

Rapid *in vitro* propagation of the threatened endemic orchid, *Ipsea malabarica* (Reichb.f.) J D Hook through protocorm-like bodies

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Received 10 January 2005; revised 16 March 2005

Rapid propagation of *I. malabarica* (Reichb. f.) J D Hook, an endemic and endangered orchid of the Western Ghats of Kerala, India through conversion of axillary buds to protocorm-like bodies (PLBs), and subsequent plant regeneration was achieved. Growth regulators and sugar displayed significant influence in the induction of PLBs. *In vitro* derived shoots from field grown rhizomes of *Ipsea* cultured on Murashige and Skoog (MS) medium with 13.3 μM N⁶-benzyladenine (BA) containing 2% commercial grade sugar turned the axillary buds to PLBs within 25 days, and developed a mean of 33.1 PLBs within 50 days. Kinetin (KIN) did not induce PLBs, but facilitated axillary bud proliferation. Transfer of PLBs on medium having same concentration of BA and sugar facilitated rapid multiplication, and developed a mean of 47.5 PLBs. No decline of PLB proliferation was observed up to 10th subculture. Half strength MS medium with 6.97 μM KIN facilitated conversion of 98% PLBs to plantlets. On this media, a mean of 5.8 roots were also developed per shoot. Shoots developed bulbs during culture were grown to rhizomes. Increase of sugar to 6 or 8% hastened the development of bulbs/rhizomes. Reintroduction of PLB-derived plantlets in the natural habitat i.e. at Vellarimala (at 1300 m height) of the Western Ghats of Kerala was attempted as a means to assist *in situ* conservation. This is the first report of conversion of axillary buds to PLBs. The protocol enables to surmount the threat of extinction of this endemic and endangered orchid.

Keywords: Bulbs, *Ipsea malabarica*, Malabar Daffodil orchid, Rhizome

Ipsea malabarica (Reichb.f.) J. D. Hook., well known as "the Malabar Daffodil Orchid", is an endemic and endangered rhizomatous terrestrial orchid of the Western Ghats of Kerala, India. The species endemic to India, once and believed to be extinct, was rediscovered in 1982 after a span of 132 years¹ and has now very restricted distribution along the rocky grasslands at high elevations (above 1200 m) in Silent Valley, Vellarimala, Chembra Peak and Siruvani hills of the Western Ghats of Kerala. Extinction of plant species is influenced by its propagation rate. Rhizomes serve as the propagules for vegetative propagation. Multiplication through rhizomes is time consuming and cumbersome, and is inadequate to cut off the threat of extinction. The mandatory mycorrhizal association restricts its natural propagation through seeds. Propagation through seed is further hampered by low viability and germination rate. Besides, the seed derived progenies are heterozygous and not true-to-type. Low propagation rate coupled with habitat loss along the Western Ghats of Kerala has moved this horticulturally

desirable (breeding for its attractive golden yellow flowers) terrestrial orchid to the endangered category. *In vitro* propagation is a viable alternative for rapid clonal propagation as well as conservation of many endemic and endangered species as has been reported in many orchids of this category²⁻⁴.

In vitro propagation of *Ipsea malabarica* through axillary bud proliferation from rhizome⁵, and seed culture⁶ has been reported. The present endeavour emphasizes the conversion of axillary buds to protocorm like bodies (PLBs) and subsequent high frequency plant regeneration of this threatened orchid, and reintroduction of the plantlets in the natural habitat at high elevation.

Materials and Methods

Shoots derived from field grown rhizome explants through axillary bud proliferation on half strength MS (only macro- and micronutrients at half strength) medium with 6.97 μM kinetin⁵ were cultured on Murashige and Skoog⁷ medium with N⁶-benzyladenine (BA) or kinetin (KIN) singly or in combination and also BA in combination with different auxins [α -naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA)] (Table 1). Conversion of protocorm-like

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bodies (PLBs) was carried out on half strength MS medium with various concentrations of different growth regulators (Table 2). The media were supplemented with 1-10% (w/v) commercial grade sugar (as described in the text), and gelled with 0.6% (w/v) agar. The pH of the media was adjusted to 5.8 before the addition of agar. Test tubes (25 × 200 mm), 100 ml conical flasks, and jam bottles (53 × 125 mm; 2 mm thick) were used as culture vessels. Culture tubes and conical flasks were closed by cotton plugs, while polypropylene autoclavable lids (14 mm ht. × 48 mm inner diam and 2 mm thick) were used for jam bottles. The media were sterilized at a pressure of 1.06 kg cm⁻² (121°C) for 20 min in an autoclave. Subcultures were carried out at an interval of 50 days. All cultures were incubated at 25±2°C under 16 hr light (at an irradiance of 25 μmol m⁻² s⁻¹)/8 hr dark cycle.

