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Simple and Rapid Regeneration of *Plumbago rosea* using BAP for Direct and Indirect Plant Regeneration from Leaf and Nodal Explants

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ABSTRACT

A rapid and highly effective method for plant micropropagation from leaf bits and vegetative shoot buds was established for the medicinal plant *Plumbago rosea* Linn. The number of multiple shoots per explant ranged from 8-10 when cultured on Murashige and Skoog (MS) basal medium supplemented with 0.5 mgl^{-1} indole-3-butyric acid (IBA) and 6 mgl^{-1} 6-benzylamino purine (BAP). Nodal explants initially produced basal callus followed by 10 ± 0.71 multiple shoots. Recently matured leaves i.e., third leaves from the top, when cultured on MS with 0.25 mgl^{-1} BAP, 0.25 mgl^{-1} kinetin and 0.5 mgl^{-1} ascorbic acid and maintained in the dark, produced dark green calli which in later stages produced shoot buds in a 16-h photoperiod regime. About 12 ± 1.22 multiple shoots also developed directly within 15 days from the leaf margin as well as from the wounds when maintained at a 16-h photoperiod regime. More than 85% of the matured leaf explants regenerated shoot buds, while very young or fully mature leaves did not respond. Excised shootlets cultured on half-strength MS with 0.2 mgl^{-1} IBA rooted within 45 days. Successful transfer of the rooted shots to pots was accomplished with 100% survival.

Keywords: acclimatization, adventitious shoots, basal callus, leaf explant, nodal explant, photoperiod, *Plumbago indica*, rooting
Abbreviations: BAP, 6-benzylamino purine; **FYM**; farm yard manure; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **MS**, Murashige and Skoog medium; **NAA**, α-naphthalene acetic acid; **PGR**, plant growth regulator

INTRODUCTION

Plumbago indica (syn. *Plumbago rosea* L.) is a medicinal plant belonging to the Plumbaginaceae. The members of this family are characterized by the presence of an acrid crystalline principle called '*plumbagin*' (2-methoxy-5-hydroxy-1,4-naphthoquinone) (Harborne 1966). Plumbagin is extracted from roots and is a natural naphthoquinone used as antimalarial (Likhitwitayawuid *et al.* 1998), antimicrobial (Didry *et al.* 1994), anticancer (Parimala and Sachdanandam 1993), cardiotonic (Itoigawa *et al.* 1991) anti fertility agent (Devi *et al.* 1998; Sharma and Mahanta 2000) and abortifacient (Munavar *et al.* 2007).

P. indica is also used in the treatment of leprosy, dyspepsia, diarrhea, skin diseases, etc. (Kirtikar and Basu 1975; Padua 1999). Many medicinal formulations are prepared by using the plant and the natural vegetation is the main source which is exploited to a great extent. For further research into the biochemical compositions and potential medicinal values of this plant, an efficient *in vitro* regeneration system for the production of plants is required because field grown plants may be subject to seasonal and somatic variations, infestations of bacteria, fungi and insects as well as environmental pollutions that can affect the medicinal value of the harvested tissues (Geng *et al*. 2001).

The propagation of *P. indica* is generally through seeds but since the percentage seed germination is very low it is a major constraint to traditional methods of propagation (Anonymous 1989). As an alternative, tissue culture techniques allow rapid mass propagation of elite genotypes independent from seasonal influences as well as the production of virus-free plants. This method offer powerful tools for germplasm conservation and the mass-multiplication of threatened plant species (Murch *et al.* 2000a). Earlier *in vitro* propagation of *P. rosea* was attempted through organogenesis from different explants such as leaves, shoot tips, axillary buds and callus (Satheesh Kumar and Bhavanandan 1988; Rout 2002; Satheesh Kumar and Seeni 2003) and somatic embryo (Das and Rout 2002). High frequency shoot multiplication was reported by Smita and Handique (2000).

The present study was aimed at the development of a simple protocol for efficient micropropagation through callus cultures and also by direct regeneration (adventitious shoots) methods from leaf discs.

