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IN VITRO ORGANOGENESIS AND MICROPROPAGATION OF THE ORCHID HYBRID, *CATTLEYA* NAOMI KERNS

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ABSTRACT
Orchids account for a large share of global floriculture trade both as cut flowers and as potted plants and are estimated to comprise around 10% of the International fresh cut flower trade. India accounts for nearly 7% of worlds orchid biodiversity contributing 1300 species which are distributed in five major phyto-geographical regions. India is in an excellent position to produce orchids of high quality compete with the leading nations in the orchid trade. Fortunately, India has all the potentials for the development of a successful orchid industry on scientific basis; it has varied and suitable climate and almost all the important commercial varieties of orchids including those of *Cattleya*, *Cymbidium*, *Dendrobium* and *Phalaenopsis*. For cut-flower growers *Cattleya* is extremely important crop. Clones of disease-free, photo-periodically controllable cultivars are desirable, because in cultivation they proved very profitable. *Cattleya* **Naomi Kerns (**CNK) hybrids were micropropagated using shot tips as explants. Micrpropagation of CNK was carried out in three steps viz, in vitro production of plbs & plantlets, sub-culturing of plantlets and induction of rooting & multiplication of plantlets. This paper deals with a modified and improvised technique for the large scale production of the orchid hybrids.

INTRODUCTION
Orchids have taken a significant position in the cut flower industry due to their attractiveness, long shelf life, high productivity, right season of bloom, ease of packing and transportation. In the international trade, among top ten cut flowers, orchids rank the sixth position. Orchids account for a large share of global floriculture trade both as cut flowers and as potted plants and are estimated to comprise around 10% of the International fresh cut flower trade. The average trade value of fresh cut orchids and buds trade during 2007-2012 was US$ 483 million. In 2012, there were more than 40 and 60 exporting and importing orchid countries, respectively around the world, and the total size of the global trade was US$ 504 million. The Netherlands is the top orchid exporting country in the world (39.67% of world orchid market) followed by Thailand (28.41%), Taiwan (10%), Singapore (10%) and New Zealand (6%). Importing countries are mainly Japan (30%), UK (12%), Italy (10%), France (7%) and the USA (6%). Asia is the main source of orchid to enter the world market (Jayarama Reddy 2008). India accounts for nearly 7% of worlds orchidbiodiversity contributing 1300 species which are distributed in five major phyto-geographical regionsviz. North eastern Himalayas, Peninsular region, Western Himalayas, Western Ghat and Andaman andNicobar Islands Majority of the cultivated orchids in Indian are native of tropical climates and are found in abundance in India in the state of Assam, Meghalaya, West Bengal, Karnataka and Kerala. Kalimpong, Shillong, Trivandrum, Bangalore, Yercaud and almost all the coastal areas of India are the places most suitable for the for the cultivation of orchids. India is in an excellent position to produce orchids of high quality compete with the leading nations in the orchid trade. Fortunately, India has all the potentials for development of a successful orchid industry onscientific basis; it has varied and suitable climate and almost all the important commercial varieties oforchids including those of *Cattleya*, *Cymbidium*, *Dendrobium* and *Phalaenopsis*.

The species from the genus *Cattleya*are tropical epiphytes, with sympodial growth and rather large pseudobulbs. The genus is named after the famous botanist William Cattley (Jayarama Reddy, 2008), it includes 40 species from Central and South America, often used in bi and trigeneric hybrids (Cullen, 1992). They are distributed in Mexico to tropical South America and West Indies (Willis, 1966). *Cattleya* is epiphytic or terrestrial with prominent pseudobulbs. Rhizomes are creeping, bearing erect, swollen or cane-like stems with several nodes. Leaves 1-4 borne on top of the stems. The flowers are solitary or in racemes. Sepals and petals are free. Lip, 3-lobed or entire. Lateral lobes or basal part rolled around the column. Column is free from lip, slightly flattened, often winged (Cullen, 1992). *Cattleya* **Naomi Kerns (CNK)** is a beautiful hybrid producing large fascinating flowers which are used for making

corsages. The flowers are bicoloured with red and orange combination. Petals and sepals are with fringed edges. The flowers are produced in pairs on a shorter stem and measure about 3 - 4 inches in diameter.

