nitrate NH_4NO_3	825
Potassium nitrate KNO_3	950
Calcium chloride $CaCl_2.2H_2O$	220

Magnesium sulphate $MgSO_4.7H_2O$	185
Potassium dihydrogen phosphate KH_2PO_4	85
Microelements	
Manganese sulphate $MnSO_4.4H_2O$	22.3
Zinc sulphate $ZnSO_4.7H_2O$	0.5
Boric acid H_3BO_3	6.2
Potassium iodide KI	8.3
Copper sulphate $CuSO_4.5H_2O$	0.025
Sodium molybdate $Na_2MoO_4.2H_2O$	0.025
Cobalt chloride $CoCl_2.6H_2O$	0.25
Iron sulphate $FeSO_4.7H_2O$	27.8
Sodium EDTA Na_2EDTA	37.3
Organic compounds	
Myoinositol	100
Niacin	0.5
Pyridoxine hydrochloride	0.5
Thiamine hydrochloride	0.1
Glycine	2.0

Table 10. Components of NDM used for orchid tissue culture

Macroelements	**Concentration (mgl^{-1})**
Ammonium nitrate NH_4NO_3	480
Potassium nitrate KNO_3	200
Calcium nitrate $Ca(NO3)_2.4H_2O$	470
Potassium chloride KCL	150
Magnesium sulphate $MgSO_4.7H_2O$	250
Potassium dihydrogen phosphate KH_2PO_4	550
Microelements	
Manganese sulphate $MnSO_4.4H_2O$	3
Zinc sulphate $ZnSO_4.7H_2O$	0.5
Boric acid H_3BO_3	0.5
Copper sulphate $CuSO_4.5H_2O$	0.025
Sodium molybdate $Na_2MoO_4.2H_2O$	0.025
Cobalt chloride $CoCl_2.6H_2O$	0.025
Iron - EDTA	21
Organic compounds	
Myoinositol	100

Niacin	1.0
Pyridoxine hydrochloride	1.0
Thiamine hydrochloride	1.0
Calcium pantothenate	1.0
Adenine	1.0
l-Cysteine	1.0
d-Biotin(crystalline)	0.1

Table 11. Components of Modified ½ MS medium used for orchid tissue culture

Macroelements	**Concentration (mgl^{-1})**
Ammonium nitrate NH_4NO_3	825
Potassium nitrate KNO_3	950
Calcium chloride $CaCl_2.2H_2O$	220
Magnesium sulphate $MgSO_4.7H_2O$	185
Potassium dihydrogen phosphate KH_2PO_4	85
Microelements	
Manganese sulphate $MnSO_4.4H_2O$	22.3
Zinc sulphate $ZnSO_4.7H_2O$	0.5
Boric acid H_3BO_3	6.2
Potassium iodide KI	8.3
Copper sulphate $CuSO_4.5H_2O$	0.025
Sodium molybdate	0.025

$Na_2MoO_4.2H_2O$	
Cobalt chloride $CoCl_2.6H_2O$	0.25
Iron sulphate $FeSO_4.7H_2O$	27.8
Sodium EDTA Na_2EDTA	37.3
Organic compounds	
Myoinositol	100
Niacin	1.0
Pyridoxine hydrochloride	1.0
Thiamine hydrochloride	1.0
Calcium pantothenate	1.0
Adenine	1.0
l-Cysteine	1.0
d-Biotin(crystalline)	0.1

Table 12. Comparison of composition of major nutrients of culture media tested for orchid hybrids

Sl.No.	**Major Nutrient (in g)**	**MS**	**VW**	**KC**	**M**	**1/2MS**	**NDM**
1	Potassium nitrate	1.9	0.53	0.18	0.18	**0.95**	0.2
2	Ammonium nitrate	1.65	-	-	-	**0.825**	0.48
3	Calcium nitrate	-	-	0.2	0.2	-	0.47
4	Calcium chloride	0.44	-	-	-	0.17	-
5	Potassium chloride	-	-	-	-	-	0.15
6	Magnesium sulphate	0.37	0.25	0.15	0.25	0.19	0.25
7	Potassium dihydrogen phosphate	0.17	0.25	0.25	0.15	0.085	0.55
8	Ammonium sulphate	-	0.5	0.1	0.1	-	-
9	Tricalcium phosphate	-	0.2	-	-	-	-

2.2 Effect of PGR on shoot regeneration in *Phalaenopsis*

Nodal segments (0.5-1cm.) bearing vegetative buds excised from both tender flower. stalks (only 2-3 opened flowers) and fully flowered flower stalks regenerated to shoots or plantlets. Shoot initiation was very slow (1-2 months) when BAP concentration was below 2mgl^{-1}. At BAP concentrations below 2mgl^{-1} induction of PLBs (protocorm like bodies) was observed. ½ MS medium with hormonal combinations BAP 3.5 mgl $^{-1}$, (NAA 0.1mgl $^{-1}$ & BAP 3.5mgl $^{-1}$) and (NAA 0.5mgl $^{-1}$ & BAP 2mgl $^{-1}$) showed the fastest plantlet regeneration in 2 months. ½ MS medium with BAP 3.5 mgl $^{-1}$, 0.2% peptone and 3% sucrose was standardized as the optimum medium for plantlet regeneration as it produced robust shoots without roots and 0% necrosis after shoot induction **(Table 13).**

NAA and BAP stimulated shoot induction in 10-30 days which approximately corresponds with data in literature (Tisserat and Jones,1999). Some authors have reported that an appropriate combination of NAA and BAP stimulated shoot formation (Tokuhara and Mii,1993; Tisserat and Jones, 1999 ; Roy and Banerjee, 2003). Mondal *et al*. (2013) reported that increased levels of BAP exhibited enhancement of shoot elongation in *D.pulcherrima*.

Table 13. Effect of NAA and BAP on shoot initiation and plantlet regeneration of *Phalaenopsis* cultures

<table>
<tr><th>NAA (mgl^{-1})</th><th>BAP (mgl^{-1})</th><th>Shoot Initiation (%)</th><th>Days to shoot induction</th><th>Days to plantlet regeneration</th></tr>
<tr><td>0</td><td>0</td><td>67</td><td>25</td><td>90</td></tr>
<tr><td rowspan="6">0</td><td>1</td><td>60</td><td>45</td><td>90</td></tr>
<tr><td>2</td><td>50</td><td>30</td><td>90</td></tr>
<tr><td>2.5</td><td>71</td><td>25</td><td>75</td></tr>
<tr><td>3</td><td>66</td><td>25</td><td>75</td></tr>
<tr><td>3.5</td><td>100</td><td>26</td><td>60</td></tr>
<tr><td>4</td><td>90</td><td>25</td><td>70</td></tr>
<tr><td rowspan="4">0.1</td><td>1</td><td>50</td><td>30</td><td>90</td></tr>
<tr><td>2</td><td>60</td><td>25</td><td>90</td></tr>
<tr><td>2.5</td><td>86</td><td>20</td><td>60</td></tr>
<tr><td>3</td><td>67</td><td>19</td><td>75</td></tr>
</table>

	3.5	67	15	**60**
	4	65	20	65
0.5	1	100	50	90
	2	86	10	**60**
	2.5	78	15	75
	3	77	16	75
	3.5	50	18	75
	4	54	20	70

NAA – Naphthalene Acetic Acid; BAP - 6-Benzyl Aminopurine

2.3 Effect of PGR (NAA:BAP hormonal combination) on direct or indirect (via PLB) multiple shoot induction in *Phalaenopsis*

Nodal segments (0.5-1cm.) bearing vegetative buds excised from both tender flower. stalks (only 2-3 opened flowers) and fully flowered flower stalks regenerated to single plantlets without multiplication.