MATERIALS AND METHODS

Plant material and *in vitro* shoot regeneration (nodal explants)

P. rosea material was collected from pot grown plants (**Fig. 1A**) at the Department of Horticulture, UAS, GKVK, Bangalore, India. All the chemicals in the present investigation were obtained from Merk (Darmstadt, Germany). The shoot tips and nodal buds excised from one-year-old plants were used as explants. The explants were kept in running tap water for 30 min and then subsequently pre-treated with solution of 0.2% carbandazime (bavistin), 0.2% cetrimide and 5-6 drops of Savlon (Johnson and Johnson's, Pvt. Ltd.) for 30 min with intermittent washes of sterile distilled water. The explants were surface sterilized with 0.1% $HgCl_2$ for 3 min followed by several washes in sterile distilled water. The explants were cultured in approximately 30 ml nutrient media in a 300 ml culture bottle (Tarsons Products Pvt., Ltd., Kolkata, India). The basic medium composed of MS (Murashige and Skoog 1962) medium with 0.1 g l^{-1} *myo*-inositol, 3% sucrose, 0.8% agar and was supplemented with different combinations and concentrations of plant growth regulators (PGRs) as mentioned in **Table 1**.

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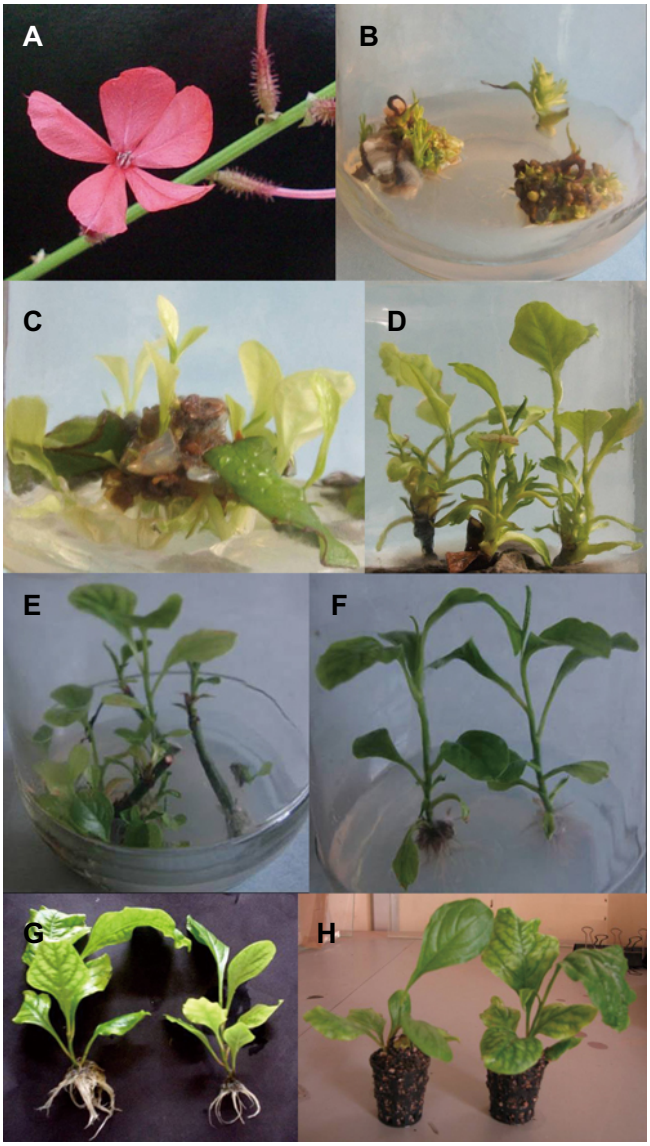

Fig. 1 Stages of micropropagation. (**A**) *P. rosea* habit (**B**) Callus derived from leaves. (**C**) Direct shoot buds from leaf explants. (**D**) Multiple shoots from the nodal explants. (**E**) Multiple shoots from the basal callus of nodal explants. (**F**) *In vitro* root initiation in shoots. (**G**) *In vitro* rooted plants. (**H**) Acclimatized plants.

Multiple shoot induction (leaf explants)

Leaves at three growth stages were used in the present investigation as reported earlier by Rout (2002). The juvenile, semi-mature and mature leaves were used in the experiment and 2-3 piercing was made on some of the leaf explants. The petiole and the apex (leaf tip) were removed, so that two cuts were made on each leaf explant. Explants were transferred with their abaxial or adaxial side down onto regeneration media (depending on the experimental design).