For cut-flower growers *Cattleya*is extremely important crop. Clones of disease-free, photo-periodically controllable cultivars are desirable, because in cultivation they proved very profitable. There are a few i*n vitro* plant and tissue culture techniques to propagate such ornamentals or even to produce new cultivars (Pindel and Miczyński 1996, Aldelberg et al. 1997, Peres and Kerbauy 1999, Lin et al. 2000, Prażak 2001, Arditti 2008, Petchthai et al. 2015). This paper deals with a modified and improvised technique for the large scale production of the orchid hybrids.

fig-1: a) CNK hybrid; b) CNK hybrid showing good growth, multiplication and rooting; c) CNK showing good Multiplication ratio.

MATERIALS AND METHODS

Micrpropagation of CNK was carried out in THREE steps

a) In vitro production of plbs and plantlets.
b) Sub-culturing of plantlets and induction of rooting.
c) Multiplication of plantlets

a. In vitro morphogenesis of plbs and plantlets

Cattleya **Naomi Kerns** (fig-1;a) hybrids were grown in green house before being used as a source of explants. Shoot tips were cleaned with water and washed with detergent and scrubbed gently with a soft brush under running water and finally placed in autoclaved distilled water before taking inside the inoculation chamber. Under ascetic conditions the explants were surface sterilized using 0.1% (v/v) $HgCl_2$ for 2 minutes, followed by washing five times with autoclaved distilled water. They were cut into small pieces and inoculated on Vacin and Went medium to obtain embryogenic callus segments. Initially Murashige and Skoog (MS) Medium was prepared with varied concentrations of NAA, BAP and 1 mgl^{-1} 2,4 D. 1% Activated charcoal (AC) was used for the absorption of oxidation products into the medium. Cultures were kept in growth rooms with illuminated (2000 lux) conditions. Within thirty days of culture, plbs were formed. Theseplbs were maintained in the same medium for 20 to 45 days till they developed into small plantlets.

b. Sub-culturing of plantlets and induction of rooting

The tiny plantlets formed in the previous step, with at least a pair of leaves and with or without roots, were transferred and sub-cultured in this second step. Modified VW medium with 1 mgl^{-1} BAP and varied concentrations of NAA, 2, 4-D and IBA. Cultures were maintained separately in growth rooms under illuminated (2000 lux) conditions (fig-1;c).

c. Multiplication of plantlets

Multiplication of plantlets is an essential step in order to obtain more number of plants for the commercial purpose. The plantlets raised in the second step were sub-cultured on VW medium with 150 ml^{-1} coconut water and varied concentration of BAP and NAA. Cultures were maintained separately in growth rooms under illuminated (2000 lux) conditions and observed for 12 weeks (fig-1;b).

Experimental design and data statistical analysis

Protocorm explants were cultured in 100 ml culture bottles with five explants, each treatment comprised of five bottles and the experiment was repeated twice. Regular observations of cultures were made once a week. Total number of protocorms responding with healthy shoots was recorded after every week weeks and the total number and length of shoots as well as roots were recorded after 12 weeks of culture. The Calculations were done as follows.

$$\text{Percentage of plantlet formation} = \frac{\text{No. of segments giving palntlets or PLBs}}{\text{No. of segments cultured}} X100$$

$$\text{Multiplication Ratio, (MR)} = \frac{\text{No. of plants produced}}{\text{No. of plants inoculated}} X100$$

Standard deviation (SD) is the positive square root of the variance. It is the value of the deviation from the mean of the two replicants. SD was calculated as follows:

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2}$$

Acclimatization

Well rooted plantlets with four to five fully expanded leaves were hardened on 1/2 MS basal medium without sucrose for about 5 weeks then transferred to small pots containing 1:1 (w/w) mixture of brick pieces and charcoal. Moss was used to lay over the potting mixture to maintain the moisture and polyethylene bags were used to cover the pots to maintain high humidity. Plantlets were moistened using diluted 1/2 MS macro nutrients alone and kept at 25 ± 1°C in artificial light provided by cool white fluorescent tubes for 4–6 weeks. Well acclimatized plantlets were then transferred to green house.

RESULTS

Micrpropagation of CNK was carried out in three steps viz, in vitro production of plbs and plantlets, sub-culturing of plantlets and induction of rooting and multiplication of plantlets.