Leafbases (85.7%) of *in vitro* plantlets when inoculated in half strength MS medium with hormonal combination (0.1mgl^{-1} NAA & 20mgl^{-1} BAP) supplemented with 20% coconut water also regenerated to single plantlets without multiplication **(Table 14).**

Although PLBs had initiated from 25% of roottips of *in vitro* plantlets 4 months after inoculation in New Dogashima Medium with hormonal combination (0.1mgl^{-1} NAA & 1mgl^{-1} BAP) , the time taken to induction was long **(Table 15).**

The above explants gave unsatisfactory results on initiation medium with respect to parameters such as earliness to induction of multiple shoots or PLBs, rate of induction and number of multiple shoots or PLBs induced. Multiplication was also poor during subsequent subculturing. Hence axenic shoottips were tested as explants.

Since experiments to optimize micropropagation in *Phalaenopsis* and *Vanda* via direct or indirect regeneration of axillary shoots using hormonal combinations of NAA and BAP did not give good results on a commercial scale, we tried to induce multiple shooting via PLB induction in ½ MS and NDM media fortified with different doses of plant growth regulator Thidiazuron.

It was observed that the above hormonal combinations with NAA and BAP was not suitable for early direct regeneration of multiple shoots on a commercial scale. This is contrary to the findings by Kosir *et al*., 2004. in which they reported that ½ MS medium

fortified with hormonal combination of 0.5mgl^{-1} NAA & 2mgl^{-1} BAP was very effective in rapid propagation of *Phalaenopsis* via direct shoot regeneration from flower stalk nodes.

Table 14. Response of leaf base explants in *Phalaenopsis* after 3 months of culture period

Hormonal composition of ½ MS media	S1	S1p	S1w1	S1w2	S1w3	S1pw1	S1pw2	S1pw3
No. of leaf base explants	14	11	7	15	7	17	4	5
Direct multiple shoot formation(%)	-	-	-	6.67	14.2	-	-	-
No. of multiple shoots (2 or > 2)	-	-	-	2	2	-	-	-
Regeneration to single plantlet(%)	-	5	71.4	53.3	85.7	47.1	25	20
PLB formation(%)	-	-	-	-	-	-	-	-
Necrosis(%)	71.4	63.6	-	20	-	29.4	50	20

S1 - ½ MS (0.1mgl^{-1} NAA ;20 mgl^{-1} BAP)

w1 - 10% CW; w2 - 15% CW; w3 - 20% CW; p - 2gl^{-1} peptone

Table 15. Response of root tip explants in *Phalaenopsis* after 4 months of culture period

Hormonal composition of NDM	N1	N1p	N1w1	N1w2	N1w3
No. of roottip explants	12	10	10	5	14
Direct multiple shoot formation (%)	-	-	-	-	-
No. of multiple shoots (2 or > 2)	-	-	-	-	-

Regeneration to single shoot or plantlet (%)	-	-	-	-	-
PLB formation(%)	25	-	-	-	-
Size of PLB mass(cm.)	0.1-0.3				
Necrosis(%)	-	33.3	20	25	22.2

N1- NDM (0.1mgl^{-1} NAA ; 1mgl^{-1} BAP;)

w1 - 10% CW; w2 - 15% CW; w3 - 20% CW; p - 2gl^{-1} peptone

2.4 Effect of PGR (TDZ) on Direct Shoot bud Induction in *Vanda*

In *Vanda*, half strength MS medium supplemented with the hormones NAA (0, 0.1, 0.2, 0.5, 1, 2 & 2.5mgl^{-1}) and BAP (0, 0.5, 1, 2, 2.5, 3, 4.5, 5, 10, 20 mgl^{-1}) alone and in combination did not induce shoot buds. Hence NAA and BAP were replaced with the PGR Thidiazuron (TDZ). Direct shoot bud initiation (12- 18) was obtained in 25 to 30 days in *Vanda* hybrid Pat Delight, from shoot tip along with leaf base segments (0.9 – 1.5 cm.) implanted vertically, 2-3mm. deep in ½ MS medium fortified with TDZ 1.5 mgl^{-1}, coconut water 20% and adenine sulphate 10mgl^{-1} (**Table 16 & Fig.9**).

Gantait and Sinniah (2012) reported that among cytokinins, development of PLBs was most efficient in the presensce of TDZ from leaves of *Vanda* hybrids. Thidiazuron (TDZ: N-phenyl-N'-1,2,3-thidiazol-5-ylurea), a phenylurea derivative and a non-purine cytokinin compound, is not catabolized via cytokinin oxidase (Hare and Van, 1994; Kishor and Devi, 2009). Previously, TDZ was reported to be effective in the regeneration of a number of orchid species such as *Doritaenopsis* (Ernst, 1994), *Phalaenopsis* (Chen and Piluek, 1995), *Cymbidium* (Chang and Chang, 1998; Nayak *et al.*, 1997), *Oncidium* (Chen *et al.*, 1999; Chen and Chang, 2001), and *Dendrobium* (Roy *et al.*, 2007).

It was also observed that the addition of coconut water to culture medium promoted proliferation of shoots . Previous studies have proven that the supplementation of coconut water enhanced shoot bud initiation in '*Blue Vanda*' (Seeni & Latha,2000). This could be attributed to the notable biochemical compounds of the tender coconut milk such as potassium, sodium, calcium, phosphorous, iron, copper, sulphur, magnesium, ascorbic acid and the B group vitamins. In addition, 70% of the free amino acids of coconut milk are made

of glutamine, arginine, asparagines, alanine and aspartic acid. Coconut liquid endosperm contains a large spectrum of biochemicals that can act as growth factors individually or synergistically such as 1,3-diphenyl urea which shows cytokinin- like activity,zeatin riboside and zeatin.

Table 16 . Effect of different hormonal combinations on multiple shoot bud induction from shoot tip explants of keikis and mature plants in *Vanda* hybrids

Hormonal composition (mgl^{-1})		No.of explants	Survival (%)	Shootbud formation (%)	No. of shoots per per leafbase	Time to induction (days)
TZ	CW					
TZ 0 Control	10%	7	42.8	0	0	-
	20%	9	44.4	0	0	-
TZ 1	10%	10	50	0	0	-
	20%	8	37.5	0	0	-
TZ 1.5	10%	6	33.3	16.7	8	60
	20%	**5**	**60**	**40**	**18**	**45**
TZ 2	10%	12	58.3	16.7	12	60
	20%	9	55.5	20	15	60

TZ – Thidiazuron ; CW – Coconut water

2.5 Effect of PGR (TDZ) on PLB Induction

Since experiments to optimize micropropagation in *Phalaenopsis* and *Vanda* via direct regeneration of axillary shoots on a commercial scale did not give good results, we tested ½ MS and NDM media fortified with plant growth regulators NAA and BAP in various combinations , with and without the additives coconut water (10% & 20%) and peptone 0.2% for PLB induction. In *Phalaenopsis*, shoot tips 1-3mm. wide and 0.5 -1mm. high of flower

stalk buds from green house grown plants exhibited PLB induction in NDM supplemented with 0.1mgl^{-1} NAA & 1mgl^{-1} BAP, 0.2mgl^{-1} NAA & 2mgl^{-1} BAP and in ½ MS enriched with 1mgl^{-1} BAP after 2 months of culture. But PLB proliferation was very slow, took 5 – 6 months in the same medium.