For callus and multiple shoot induction in MS medium with different combinations of 6-benzylamino purine (BAP), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and Kinetin (Kin) were used with and without the anti-oxidants ascorbic acid and adenine sulfate. The media are labeled as V_1 to V_7 with varying media compositions as shown in **Table 1**.

Table 1 MS medium with different growth regulator combinations.

V_1	0.25 mgl^{-1} BAP + 0.25 mgl^{-1} kinetin + 50 mgl^{-1} ascorbic acid
V_2	0.5 mgl^{-1} BAP
V_3	1.0 mgl^{-1} BAP + 0.01 mgl^{-1} IAA
V_4	1.5 mgl^{-1} BAP + 0.25 mgl^{-1} IAA + 50 mgl^{-1} adenine sulfate
V_5	2.0 mgl^{-1} BAP
V_6	4.0 mgl^{-1} BAP
V_7	6.0 mgl^{-1} BAP + 0.5 mgl^{-1} IBA

Rooting and acclimatization

Eight-week-old *in vitro* shoots measuring 4–5 cm were transferred to rooting medium consisting of half-strength MS medium supplemented with 3% sucrose and 0.7% agar. Rooting was attempted with various concentrations of IBA, NAA and IAA ranging from 0.2 to 1.0 mgl^{-1}. The pH of the medium was adjusted to 5.7 prior to autoclaving. Percentage of rooting and number of roots per shoot were scored after 45 days. Rooted shoots were then washed in running tap water, dipped in 1% IBA and then transferred to thumb pots with soilrite (Khoday Biotech Ltd. Bangalore, India) and acclimatized in the greenhouse under high humidity. The plants were maintained in mist chamber for 30 days and later they were shifted to pots having sand: soil: FYM (1:1:1) with 100% survival.

All cultures were incubated in a culture room maintained at 25 ± 1°C under a 16/8 h photoperiod with a photosynthetic photon flux density of 70 µmol m^{-2} s^{-1} and were sub-cultured at 15 days interval.

Statistical analysis

The percent regeneration of roots or shoots and the number of shoots or roots per explant were determined for each culture bottle. The shoot length was measured in cm. Shoot organogenesis and *in vitro* rooting experiments were examined by one-way ANOVA followed by mean separation by the Student Newman-Keul's test (p=0.05). All analyses were performed using the SAS (1996) package. At least four replications were performed for shoot organogenesis and three for *in vitro* rooting.

RESULTS

Results of the present study of direct and indirect *P. rosea* regeneration from leaf and nodal explants are presented in **Tables 1-5** and **Figs. 1** and **2**. The reaction of explants (2 cm^2, juvenile, semi-mature and mature leaves; shoot apex and top two nodes) at different stages of growth grown in different PGR combinations is described in **Table 1**.

Multiple shoot induction (nodal explants) and regeneration

Cultures maintained in dark conditions (**Table 2**) did not produce multiple shoots in any PGR combination. Instead, they produced basal calli, maximum at 6.0 mgl^{-1} BAP and 0.5 mgl^{-1} IBA (V_7) followed by V_1 and V_5 at 45-50 days. These cultures were sub-cultured every 15 days on the same medium. Variations in the character of callus such as, callus coloration (**Table 2**) and amount of callus (**Table 3**) were also observed among treatments.

The calli obtained from these cultures were shifted to a 16-hr photoperiod. Within one month after the appearance of callus, adventitious shoots began to grow from the callus. The number of shoots ranged from 25-30. Shoot tip and nodal explants, when placed on V_7 medium, produced multiple shoots with a slight amount of basal callus by 15 days (**Fig. 1D, 1E**) whereas shoot explants placed on V_1 medium produced very stunted shoots; subsequently the amount of

Table 2 Response of explants to dark conditions.

Medium	Response		
	Callus character	Leaf bits	Nodes and shoot tips
V_1	Dark Green	L,S	Basal callus
V_2	-	-	-
V_3	White	-	-
V_4	Yellowish	L	-
V_5	-	-	-
V_6	Light green	L,S	Basal callus
V_7	Pale white	L,S	Basal callus

LS: leaf-like structures; S: shoot regeneration.