In vitro morphogenesis of plbs and plantlets

Explants of the CNK hybrids were cultured in MS medium to study the effects of NAA and BAP on the rate survival of explants and PLB formation. Observation made after 12 weeks of culture. Even the colour of the plbs formed was also observed. At 1 mgl^{-1} each of NAA and BAP the percentage of survival and formation of plbs were very high. The second best combination was found to be 5 and 1 mgl^{-1} each of NAA and BAP respectively. Formation of plantlets was found to excellent at 1 mgl^{-1} each of NAA and BAP (Table-1).

Table-1: Effects of NAA and BAP on the survival of callus segments and PLB formation from callus segments of *Cattleya*Naomi Kernsin MS medium. Observation made after 12 weeks of culture.G - Green, PG - Pale Green, PY - Pale Yellow, Y - Yellow

NAA in mgl^{-1}	BAP in mgl^{-1}	No. of explants observed	Survival rate (%)	PLB formation (%)	Colour of PLBs	No. of plantlets formed from each explant
1	1	50	79	65	G	6
	5	50	62	48	G	5
	10	50	40	12	PG	2
	15	50	0	0	-	1
5	1	50	38	22	G	4
	5	50	22	16	PG	2
	10	50	04	0	-	-
	15	50	0	0	-	-
10	1	50	16	12	Y	2
	5	50	04	0	-	-
	10	50	02	0	-	-
	15	50	02	0	PY	-
15	1	50	04	02	PY	-
	5	50	02	0	-	-
	10	50	02	0	-	-
	15	50	0	0	-	-

Sub-culturing of plantlets and induction of rooting

An effect of different concentrations on cytokinins on the growth of the plantlets of CNK in VW medium with 1 mgl-1 BAP was studied. Observations made in 12 weeks cultures. Each value represented here under is the average of two independent experiments and represents means + SE. Length of the plantlets, number of leaves and number of roots were observed to understand the effects of cytokinins. The best levels for the growth of the plantlets were found to be at 10 mgl^{-1} of NAA. At this concentration length of the plantlets, number of leaves and number of roots were found to be very good (Table-2).

Table-2: Effects of different concentrations of Cytokinins on the growth of the plantlets of *Cattleya* Naomi Kerns in VW medium with 1 mgl^{-1} BAP. Observations made in 12 weeks cultures. * Each value is the average of two independent experiments and represents means + SE.

Auxins	Mgl^{-1}	Length of plantlet cm + SE	No. of leaves + SE	No. of roots + SE
NAA	2	3.1 + 0.79	4.0 + 0.49	2.2 + 1.19
	4	4.6 + 1.19	6.1 + 0.79	3.2 + 1.09
	6	3.3 + 1.09	4.2 + 1.19	4.1 + 1.34
	8	3.9 + 1.27	4.3 + 1.09	5.0 + 2.39
	10	7.0 + 2.29	6.2 + 1.97	5.1 + 2.29
1 BA	2	2.8 + 0.56	4.2 + 0.39	2.0 + 0.98

	4	3.3 + 0.89	6.4 + 0.69	2.2 + 0.98
	6	3.1 + 1.19	4.3 + 1.69	3.1 + 1.27
	8	4.2 + 1.59	4.3 + 1.59	4.2 + 1.49
	10	5.1 + 1.8	4.4 + 1.8	4.3 + 1.69
2,4-D	2	2.2 + 0.89	3.1 + 1.19	2.1 + 0.98
	4	3.1 + 1.49	4.0 + 1.97	2.2 + 1.69
	6	3.4 + 2.29	4.2 + 2.09	4.0 + 2.8
	8	4.3 + 2.5	4.3 + 2.79	4.2 + 2.79
	10	5.6 + 2.68	5.7 + 2.8	4.3 + 2.9

Multiplication of plantlets

Effects of auxins and cytokinins on the multiplication ratio (MR) of *Cattleya* **Naomi Kerns** plantlets in MS medium with 150 ml^{-1} coconut water was stidied. Observations made in 12 weeks culture. Multiplication ratio (MR) was found to best at 4 and 1 mgl^{-1} each of BAP and NAA respectively (Table-3).

Table-3: Effects of auxins and cytokinins on the multiplication ratio (MR) of *Cattleya*Naomi Kernsplantlets in MS medium with 150 ml^{-1} coconut water. Observations made in 12 weeks culture.