Experiments to induce PLB was also done by culturing small axenic shoot tips and leaf bases of 0.3-0.5cm. length from two-month-old to eight-month-old *in vitro* plantlets of *Phalaenopsis* and *Vanda* respectively**.** The results showed that in *Phalaenopsis* PLBs were induced from shoot tips derived from *in vitro* plantlets above 6 months of growth in media fortified with coconut water .

Mondal *et al* (2013) has reported PLB formation from shoot tip explants derived from six month old axenic seedlings in *Doritis pulcherrima* when cultured on Knudson's C medium containing 2mgl^{-1} NAA. They were able to induce an average 7.33 plbs per explant.

It was found that when NAA and BAP were replaced with the plant growth regulator Thidiazuron (TDZ), it reduced the time span to PLB induction and increased PLB proliferation. TDZ in varying concentrations along with 10% coconut water was used to fortify ½ MS basal medium for culturing shoot tips from 2 - 8 month-old *in vitro* plantlets of *Phalaenopsis* and *Vanda* hybrids. In the present study we have optimized the growth regulator concentration and obtained the highest number of PLBs (18 per explant in *Phalaenopsis*) and (31.5 per explant in *Vanda*) on ½ MS medium with 1mgl^{-1} TDZ and 10% coconut water**. (Table 17 & 18)**

Tokuhara and Mii(1993) reported induction of PLBs in 5 months from shoot tips of flower stalk buds of *Phalaenopsis* and *Doritaenopsis* using NDM containing NAA 0.1mgl $^{-1}$ & BAP 1mgl $^{-1}$. Substituting NAA and BAP with TDZ in the present study reduced the time to PLB induction from explants by 3-3.5 months and the use of axenic shoots instead of shoot tips from flower stalk buds eliminated the major problem of microbial contamination resulting in 100% survival of explants. More than 50% increase in PLB and shoot bud induction was obtained through culturing vertically cut explants.

2.6 Effect of PGR on *in vitro* flowering in *Phalaenopsis*

` Direct regeneration of dormant buds to multiple flower stalks (2-3) was induced in a few random cultures of *Phalaenopsis* in NDM and 1/2 MS medium fortified with BAP 5mg^{-1} **(Fig.4)**. Early flowering is of major commercial interest, since at least three years are required from sowing seed to flower development under greenhouse conditions. The

combination and concentration of plant hormones and the nitrogen and phosphorus content have a major impact on early flowering induction under *in vitro* conditions. A combined treatment of BAP, restricted nitrogen supply with phosphorus enrichment and root excision, induced a transition of *Cymbidium* shoots from vegetative to reproductive stages (Kostenyuk et al., 1999). The formation of floral buds and the development of shoots need different levels of nitrogen and BAP. A higher BAP concentration (5 mg/l) and lower nitrogen content (4.5 mM) induced more flower buds in *Phalaenopsis* (Duan and Yazawa, 1995). In the present study, ½ MS medium was supplemented with approximately 6.5× higher nitrogen and NDM with approximately 4.5× higher nitrogen . So these media with slight changes in the media composition (lower nitrogen content) could serve for earlier flowering induction in tissue culture and later *in vivo*.

Fig.4 *In vitro* flowering induction in *Phalaenopsis*

Table 17 . Effect of TDZ on PLB induction in axenic shoot tip explants of *Vanda* after 3 months of culture

TDZ	**No.of explants**	**PLB formation (%)**	**Size of PLB mass (cm.)**	**Approx. no. of PLBs per shoot tip**	**Colour of PLBs**
TZ 0 (Control)	3	0	-	-	-
TZ 1	5	80	**2- 2.5**	**28-35**	green
TZ 1.5	3	50	0.6 – 0.8	12 - 15	green
TZ 2	4	42.8	0.6 – 1	12 - 18	green

2.7 Effect of Vertical Excision on PLB Induction in *Phalaenopsis*

It was observed that when vertical excision was not given, PLBs were induced only in shoot tips derived from older axenic plantlets above 5 months old. Intact shoot tips took a longer time for shoot induction (1.5 – 2months) and another 2 – 2.5 months to proliferate to about 5-8 shoot buds per shoot tip. Rate and earliness of induction and proliferation was very less compared to vertically cut shoot tips (12-18 shoot buds/shoot tip). The values are given in a range because PLBs and shoot buds appeared as a mass of structures making it impossible to count **(Table 19)**.

It was observed that 0.3-0.5cm. long vertically cut shoot tips excised from 3-3.5 month-old axenic plantlets showed early induction of PLBs and shoot buds in all treatments with TDZ (1, 1.5 & 2 mgl^{-1}), 10% coconut water and 1% sugar. Induction initiated after 20 – 25 days which proliferated further for another 1 – 1.5 months. Approximately 0.7 -1.2 cm. shoot bud masses were produced without callusing in 2 months after which symptoms of regeneration of small shoots was observed **(Table 18)**.

Earliness and more number of PLBs may be due to the direct contact of the inner cells of the shoot tip with the hormone fortified medium upon vertical excision of the shoot tip from top to bottom. Histological observations on thin flower stalk intermodal sections (1-1.5mm.) of *Phalaenopsis* cultured *in vitro* by Lin (1987) **revealed that a number of initial cells with large nuclei and dense protoplasm exist in the epidermis, cortex and immature vascular**

bundles and that these divide or are capable of dividing in the early stage of the internodes. These cells 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.

Plant regeneration may occur via direct shoot bud formation or PLB mediation.The proximal part of the leaf in orchids is meristamatic and on excision and culturing differentiates into plantlets (Zimmer and Pieper,1975). In the present study, the shoot tips were excised from the axenic plantlets in such a way that the proximal end of the leaf base is retained on the shoot tip. According to Seeni and Latha (2000), **the resident axillary meristems of the condensed nodes in the basal part of the shoot tips, relieved of the apical meristem, proliferates to produce callus-free shoot buds. On the other hand, the adventive meristematic cells spread all over the surface of the leaf base responds to favourable culture conditions initially by random mitotic divisions and then organizes into PLBs before the emergence of shoot initials.**

Mondal *et al* in 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) on vertically cut shoot tips cultured in all the three TDZ fortified media in the present study. Udomdee *et al*. in 2012 also observed enhanced multiple shooting from vertically cut axenic shoot tips compared to intact shoot tips in *Paphiopedilum*, a terrestrial orchid. Subsequently, young shoots emerged efficiently by this cutting method to give higher multiplication rate than others.

In the present study we have optimized the plant growth regulator concentration and obtained a maximum of 18 shoot buds per explant on ½ MS medium with $1mgl^{-1}$ TDZ and 10% coconut water. However, 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 $1.5mgl^{-1}$. 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 .