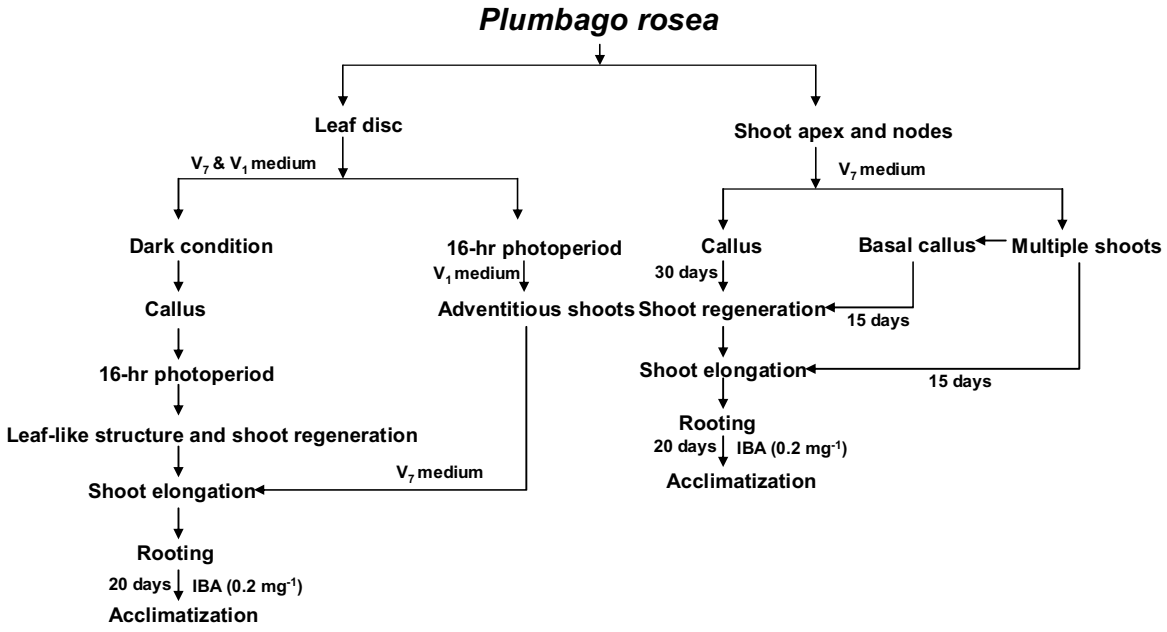

Fig. 2 Summary of *Plumbago rosea in vitro propagation* protocol.

Table 3 Response of shoot and node explants to 16-hr photoperiod.

Medium	Nodes and Shoot tips			
	Callus	Number of multiple shoots	Height of shoots (cm)	No. of nodes
V_1	++	10 ± 0.71 a	3.20 ± 0.16 b	5 ± 1.58 a
V_2	+	2 ± 0.71 e	0.45 ± 0.02 g	1 ± 0.71 c
V_3	+	2 ± 0.71 e	0.64 ± 0.01 f	1 ± 0.71 c
V_4	-	3 ± 1.00 de	0.90 ± 0.07 e	1 ± 0.71 c
V_5	++	4 ± 0.71 d	1.50 ± 0.16 d	2 ± 0.0 c
V_6	+	7 ± 1.58 bc	2.30 ± 0.12 c	3 ± 0.71 b
V_7	++++	8 ± 0.71 b	4.00 ± 0.16 a	5 ± 0.71 a
		CD = 1.20	CD = 0.15	CD = 1.10
		S.Em± = 0.41	S.Em± = 0.05	S.Em± = 0.37

Different letters in a column exhibit significant differences at P = 0.05 according to DMRT.

Table 4 Response of leaf explants to 16-hr photoperiod.

Medium	Leaf explant		
	Number of multiple shoots	Height of shoots (cm)	No. of nodes
V_1	12 ± 1.22 a	4.4 ± 0.22 a	4.2 ± 0.19 a
V_2	2 ± 0.0 d	0.6 ± 0.16 e	1.2 ± 0.07 d
V_3	2 ± 0.0 d	0.6 ± 0.14 e	1.2 ± 0.15 d
V_4	5 ± 0.71 c	1.0 ± 0.03 d	1.0 ± 0.14 d
V_5	5 ± 0.71 c	1.8 ± 0.16 c	2.0 ± 0.12 c
V_6	7 ± 1.73 b	2.8 ± 0.24 b	3.4 ± 0.28 b
V_7	8 ± 0.71 b	4.6 ± 0.28 a	2.0 ± 0.12 c
	CD = 1.40	CD = 0.29	CD = 0.24
	S.Em± = 0.47	S.Em± = 0.09	S.Em± = 0.08

Different letters in the column exhibit significant differences at P = 0.05 according to DMRT.

basal callus increased. Each culture produced at least 15 shoots by 60 days on V_7 medium (**Table 3**).