The plantlets transplanted to small cups containing sand and vermicompost (1:1), were acclimatized, and grown in greenhouse. Cultures with protocorm-derived plantlets taken to Vellarimala (at 1300 m height) were opened there and the propagules were planted directly in the black clay soil. The survival rates of the plantlets in greenhouse as well as at the natural habitat (Vellarimala) were recorded.

The experiments were run in a completely randomised design. Twenty replicates were raised for each treatment, and the best treatments were repeated thrice (10 replicates each). Mean values of different treatments were compared using Duncan's multiple range test.

Results and Discussion

Growth regulators and sugar significantly influenced the induction and multiplication of PLBs, and plantlet regeneration (Table 1). BA induced PLBs, while KIN did not induce PLBs, but favoured axillary bud proliferation. MS medium with 13.3 μM BA and 2% sugar was the best for induction and proliferation of PLBs (Table 1). On this medium, the axillary buds of shoots were converted to PLBs within 25 days, which underwent proliferation and developed a mean of 33.1 PLBs within 50 days (Table 1). Level of sugar above and below 2% delayed and decreased the number of PLBs. On MS medium containing 13.3 μM BA and 1% sugar, a mean of 12.9 PLBs were developed, and which initiated after 35 days. Medium having 13.3 μM BA and 3% sugar produced a mean of 19.1 PLBs, and the initiation of PLBs occurred

after 30 days. The PLBs were cream in colour. Subculture of isolated PLBs on medium with 13.3 μM BA and 2% sugar facilitated rapid proliferation, and induced a mean of 47.5 PLBs within 50 days. On this medium, less than 5% of the PLBs differentiated to shoots. On media with higher concentration of BA (above 13.3 μM), there was a reduction in number of PLBs (Table 1). Culture of protocorm-derived shoots on the above medium facilitated development of PLBs around the node region including the axillary bud (Fig. 1A). PLBs at the sixth subculture developed more than 75 PLBs (Fig. 1B). No decline in the proliferation of PLBs was observed up to 10th subculture. Induction of PLBs from different explants by the influence of BA has been reported in several orchids^{8,9}. The present study emphasizes the conversion of axillary buds to PLBs for the first time.

Of the various concentrations of KIN tested with MS medium, 6.97 μM KIN was superior for shoot propagation, and induced a mean of 10.3 shoots per axillary bud (Table 1; Fig. 1C). Increase or decrease of KIN reduced the number of shoots (Table 1). In addition to the shoots, KIN enriched medium later developed a mean of 5.4 roots per shoot. KIN at higher concentrations increased the number of roots. Combination of BA and auxins (NAA, IAA, IBA) was not beneficial for the development of PLBs when compared to medium with 13.3 μM BA (Table 1). Though, the induction of shoots through axillary bud multiplication by KIN supplemented medium was consistent with the previous report⁵, the present study emphasizes the rapid propagation of the plant through the conversion of axillary buds to PLBs with a reduction in cost by the use of commercial grade sugar.

KIN provided better results for the conversion of PLBs to plantlets compared to auxins alone (Table 2). On half strength MS medium supplemented with 2.69 μM NAA, the conversion of PLBs to plantlets was slow, and the plantlets were very small. NAA supplemented medium induced proliferation of PLBs. The roots developed on this medium were dark green and < 1.5 cm. Half strength MS medium with KIN favoured both rooting and shoot multiplication. Half strength MS medium containing 6.97 μM KIN facilitated conversion of 98% PLBs to plantlets (Table 2; Fig. 1D, E). On this medium, the plants grew well and developed 3-5 dark green leaves. In addition, the shoots underwent multiplication through axillary bud proliferation. Each shoot developed a

mean of 5.8 roots per shoot (Table 2). Roots developed from the base of the shoots were with a mean of 3.7 cm length.

Upon PLB conversion medium (half strength MS with 6.97 μM KIN), the base of the shoots swelled and formed bulbs. Increase of sugar significantly influenced the formation of bulbs, and thereafter the development of rhizomes. Induction of rhizome from PLB derived plants as to the level of carbon source was concurrent with the *in vitro* studies of *Ipsea* using axillary bud proliferation⁵. Of the different levels of sugar (1-10%) supplemented to half strength MS

medium having 6.97 μM KIN, the bulbs on 6 or 8% sugar containing medium were grown to rhizomes within 50 days. At low concentration of sugar (2%), bulb and rhizome formation was delayed. The bulbs and rhizomes formed *in vitro* varied in size and they bore scale leaves. According to Wellensiek¹⁰, supply of carbohydrates is the causal factor for tuberization. In concurrence with the present study, *in vitro* rhizome formation by an increase of carbon source has been documented in *Curcuma amada*¹¹. Influence of Kn, carbohydrates, and other factors in tuberization has been reviewed¹². Growing of bulbs to rhizomes