In the present study we have optimized the growth regulator concentration and obtained the highest number of PLBs (18 per explant in *Phalaenopsis*) and (31.5 per explant in *Vanda*) on ½ MS medium with 1mgl^{-1} TDZ and 10% coconut water **.** This study is in agreement with the work of Sujjaritthurakarn and Kanchanpoom (2011) in which TDZ gave a superior response to BA for inducing PLBs in dwarf *Dendrobium*. 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 cytokinin TDZ (thidiazuron). He also reported that that shoot and root development were reduced with increasing concentrations of TDZ .In the present study also, shoot and root development was not observed in any of the treatments with TDZ.

It was also observed that the addition of coconut water to culture medium promoted proliferation of PLBs and axillary shoots from shoot tips. Previous studies have proven that supplementation of coconut water in both liquid and solid medium enhanced the survival of PLBs of Cattleytonia (Uesato and Sagawa,1986). Very recently, Ichihashi (1992) also reported the importance of coconut water for inducing PLB formation from shoot tip explants of *Phalaenopsis* without using PGR. In the present study, PLBs were not induced with coconut water alone . However, the rate and earliness of PLB formation he obtained was not as high as we achieved in the present study. Therefore the use of PGR might be indispensable for optimum induction of PLB from shoot tip explants.

Tissue blackening and necrosis of the explants have been frequently encountered in this study; necrosis might have been caused by the oxidation of phenolic compounds. Similar findings have been reported by Mondal *et al*. in 2013. It has been reported earlier that the addition of organic acids and frequent subculturing of the propagules exhibited satisfactory results (Morel,1974, Ernst,1994). Specifically in orchids, oxidation of phenolics was reported to account for browning of explants followed by necrosis (Morel 1974, Vendrame and Maguire 2007).

Addition of activated charcoal was avoided in the initiation medium for PLB induction since it was observed that PLB induction was affected. This must be because charcoal also absorbs plant growth regulators and other compounds such as vitamins in the medium.

Table 18. Effect of TDZ on PLB and shoot bud induction in intact and vertically cut axenic shoots.

Culture medium (mgl^{-1})	Nature of shoot tip	No. of explants	PLB & shoot bud forming explants (%)	Mean diameter of PLB/shoot bud mass (cm.)	No. of PLBs/ shoot buds per shoot tip	Time to proliferation	Colour of shoot buds
TZ 0 (Control)	v.nc	7	0	-	-	-	-
	v.c	7	0	-	-	-	-
TZ 1	v.nc	9	11.1	0.15	5-6	4.5	Yellowish green & Green
	v.c	9	77.7	1.0	12-18	2-2.5	Green
TZ 1.5	v.nc	8	25	0.2	6-8	3.5-4	Green
	v.c	8	75	0.7	12 - 15	2.5-3	Green
TZ 2	v.nc	7	14.3	0.25	6-7	4	Green
	v.c	7	71.4	0.8	12 - 18	2.5-3	Green

2.8 Effect of Age of Axenic Plantlet on PLB Induction in *Phalaenopsis*

PLB induction percentage was very less (only 14.2%) when shoot tips were excised from *in vitro* plantlets less than 3 months of growth **(Table 19).** But shoot tips from *in vitro* grown plantlets above 3 months of age exhibited an approximately a five-fold increase in plb induction percentage. (72.7 – 77.7%). This shows that the physiological age of the planting material used for explant excision is an important factor in PLB formation.

Necrosis of the explants was commonly observed in all treatments in varying amounts. The frequency of occurrence of necrosis in the explants was high when the explant source was from *in vitro* plantlets below 3 months old (**Table 19**). Taking into account all the above

mentioned factors, in the present study 3-3.5 month old *in vitro* plantlets were standardized as the source for axenic shoot tips.

Table 19. Effect of age of axenic plantlet (explant source) on PLB induction of shoot tips after 3 months of culture in *Phalaenopsis.*

Age of axenic plantlet (explant source)	**PLB formation (%)**	**Direct multiple shoot formation (%)**	**Necrosis (%)**	**Time taken for PLB induction (days)**	**Time taken for PLB proliferation (months)**	**Size of PLB mass (cm.)**
< 3 months	14.2	-	42.8	30	3	0.2 – 0.5
3 -6 months	**77.7**	**-**	**27.2**	**20 - 25**	**2.5 – 3**	**0.8 - 1**
>6 months	**72.7**	**28.6**	**22.2**	**15 - 25**	**2 – 2.5**	**0.8 - 1**

3. EFFECT OF MULTIPLICATION MEDIA

Comparative analysis from preliminary experiments with ½ MS and NDM showed that New Dogashima Medium fortified with and without NAA:BAP hormonal combination along with 0.075% activated charcoal was more efficient for formation and proliferation of PLBs and shoot buds for both *Phalaenopsis* and *Vanda*. Hence optimization of hormonal combinations was done in NDM.

Addition of activated charcoal (0.03% – 0.1%) was done as charcoal absorbs ethylene and phenolic inhibitors and improves aeration. However charcoal also absorbs plant growth regulators and other compounds such as vitamins in the medium and therefore from different trials the dosage was optimized at 0.075% .

3.1 Effect of PGR on direct axillary shoot multiplication in *Phalaenopsis*

Based on preliminary experiments in *Phalaenopsis*, half strength MS medium fortified with the hormonal combinations (0.5mgl^{-1} NAA & 3mgl^{-1} BAP) ; (0.1mgl^{-1} NAA & 5mgl^{-1} BAP) ; (0.1mgl^{-1} NAA & 20mgl^{-1} BAP) ; (1mgl^{-1} NAA & 5mgl^{-1} BAP) and (1mgl^{-1} NAA &

20mgl^{-1} BAP) with 10% coconut water were found to induce multiple shoots in six-month-old to one-year-old regenerated plantlets or axillary shoots when subcultured after excising leaves. Maximum number of multiple shoots (7-12) was induced in the medium with hormonal combination (1mgl^{-1} NAA & 20mgl^{-1} BAP) with 10% coconut water. It was observed that coconut water enhanced multiple shooting **(Table 20)**.

Table.20. Response of axillary shoot explants in *Phalaenopsis* after 2 months of culture

Hormonal composition ½ MS media	**S**	**Sp**	**Sw1**	**Sw2**	**Sw3**	**Spw1**	**Spw2**	**Spw3**
No. of cultures	7	5	9	8	9	7	3	5
Direct multiple shoot (2 or > 2) formation (%)	-	20	11.1	12.5	77.7	28.6	-	20
No. of multiple shoots	-	3-4	7-12	3-5	5-7	7-8	-	2-3
Regeneration to single shoot or plantlet (%)	14.2	20	44.4	37.5	33.3	14.3	-	20
PLB formation(%)	-	-	-	-	-	-	-	-
Necrosis(%)	71.4	4	2	3	4	14.3	2	20

S - ½ MS (1mgl^{-1} NAA ;20 mgl^{-1} BAP)

w1 - 10% CW; w2 - 15% CW; w3 - 20% ; CW; p - 2gl^{-1} peptone

3.2 Effect of PGR on PLB and shoot bud proliferation

In *Phalaenopsis*, secondary PLBs and shoot buds were formed on the basal and cut surfaces of 0.3-0.4cm. shoot tip portions, primary PLBs and shoot buds after 2 weeks of subcultivation in NDM without hormones and in NDM supplemented with (NAA 0.1mgl $^{-1}$ & BAP 1mgl $^{-1}$) and (NAA 0.2mgl $^{-1}$ & BAP 2mgl $^{-1}$) and 1% sugar. 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. Direct axillary shoot buds and shootbuds via PLBs multiplied and covered the medium that it was unable to distinguish between the direct shoot buds and shoot buds from

PLBs. At the end of 2 months PLBs and shoot buds appeared as a mass of structures making them impossible to count. Hence the experiment was evaluated at 3 months of culture when shoot buds gave rise to shoots. The multiplication factor of axillary shoots ranged from 34.3 in NDM fortified with NAA 0.2mgl $^{-1}$ & BAP 2mgl $^{-1}$, 37.3 in NAA 0.1mgl $^{-1}$ & BAP 1mgl $^{-1}$ and the highest mean of 46.3 shoots was observed in the medium NDM with no hormones for hybrids. **(Table 21)**.