BAP in mgl^{-1}	NAA in mgl^{-1}						
	0	**1**	**2**	**3**	**4**	**6**	**10**
0	1.4	1.5	1.5	1.6	1.8	1.8	1.9
1	2	2.5	2.5	2.5	1.5	1.5	1
2	5	9.5	7.5	5	4.5	2.5	2
4	6	9	6.5	4	4	2	1.5
6	4.5	4	3	2.5	2	1.8	1.8
8	4	3.5	3	3	2.5	2	1.5
10	4	3	2.5	2	2	1.5	1.8

DISCUSSION

Owing the significance of Cattleya orchid hybrids to meet the ever-increasing demand for orchids in the multi-billion dollar orchid industry. The present protocol was developed to achieve the maximum result in terms of high rate of production of plantlets. The current method a three step process was used. Micrpropagation of CNK was carried out in three steps viz, in vitro production of plbs and plantlets, sub-culturing of plantlets and induction of rooting and multiplication of plantlets. This was found to be successful as orchids required different concentrations of growth regulators at different levels of growth. MS medium was used only in the first stage as contains main salts and proves expensive. It is advisable to use a less expensive medium especially when we are producing plants on a commercial scale. Activated charcoal was used in the first stage of the protocol. Activated charcoal has a very fine network of pores with large inner surface area on which many substances can be adsorbed. Activated charcoal is often used in tissue culture to improve cell growth and development. It plays a critical role in micropropagation, orchid seed germination, somatic embryogenesis, anther culture, synthetic seed production, protoplast culture, rooting, stem elongation, bulb formation etc. (Thomas, 2008).

As it is shown by many workers supply of growth regulators promoted the production of plantlets from the shoot tip explants cultured on all the media used. This is probably an outcome of the habituated nature and juvenility of plants. The juvenility of tissues is thought to be an important factor controlling cell proliferation in several orchids (Arditti and Ernst 1993; Vij et al. 1997; Vij et al. 2000). The addition of complex mixtures to the culture medium, such as coconut water, malt extract, and banana juice, has been successfully used for different species since the beginnings of tissue culturing (Loewenberg; Skoog, 1952), and the culture medium has been supplemented with amino acids, vitamins and growth regulators (George, 1993). The function of complex mixture, into culture medium, has been promoted growth of micropropagated plants (Silva et al., 2005). Hence in order to get good morphogenetic response use of the growth regulators, coconut water and other adjutants is very much essential.

Optimization of in vitro rooting of *Cattleya* shoots with the aim to increase survival rate during acclimatization was carried out by Dewir et al (2015). In which shoots (2.0–2.5cm) which were regenerated in vitro were cultured on Murashige and Skoog (MS) medium and the effect of various parameters such as type and concentration of auxin, medium strength (full and half strength), sucrose concentration (0, 15 and 30 $g \cdot L^{-1}$) and light intensity [photosynthetic photon flux density (PPFD) of 30, 60, 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$] were tested. Similarly in the present study also in the second stage of the protocol effects of growth regulators on the formation of roots was studied. Coconut water and higher levels of NAA was found to be good for the formation of roots.

The response of the explants to PLB formation varies from species to species and from explant to explant used (Teng *et al*. 1997). A high ratio (12.2) of NAA to BAP in *Spathoglottis plicata* was reported to be best for the induction of PLBs from nodal explants (Teng *et al*. 1997). However, a low ratio of NAA to BAP, 0.12 in the case of *Phalaenopsis amabilis* (Tanaka and Sakanishi 1985) and 0.42 in *Dendrobium antennatum*, was reported to be suitable (Kukulczanka and Wojciechowska 1983). Also, in several hybrid species of *Aranda*, a ratio of 1.23 for NAA to BAP has been found to be most effective (Khaw *et al*. 1978).

In the current method shoot explants with an axillary bud cultured *in vitro* on MS solid medium supplemented with the cytokinin BAP and the auxin 2,4-D, each at different levels. Regeneration of viable rooted shoots is mediated by combination of direct shoot bud formation and indirectly via PLBs. The technique is likely to be widely applicable but the growth regulator component may need adjustment depending on the species and the physiological state and nutrient environment of the source material.

The survivability of the micropropagated plantlets on being transferred to pots depends on their proper acclimatization. CNK needs a substratum containing water-holding capacity with good drainage. Crushed charcoal and brick pieces were therefore adopted as the basis for the six compost mixes we tested. The highest survival levels (over 60%) were achieved when this combination was augmented with shredded bark or decaying litter plus a covering layer of moss for water retention (Dohling*et al*. 2008).

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