Table 1 — Effect of MS medium supplemented with different growth regulators in the induction of PLBs or shoots *I. malabarica* [Values are mean of 20 replicates]

Growth regulators (μM)					Cultures induced PLBs or shoots (%)	Number of PLBs per shoot	Number of shoots per shoot
BA	KIN	IBA	IAA	NAA			
0	0	0	0	0	45	00	1.2 ^d
2.22					75	00	2.0 ^d
4.44					85	00	3.6 ^c
6.66					90	7.5 ^f	00 ^c
8.87					100	19.8 ^d	00 ^c
13.3					100	33.1 ^a	00 ^c
15.52					100	21.2 ^{cd}	00 ^c
17.74					85	7.3 ^f	00 ^c
22.22					90	4.4 ^b	00 ^c
	2.32				70	00 ^h	3.3 ^c
	4.65				95	00 ^h	7.4 ^b
	6.97				100	00 ^h	10.3 ^a
	9.29				85	00 ^h	7.2 ^b
	13.94				80	00 ^h	4.4 ^c
13.3	2.32				90	00 ^h	3.7 ^c
13.3	4.65				85	00 ^h	6.3 ^b
13.3	6.97				85	00 ^h	7.6 ^b
13.3		2.46			90	23.7 ^c	00 ^c
13.3		4.90			90	16.2 ^e	00 ^c
13.3			2.85		85	22.4 ^c	00 ^c
13.3			5.71		90	17.6 ^d	00 ^c
13.3				2.69	85	26.7 ^b	00 ^c
13.3				5.37	90	18.3 ^d	00 ^c

Mean values followed by different letters within column are significantly different at 5% level. Growth period 50 days