Depending upon the media composition subcultured PLBs and shoot buds followed two different pathways of proliferation, growth and morphogenesis. In one pathway, proliferation of shoot buds and PLBs occured in 1.5 – 2 months in all the above hormonal combinations followed by morphogenesis to vegetative regenerants with small shoots in 2-2.5 months. In the second pathway, vegetative regenerants with large shoots were produced without PLB proliferation in 1.5-2 months in ½ MS fortified with (NAA 0.5mgl $^{-1}$ & BAP 4.5mgl $^{-1}$), (NAA 1mgl $^{-1}$ & BAP 5mgl $^{-1}$) and (NAA 2mgl $^{-1}$ & BAP 5mgl $^{-1}$) with 0.2% peptone and 2% sugar. Robust rooted plantlets were obtained in the same media at the end of 3 months. The multiplication factor ranged from 18.3 – 29.5; the highest mean of 29.5 axillary shoots was observed in the treatment NAA 1mgl $^{-1}$ & BAP 5mgl $^{-1}$ **(Table 21)**.

Similar results were observed by Kosir *et al.*, 2004 also in *Phalaenopsis* depending upon the chemical composition of the media. According to him, **the first pathway proved to be more appropriate when it is desired to carry forward PLB multiplication for many generations by subculturing every 2 months or when regenerants at different developmental phases are desired. The second method can be adopted when plantlets for field planting are desired in a short time by easily rooting the large shoots in any hormone free medium.**

It was observed that inoculation of five clumps of primary shoot bud mass of approximately 0.3-0.4 cm. diameter resulted in optimum proliferation. When the inoculation density and clump size was less, the necrosis percentage was higher and the primary shootbuds only regenerated to small plantlets without further multiplication. According to Street (1969), certain essential substances can pass out of plant cells by diffusion. Substances known to be released into the medium by this means include alkaloids, amino acids, enzymes, growth substances and vitamins. The loss is of no consequence when there is a large cluster of cells growing in close proximity or where the ratio of plant material to medium is high. However, when cells are inoculated onto an ordinary growth medium at a low population density, the concentration of essential substances in the cells and in the

medium can become inadequate for the survival of the culture. For successful culture initiation, there is thus a minimum size of explant or quantity of separated cells or protoplasts per unit culture volume. Inoculation density also affects the initial rate of growth in vitro. Large explants generally survive more frequently and grow more rapidly at the outset than very small ones.

In the present study, high proliferation rate of PLBs and direct shoot buds was observed in the control medium without hormones in 2 months. As this propagation process does not involve exposure to exogenous PGRs, secondary PLBs with the lowest chance of somaclonal variation can be obtained. During *in vitro* culture of plants, variations can happen due to different reasons such as modifications in DNA methylation, gene amplification, chromosomal abnormality and point mutation. Most orchid researchers prefer to use PGR-free media to obtain genetically stable PLBs (Huan *et al*.,2004). Conversion of PLBs into plantlets has often been reported to occur without the influence of PGRs (Hong *et al*.,2010, Gantait *et al*.,2012). After the formation of secondary PLBs and at the time of organogenesis, the endogenous hormonal synthesis system is triggered on and the increased level of endogenous PGRs in PLBs allowed the proliferation and differentiation of cells even in the absence of exogenous PGRs (Smith and Krikorian,1990). Lower concentrations of both NAA and BAP also induced optimum number of secondary PLBs and shoot buds. Higher concentrations of NAA (0.5-2mgl $^{-1}$) ,BAP (4.5-5mgl $^{-1}$) and sugar (2%) in the media produced large shoots and leaves and less PLB proliferation. Mondal *et al*.(2013) reported that PLB production was severely reduced in the medium containing BAP 4mgl $^{-1}$in *Doritis pulcherrima*. Increased levels of BAP exhibited suppression of shoot proliferation and enhancement of shoot elongation in the endangered terrestrial orchid *Anoectochilus elatus* (Sherif et al.,2012).

In ***Vanda*** hybrids, PLB proliferation was much faster compared to *Phalaenopsis*. The dark green PLBs proliferated to 1- 1.5 cm. size in 2 weeks in NDM without hormones and sugar. But in no–hormone-NDM with 10% sugar although proliferation occurred, the PLB clumps showed yellowing after 2 weeks **(Fig.5 & 6).** Regeneration to robust rooted plantlets was obtained in modified ½ MS medium supplemented with (NAA 0.1mgl^{-1} + BA 5 mgl^{-1}) and (NAA 1mgl^{-1} + BA 5 mgl^{-1}) with 2% peptone or 20% coconut water within 2 months.

Table 21. Effect of NAA and BAP in varying concentrations on *in vitro* response of primary PLBs and shoot buds after 2 months of culture in *Phalaenopsis*

Culture media	Plantgrowth regulators (mgl^{-1})		No. of multiple shoots	Nature of shoots	Response of explants after subsequent subcultivation
NDM	NAA	BAP			
N0B0	-	-	46.3	Small	m+s+r
N0.1B1	0.1	1	37.3	Small	m+s+r
N0.2B2	0.2	2	34.3	Small	m+s+r
Modified ½ MS					
N0.5B2.5	0.5	2.5	27.5	Large	s+r
N0.5B4.5	0.5	4.5	27.5	Large	s+r
N1B5	1	5	29.5	Large	s+r
N2B5	2	5	18.3	Large	s+r

s-shoot formation ; m- PLB and shoot bud multiplication; r- root initiation

Fig.5 PLB proliferation in *Vanda* after 2 weeks of culture in NDM without hormones and sugar

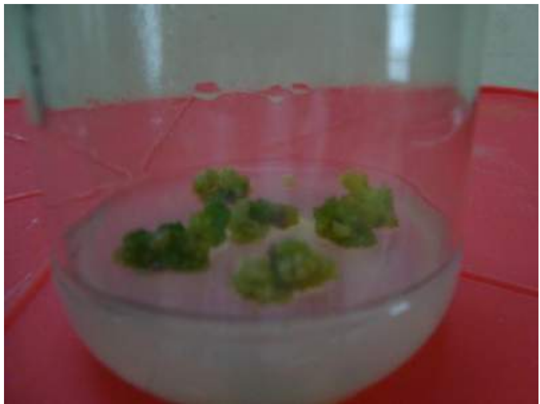


Fig.6 Yellowing of PLBs after 2 weeks of culture in no hormone NDM with 10% sugar

4. Effect of PGR on Rooting

Rooting was not a problem in *Phalaenopsis* and *Vanda* and rooted plantlets with three to six thick and long roots with length ranging from 2.5 - 6cm. were produced in all the optimized multiplication media for axillary shoot proliferation, viz., ½ MS fortified with (NAA 0.5 mgl^{-1} & BAP 2.5 mgl^{-1}), (NAA 0.5mgl^{-1} & BAP 4.5mgl^{-}1), (NAA 1mgl^{-1} & BAP 5mgl^{-1}) and (NAA 2mgl^{-1} & BAP 5mgl^{-1}) with 0.2% peptone and 2% sugar in 3 months. But in *Phalaenopsis*, satisfactory elongation could be obtained only when the above mentioned media was supplemented with either coconut water @ 10% or 20%. It was observed that vegetative regenerants with optimum shoot elongation (0.5-0.8cm.) and thick roots (2 - 6cm. in length) ready for hardening were produced after 2.5 months in Modified ½ MS medium fortified with (NAA 0.5mgl^{-1} & BAP 2.5mgl^{-}1) with 0.2% peptone and 3% sugar(**Fig.7**). This protocol is cost effective too since it avoids the use of coconut water.