Throughout the experiment the formation of basal callus in nodal cultures was very evident, although it did not appear to hinder the growth of shoots.

Multiple shoot induction (leaf explants)

The response of leaf explants to dark conditions (**Table 2**) indicates that five out of the seven experiments in which PGR combinations and concentrations were screened were totally ineffective in forming callus. Leaf explants in the dark started to produce callus from cut ends and wounded regions (**Fig. 1B**). No callus formed on medium without PGR (the control) or in medium containing 0.5 mgl^{-1}, 2 mgl^{-1} BAP or in combination with 0.01 mgl^{-1} and 0.25 mgl^{-1} IAA. Callus was observed only in semi-mature leaf samples when inoculated on 0.25 mgl^{-1} BAP, 0.25 mgl^{-1} Kin and 0.5 mgl^{-1} ascorbic acid (V_1) and 6.0 mgl^{-1} BAP with 0.5 mgl^{-1} IBA (V_7) medium and maintained in complete darkness for a period of 60 days (**Table 2**). The callus at this stage appeared embryogenic as it was friable and nodular. When these cultures were sub-cultured on the same medium and maintained under a 16-hr photoperiod, leaf-like structures and shoot buds were produced. Variations in callus character were also observed among treatments (**Table 2**).

The number of shoot buds ranged from 18-20 in V_7 and 10 shoots were recorded in V_1. Other media combinations did not give significant results except for V_4 where whitish callus was formed without further differentiation. V_6 also successfully produced light green calli followed by leaf-like structures and later formed shoot buds (7) which were very much less when compared to V_7 (data not shown).

The response of leaf explants to the 16-hr photoperiod is depicted in **Table 4**. The leaf explants placed on MS medium with different PGR combinations produced adventitious shoots directly without callus. V_1 and V_7 produced a maximum of 12 and 8 shoots per leaf explant from cut ends and wounded areas (**Fig. 1C**), 4.36 and 4.6 cm, respectively. The number of nodes ranged from 3 to 4. Medium containing 4.0 mgl^{-1} BAP could induce only 7 shoot buds which attained 2.8 cm in height, while BAP at 0.5 mgl^{-1} and 1.0 mgl^{-1} with IAA produced fewest (2) and highly stunted (0.6 cm) shoots. However, these shoots were allowed to grow until they reached 3-4 cm in length, were excised and sub-cultured on V_7 medium. No response was observed in basal medium without any PGRs (**Table 4**).

In vitro rooting and acclimatization

Shoots were excised and placed on V_7 medium to attained optimum height (3.2 cm). Regenerated leafy shoots derived from callus cultures and nodal cultures that were transferred to half-strength MS media without PGRs did not induce roots. Roots emerged on excised shoots grown on half-strength MS media containing IAA, IBA or NAA (**Fig. 1F**). Rooting began within 10 days and developed into a well-developed rooting system within 20 days of culture. A high percentage of shoots (90%) rooted on medium containing half-strength MS basal salts with 0.2 mgl^{-1} IBA (**Table 5**).

Table 5 Effect of growth regulators on rooting.