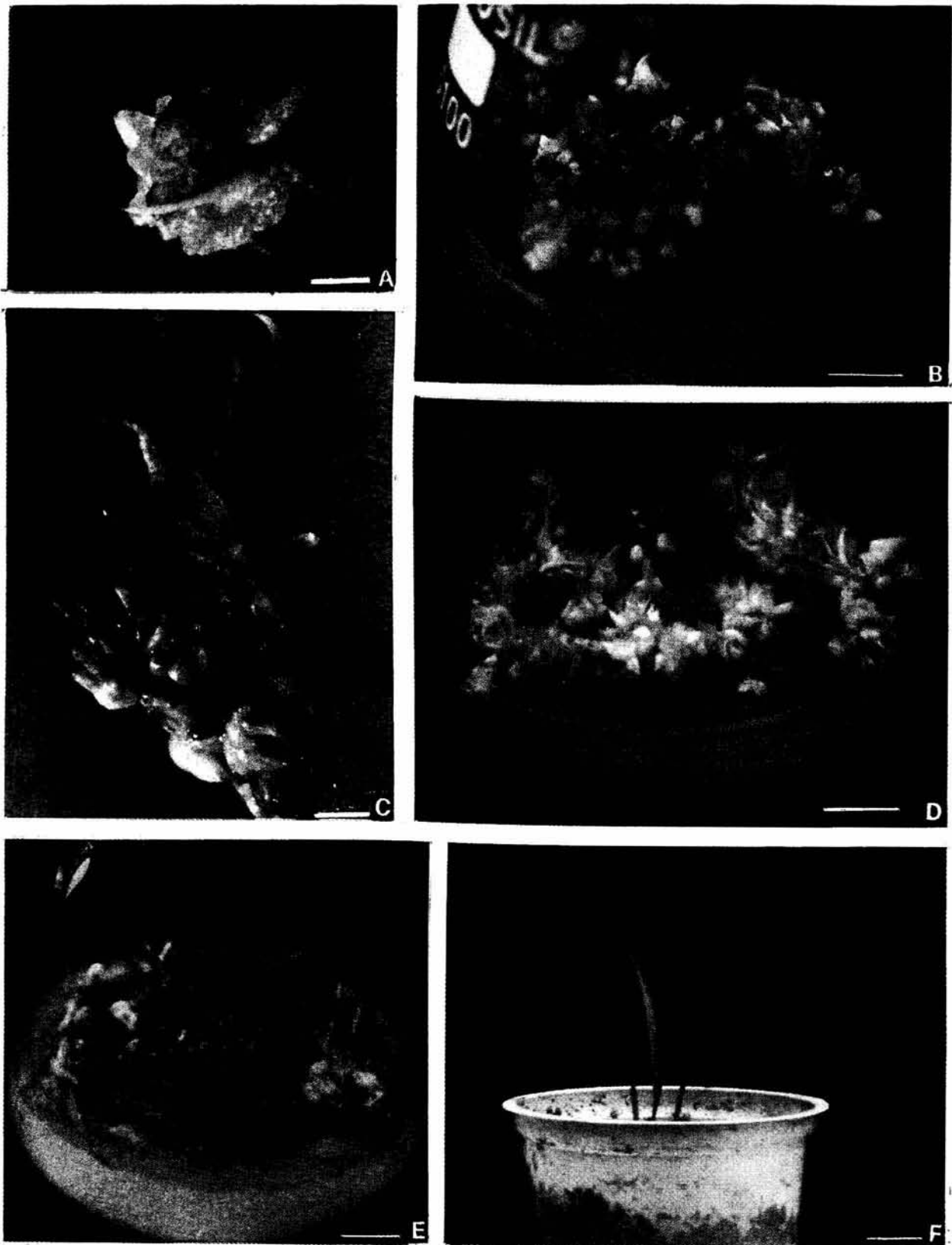


Fig 1A-F — *In vitro* propagation of *Ipsea* through the induction of shoots and PLBs. (A) — Induction of PLBs on MS medium with 13.3 μM BA and 2% sugar around the node region of PLB derived shoot (bar = 2.5 mm); (B) — PLBs proliferation on MS medium containing 13.3 μM BA and 2% sugar (6th subculture; bar = 10 mm); (C) — Axillary bud multiplication on MS medium with 6.97 μM KIN (bar = 2.5 mm); (D) — PLB conversion on half-strength MS medium containing 6.97 μM KIN (within 20 days; bar = 10 mm); (E) — Plantlets developed on half strength MS medium with 6.97 μM KIN (bar = 10 mm); and (F) — Greenhouse established PLB derived plantlet (45 days; bar = 8 mm).

Table 2 — Efficacy of different growth regulators in the conversion of PLBs to shoots or plantlets
[Values are mean of 20 replicates with 5 PLBs]

Growth regulators (μM)					Conversion of PLBs	Number of roots
BA	KIN	IBA	IAA	NAA	(%)	per shoot
0	0	0	0	0	16 ⁱ	3.7 ^c
0.22					69 ^e	2.1 ^d
0.44					58 ^f	0.8 ^e
2.22					49 ^g	00 ^f
4.44					38 ^h	00 ^f
	2.32				91 ^{bc}	6.4 ^a
	4.65				94 ^b	6.2 ^{ab}
	6.97				98 ^a	5.8 ^b
	9.29				89 ^c	4.1 ^c
		0.49			88 ^{cd}	3.5 ^c
		2.46			71 ^e	4.0 ^c
			0.57		86 ^d	3.6 ^c
			2.85		73 ^e	4.1 ^c
				0.54	90 ^c	4.3 ^c
				2.69	83 ^d	3.8 ^c

Mean values followed by different letters within column are significantly different at 5% level. Growth period 50 days.

was similar to that on *ex vitro* germinated seed derived progenies of *Ipsea*. After germination, the base of the seedlings of *Ipsea* resembled bulbs, and which later grown to rhizomes as to age.

PLB conversion medium (half strength MS with 6.97 μM KIN) was appropriate for shoot multiplication and induction of roots sufficient for field establishment (Table 2). Induction of roots and shoot multiplication on KIN supplemented medium was in agreement to that obtained with axillary bud proliferation of the previous study on *Ipsea*⁵. Induction of roots on PLB conversion medium eliminates separate rooting stage of micropropagation, and reduces the time from laboratory to field along with reduction in cost. *Ipsea* is a high altitude plant, however, 80% (40 out of 50) of the plantlets acclimatized in greenhouse in small cups containing sand and vermicompost (1:1) (Fig. 1F) survived even two years after establishment. Fifty PLB derived plantlets were introduced in black clay soil at Vellarimala (at an elevation of 1300 m) of the

Western Ghats of Kerala, and all the established plantlets survived. As observed in the culture, the bulbs developed to rhizomes as growth proceeds. The plantlets established in greenhouse as well as in natural habitat were similar in morphological characters to that of the source plant. The plantlets reintroduced at natural habitat underwent flowering normally. The plantlets grown in greenhouse, though developed rhizome were less vigorous in growth, and did not undergo flowering compared to the plantlets established in natural habitat at high elevation and this may be due to the climatic variation.

Conservation of *Ipsea malabarica* is mandatory to keep it off from extinction. Impact of low propagation on extinction can be minimized by rapid propagation. Thus propagation at an increased pace compared to the vegetative propagation (irrespective of the season, temperature, and elevation), using *in vitro* strategies and, its timely reintroduction at the natural habitats, is the simple way to uphold this endemic endangered

orchid from extinction. The present protocol through the conversion of axillary buds to PLBs on MS medium with 13.3 μM BA, its multiplication on medium having the same concentration of BA, and the development of these protocorms to plantlets on half strength MS medium with 6.97 μM KIN helps to accomplish rapid propagation and conservation of this threatened orchid.

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