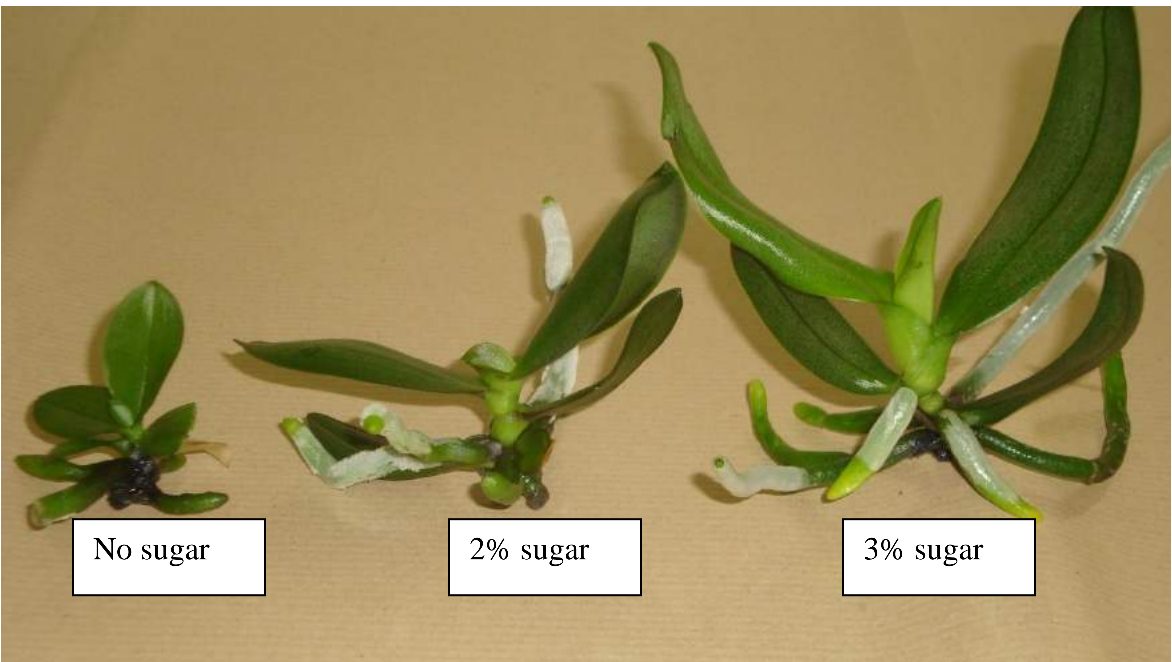


Fig. 7 Comparison of shoot elongation in *Phalaenopsis* plantlets in rooting media Modified ½ MS fortified with (NAA 0.5mgl^{-1} & BAP 2.5mgl^{-}1) without sugar and with 2% and 3% sugar

5. Effect of Hardening

In *Phalaenopsis*, it was observed that transplantation loss was least in plastic pots (15%) followed by clay pots (19.7%) and husk (23.1%). One or two fresh roots initiation started in 32.5% plantlets in plastic pots, 10.9% plantlets in husk and 6% plantlets in clay pots at the end of 2 months. (Fig.)After 4 months of primary hardening, plants were transferred to individually to claypots for 1 month of secondary hardening.

The higher percentage of loss in husk was mainly due to husks with less fibre and decaying of plants due to over watering. If these two problems are corrected, husk may be optimized as the hardening medium for *Phalaenopsis* as the plantlets which survived were healthy and established well to field conditions with the initiation of fresh roots which held tightly to the coir compared to pots **(Fig.8).**

In *Vanda*, loss due to transplantation was 23.9% and growth was slow compared to *Phalaenopsis* **(Fig.9).**

Primary hardening in clay pots

Primary hardening in coconut husk

Primary hardening in plastic pots

Secondary hardening in clay pots

Fig.8 Different methods of hardening

Keiki (explant source)

Shoot bud initiation in shoot tip with leafbase (explant)

Multiple shooting

Plantlet regeneration in rooting medium

Hardened *Vanda* plants after 6 months

Trays filled with water placed under the *Vanda* plantlets in the hardening shed

Fig. 9 Micropropagation in *Vanda* via direct shoot bud formation

PLB Induction — PLB multiplication

Shoot multiplication — Elongation & Rooting

Plantlet ready for hardening in field — 6 months after hardening

Fig. 10 Micropropagation in *Phalaenopsis* via PLB formation in axenic shoot tips of *Phalaenopsis* hybrids.

SUMMARY

The present study describes an efficient micropropagation method for axillary shoot proliferation via PLB mediated and direct shoot buds in *Phalaenopsis* and via direct shoot buds in *Vanda* without callus formation which ensures the genetic stability of plants. The success of micropropagation has been limited mainly due to the difficulties in decontaminating the explants. In the present study, the aseptic nature of axenic plantlets used as explant source ensures lack of microbial contamination and protects their meristem-derived juvenility status, rendering them responsive to tissue culture regeneration. Selection of appropriate mineral composition is essential for successful shoot bud formation.

Optimal results for initiation of PLB (protocorm-like-bodies) and direct shoot buds for mass multiplication was got in *Phalaenopsis* by culturing small axenic shoot tips of 0.3-0.5cm. length from three-month-old *in vitro* plantlets in ½ MS medium fortified with TDZ. The age of the axenic plantlets from which the shoot tips were excised markedly influenced the explant response in *Phalaenopsis*. Explants from three-month-old and above *in vitro* plantlets exhibited optimum PLB and shoot bud induction and less necrosis in TDZ fortified media. PLBs and shoot buds emerged efficiently by the vertical cutting method to give earlier and higher multiplication rate than shoot tips without vertical cutting in *Phalaenopsis*. Optimum PLB proliferation was observed in NDM with 10% sugar and without hormone or with low concentrations of NAA: BAP, namely, 0.1mgl $^{-1}$: 1mgl $^{-1}$ and 0.2mgl $^{-1}$: 2mgl $^{-1}$ depending on the variety. Large vegetative regenerants were obtained at higher sugar and NAA : BAP concentrations.

In *Vanda,* early and high rate of PLB induction was got when shoot tips with leaf bases from mature plants, keikis and axenic plantlets were used as explants. Interestingly in *Vanda*, optimum secondary PLBs, shoot buds and axillary shoot proliferation was observed on subcultivation to a sugar and hormone free New Dogashima Medium which reduces further the chances of somaclonal variation due to continued exposure to synthetic hormones and also makes the protocol more cost effective.

By repeated bimonthly subculture of PLB and shoot bud sections on proliferation medium it is possible to maintain continuous production plantlets which makes the present protocol an efficient means for commercial proliferation of genetically stable *Phalaenopsis* and *Vanda* hybrids.

SALIENT OUTCOME OF THE PROJECT

1. Germplasm collection of 190nos. of 30 different *Phalaenopsis* hybrids and 15 *Vanda* hybrids are maintained at RRS, Vyttila.

2. Cost effective protocols were standardized by substituting

i) Branded sodium hypochlorite solution @ Rs.40/ l. by household commercial bleach @ Rs.15/ l. for explant sterilization.

ii) Sucrose(Rs.650/kg.) by ordinary sugar (Rs.40/kg.).

iii) High grade agar(Rs.4050/kg.) by industrial agar(Rs.1100/kg.).

3.Two innovations developed were

(i) Vertical cut of axenic shoot tip explants in *Phalaenopsis* which enhanced rate and earliness of PLB induction in *Phalaenopsis*.

(ii) A new media composition , namely, Modified ½ MS was developed which was effective in producing optimum elongation along with rooting.

4. An efficient protocol for *in vitro* propagation of 3 *Phalaenopsis* hybrids namely Tying Shin Zebra, Jinseng Sun and Fuller's Cheese through direct protocorm-like-body formation from axenic shoot tip explants has been developed. The anticipated figures of production on a large scale is given below: Approximately 5000 plantlets can be produced in 2 years from a single shoot tip of the above mentioned *Phalaenopsis* hybrids .

Projected figures of production on a commercial scale in *Phalaenopsis* hybrids

Stages of micropropagation in *Phalaenopsis*	Production per shoot tip (No.)	Total time period taken(weeks) (at a subculturing frequency of 7 weeks)
PLB Induction		
S0 (0.8 – 1 cm. mass)	1	7
PLB Multiplication (5 clumps per bottle)		
S1 (0.3cm. clumps)	5	14
S2 (0.3cm. clumps)	5	28
S3-S8 Multiplication factor-2.5		
S3 (0.3 – 0.5cm. clumps))	13	35

S4 (0.5 – 0.8cm. clumps)	31	42
S5 (0.5 – 0.8cm. clumps)	78	49
S6 (0.5 – 0.8cm. clumps)	195	56
Shoot Multiplication		
S7	488	63
S8	1221	70
Elongation and rooting Multiplication factor - 5		
S9 (Plantlets)	6105	77
S10 (Plantlets)	6105	84
Hardening	6105	96
Total no. of plantlets produced after an anticipated loss of 20% due to contamination and during acclimitisation	**4884**	96

About 50,000 hardened plantlets can be produced from a single flowerstalk with an average of 10 buds, within 2 years from initiation of axenic shoot tip cultures using this protocol. Thus the present protocol of PLB multiplication should serve as an efficient means for commercial propagation of *Phalaenopsis gigantea*.

4. In *Vanda* , rapid multiplication on a commercial scale can be acieved through culture of shoot tips of axenic plantlets, on ½ MS medium fortified with TDZ 1 mgl^{-1}, coconut water 10% and adenine sulphate $10mgl^{-1}$ followed by rapid PLB proliferation in 2 weeks in New Dogashima Medium without hormones and sugar.

5. Induction of flower buds was obtained in *Phalaenopsis* in media fortified with high BAP concentrations (5 mgl^{-1}) and restricted nitrogen supply. In the present study, ½ MS medium was supplemented with approximately 6.5 × higher nitrogen and NDM with approximately 4.5 × higher nitrogen than needed for *in vitro* flowering . So these media with slight changes in the media composition (lower nitrogen content) could serve for earlier flowering induction in tissue culture and later *in vivo*.

SCOPE FOR FUTURE LINE OF WORK

Healthy mother plants of *Phalaenopsis* and *Vanda* hybrids (190 nos.) which serve as the explant source are maintained under green house conditions with regular management practices. The protocols optimized for mericloning of the selected *Phalaenopsis* and *Vanda* hybrids in the present study can be applied to the other varieties of these genera maintained in the Institute. Thus commercial level production of any variety of these genera can be taken up. Based on the standardized protocols of the selected *Phalaenopsis* and *Vanda* hybrids, mass production of these hybrids can be done at Rice Research Station, Vyttila.

SIGNIFICANCE OF THE OUTCOME WITH RESPECT TO THE BENEFIT OF SOCIO-ECONOMIC SCENARIO OF THE STATE OF KERALA

Although orchid culture has been identified as a lucrative agri-business in India and Kerala'a agroclimatic conditions are ideal for growing orchids , farmers are reluctant to exploit the prospects of commercial orchid cultivation. Non-availability of locally adapted and reasonably priced varieties is the major bottle neck which restricts large scale orchid cultivation in Kerala. At present, the planting material is being imported from other countries at exhorbitant rates. Presently in Ernakulam, consumers depend mainly on the few (4 or 5) private nurseries for purchase.

For seedling size / young plants the average market price is as follows:

Phalaenopsis	Rs 250 - Rs 350 per plant
Vanda	Rs 150 - Rs 200 per plant

For medium/flowering sized hybrids,the average market price is as follows:

Phalaenopsis	Rs 500 - Rs 1500 per plant
Vanda	Rs 600 - Rs 1000 per plant

It is anticipated that about 50,000 hardened *Phalaenopsis* plantlets can be produced from a single flowerstalk with an average of 10 buds, within 2 years from initiation of axenic shoot tip cultures using the protocols optimized in the project.

Follow up works of the project have already been initiated at Rice Research Station, Vyttila under Kerala Agricultural University to apply the protocols which have been optimized for mericloning of *Phalaenopsis* and *Vanda* hybrids in this project for the commercial production of these hybrids. By mass multiplication, these hybrids can be sold at much lower prices compared to private nurseries which in turn will benefit both the Institute and the farmers. It is estimated that even after anticipating a loss of 20% due to handling errors

and contamination problems, it will still be profitable to sell the secondary hardened *Phalaenopsis* and *Vanda* plants at 40% cost of the present market price. Thus commercial production and distribution of elite hybrids at reasonable prices to the farming community can be achieved. Availability of good quality planting material at reasonable prices will also encourage farmers to take up large scale cultivation of orchids as a viable business.

Greenhouse in which mother plants and hardened plantlets are maintained

REFERENCE

Chang,C. and Chang,W.C. (1998). Plant regeneration from callus culture of *Cymbidium ensifolium* var. *misericors*. Plant Cell Rep. 17:251-255.

Chen, J.T., Chang, C. and Chang,W.C. (1999). Direct somatic embryogenesis on leaf explants of *Oncidium* 'Gower Ramsey' and subsequent plant regeneration. Plant Cell Rep. 19:143-149.

Chen,J.T. and Chang, W.C. (2001). Effects of auxins and cytokinins on direct somatic embryogenesis on leaf explants of *Oncidium* 'Gower Ramsey'. Plant Growth Reg. 34:229-232.

Chen,Y.and Piluek,C. (1995). Effect of thidiazuron and N6-benzylaminopurine on shoot regeneration of *Phalaenopsis*. Plant Growth Reg. 16:99-101.

Chung JD, Leu JH, Lee S and Kim CK .1998. Effect of medium composition on multiple sho oting growth of mericlone from rhizome of shoot tip cultures of temperate Cymbidium species. In: Biological Abstract, 105(4): 50.

Duan, J.X and Yazawa,S.1995. Floral induction and development in *Phalaenopsis in vitro*. Plant cell, tissue and organ culture 43: 71-74.

Ernst, R. 1994. Effects of thidiazuron on in vitro propagation of Phalaenopsis and Doritaenopsis (Orchidaceae). Plant Cell Tiss. Organ Cult.39:273–275.

Gantait,S., Bustam, S and Sinniah UR . 2012Alginate-encapsulation, short-term storage and plant regeneration from protocorm-like bodies of Aranda Wan Chark Kuan 'Blue'× Vanda coerulea Grifft. ex. Lindl. Plant Growth Regulators 68: 129-140.

George, E.F. 1993. Plan propagation by Tissue Culture: Part 1, The Tchnology. 2nd Edn. Exegetics, Edington, UK., pp 337 – 356 .

Hare, P.D. and Van, S.J. (1994). Inhibitory effect of thidiazuron on the activity of cytokinin oxidase isolated from soybean callus. Plant Cell Physiol. 35:1121-1125.

Hong P.I.,Chen,J.T. and Chang,W.C.2010.Shoot development and plant regeneration from protocorm-like-bodies of Zygopetalum mackayi.Invitro.Cell.Dev.Bio.Plant.46:306-311

Huan, L.V.T.,Takamura,T.and Tanaka, M. (2004) Callus induction from protocorm-like body segments and plant regeneration in *Cymbidium* (Orchidaceae). J. Hortic. Sci. Biotechnol. 79: 406- 410

Ichihashi, S. (1992). Micropropagation of *Phalaenopsis* through the culture of lateral buds from young flower stalks. Lindleyana, 7: 208-215.

Kishor,R.and Devi, S. (2009). Induction of multiple shoots in a monopodial orchid hybrid (*Aerides vandarum* Reichb.f *X Vanda stangeana* Reichb.f) using thidiazuron and analysis of their genetic stability. Plant Cell, Tissue Organ Cult. 97:121-129.

Kosir, P., Skof, S. and Luthar, Z., 2004. Direct shoot regeneration from nodes of Phalaenopsis orchids. Acta Agric. Slov. 83, 233–242.

Kostenyuk, I., Oh,B.J. and So,I.S.. 1999 .Induction of early flowering in Cymbidium niveo-marginatum Mak in vitro. Plant Cell Reports 19:1–5.

Lin,C.C. 1987. Histological observations on in-vitro formation of protocorm-like bodies from flower atalk internodes of *Phalaenopsis*. Lindleyana 2(1): 58-65.

Mondal,T; Aditya,S and Banerjee,N.2013. *In vitro* Axillary Shoot Regeneration and Direct Protocorm-like Body Induction from Axenic Shoot Tips of Doritis pulcherrima Lindl. Plant Tissue Cult. & Biotech. 23(2): 251-261.

Morel G .1974. Clonal multiplication of orchids. In: Withner C.L. (Ed.). The orchids: scientific studies. Wiley, New York.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

Murthy, H.N. and Pyati, A.N. 2001. Micropropagation of *Aerides maculosum* Lindl. (Orhidaceae). In Vitro Cell. Dev. Biol. Plant., 37: 223-226.

Nayak,N.R.,Rath,S.P. and Patnaik, K. (1997). *In vitro* propagation of three epiphytic orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch-Ham) Sw. through thidiazuron-induced high frequency shoot proliferation. Sci Hortic. 71:243-250.

Pant B and Gurung R .2005. In vitro seed germination and seedling development in Aerides odorata Lour. J. Orchid Soc. India 19(1&2): 51-55.

Park, S.Y., Murthy, H. N. and Paek, K.Y. 2002. Rapid propagation of *Phalaenopsis* from floral stalk-derived leaves. In Vitro Cell. Dev. Biol. Plant., 38: 168-172.

Roy, J. and Banerjee, N. 2003. Induction of callus and plant regeneration from shoot-tip explants of *Dendrobium fimbriatum* Lindl. var. *oculatum* Hk. F. Scientia Hortic., 97: 333-340.

Roy, J., Naha, S., Majumdar, M. and Banerjee, N. (2007). Direct and callus-mediated protocorm-like body induction from shoot-tips of *Dendrobium chrysotoxum* Lindl. (*Orchidaceae*). Plant Cell, Tiss Organ Cult. 90:31-39.

Seeni,S and Latha,P.G. 2000. *In vitro* multiplication and ecorehabilitation of the endangered Blue Vanda.Plant Cell, Tissue and Organ Culture 61: 1–8, 2000.

Sherif,N.A., Benjamin,J.H.F., Muthukrishnan,S., Kumar,T.S. and Rao,M.V. 2012. Regeneration of plantlets from nodal and shoot tip explants of *Anoectochilus elatus* Lindley, an endangered terrestrial orchid. Afr.J.Biotechnol.11(29): 7549-7553.

Smith,D.L.and Krikorian,A.D.1990. Low external pH replaces 2,4-D in maintaining and multiplying 2,4_D initiated embryogenic cells of carrot.PlantPhysiol.72:329-336.

Sujjaritthurakarn,P and Kanchanapoom,K. 2011. Efficient Direct Protocorm-Like Bodies Induction of Dwarf Dendrobium using Thidiazuron. Not Sci Biol, 2011, 3(4):88-92.

Sunitibala, H.and Kishor R .2009. Micropropagation of Dendrobium transparens L. from axe nic pseudobulb segments. Indian J. Biotechnol. 8: 448-452.

Talukdar SK, Narsiruddin KM, Yasmin S, Hassan L and Begum R .2003. Shoot proliferation of Dendrobium orchid with BAP and NAA. J. Biol. Sci. 3(11): 1058-106 2.

Tisserat, B. and Jones, D. 1999. Clonal propagation of orchids. In: Hall, R.D. (Ed.): Plant Cell Culture Protocols. Methods in Molecular Biology, 111. Humana Press Inc., Totowa, NJ, USA, 127-134.

Tokuhara, K. and Mii, M. 1993. Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. Plant Cell Rep., 13: 7-11.

Udomdee,W., Pei-Jung Wen, Shih-Wen Chin and Fure-Chyi Chen.2012. Shoot multiplication of *Paphiopedilum* orchid through *in vitro* cutting methods. African Journal of Biotechnology Vol. 11(76):14077-14082

Uesato**,** K and Sagawa,Y.1986. Effects of sucrose and CW on the survival of PLB tissues of Cattleytonia (Orchidaceae) under cold storage.Science Bulletin of the College of Agriculture,1986.

Vendrame, W.A. and Magnire, I.2007. In vitro propagation and Plantlet Regeneration from D oritaenopsis Pruple Gem ‘Ching Hua’ Flower Explants. Hort. Sci. 42(5): 1256-1258.

Webster,S.A.,Mitchell,S.A. and Ahmad, M.H. 2003. A novel surface sterilization method for reducing microbial contamination on field-grown medicinal explants intended for in vitro culture. Chemosphere 51: 701–706.

Zimmer,K. and Pieper,W.1975. Weitere Untersuchungen zur Kultur *in vitro* von Aechmea. Gartenbau uwissenschaft 40: 129–132.