½ MS			Percentage of	No. of roots	Days taken
IBA	IAA	NAA	shoots rooted		for rooting
0.0	0.0	0.0	0 ± 0.00 m	0 (0.71) k	15
0.1	0.0	0.0	30 ± 1.58 i	7 (2.74) e	12
0.2	0.0	0.0	90 ± 3.81 a	15 (3.94) a	10
0.4	0.0	0.0	21 ± 3.08 j	12 (3.54) b	11
0.6	0.0	0.0	11 ± 2.55 l	12 (3.54) b	15
0.8	0.0	0.0	17 ± 1.73 k	10 (3.24) c	15
1.0	0.0	0.0	38 ± 1.58 h	9 (3.08) d	18
0.0	0.1	0.0	0 ± 0.00 m	0 (0.71) k	15
0.0	0.2	0.0	59 ± 4.06 e	2 (1.58) i	16
0.0	0.4	0.0	48 ± 2.55 g	5 (2.35) f	16
0.0	0.6	0.0	39 ± 2.55 h	1 (1.22) j	11
0.0	0.8	0.0	50 ± 3.00 f	1 (1.22) j	15
0.0	1.0	0.0	20 ± 2.12 j	2 (1.58) i	10
0.0	0.0	0.1	0 ± 0.00 m	0 (0.71) k	15
0.0	0.0	0.2	67 ± 1.41 c	1 (1.22) j	18
0.0	0.0	0.4	72 ± 2.74 b	2 (1.58) i	16
0.0	0.0	0.6	62 ± 1.87 d	2 (1.58) i	16
0.0	0.0	0.8	59 ± 1.87 e	3 (1.87) h	19
0.0	0.0	1.0	48 ± 1.87 g	4 (2.12) g	18
			CD=1.13	CD = 0.28	
			S.Em ± =0.3	S.Em ± = 0.1	

Figures in parenthesis indicate transformed values.
Different letters in the column exhibit significant differences at P = 0.05 according to DMRT.

Rooted plantlets established well in soilrite (**Fig. 1G, 1H**).

DISCUSSION

Since *P. rosea* is the major contributor for plumbagin extraction, mass multiplication to meet demand is required. The type, concentration, and combination of PGRs are key factors influencing direct and indirect shoot organogenesis in *Plumbago* spp. V1 medium produced the highest number of shoots compared to all other tested MS combinations. Even though V7 medium produced the highest amount of callus in all three types of explants i.e. nodes, shoot tips and leaf, it could not produce as many shoots as in V_1 medium. Similar PGR effects on callus formation have been reported in other *Plumbago* species (Satheesh Kumar and Bhavanandan 1988; Rout 1999; Chetia and Handique 2000; Saxena *et al*. 2000; Rout *et al.* 2002) as shown in **Table 6** and in other genera (Khanam *et al.* 2000; Reddy *et al.* 2001; Ma and Xu 2002; Giridhar *et al.* 2004; Azad *et al.* 2005; Datta and Majumder 2005; Zhou and Brown 2005). Differential responses of genotypes and explant sources to PGR requirements are well documented (Das and Rout 2002; Satheesh Kumar and Seeni 2003). *P. rosea* shoots regenerated *in vitro* proved to be easily adaptable to *ex vitro* plant establishment with 90% survival. The *ex vitro* rooted plantlets did not require any additional acclimatization prior to transplanting to regular greenhouse conditions (Reed 1995; Gavidia *et al.* 1996; Pruski *et al.* 2000). The ability to regenerate shoots from calli has several advantages. A great number of shoots can be produced from an explant through callus induction and shoot formation (Shen *et al.* 2007).

There are not many reports on *in vitro* multiplication of *P. rosea* except for that of Satheesh Kumar and Bhavanandan (1988), which de-emphasized the importance of photoperiod; they also reported the formation of very few shoots. Satheesh Kumar and Seeni (2003) showed that a combination of 3 mgl^{-1} BA and 1.5 mgl^{-1} NAA could give nodular calli which on subsequent sub cultures produce 23-73 multiple shoots within 23 weeks.

Basal calli produced multiple shoots (20-23) (data not shown) when retained on the same medium. As examined by Rout (1999) basal callus started appearing with the increasing concentration of growth regulators (4.44-6.66 µM BA and 1.42 µM IAA) in *P. zeylanica* and there after (8.88 µM BA/9.28 µM Kinetin with 5.71 µM IAA) the plants became stunted and no observable changes were found in plants growth. A similar trend was observed in the present study (**Table 3**).

This study demonstrates high-frequency shoot regeneration for commercially important cultivars such as *P. rosea*. The method is flexible, allowing incorporation of different explant types (shoot tips, nodes and leaves) with BAP and NAA effective in both callus and multiple shoot induction. *In vitro* shoots can be rooted easily *in vitro* using IBA with 100% survival. Similar but a less efficient observation in terms of number of multiple shoots, number and frequency of rooting and most importantly transplantation success was made by Rout (2002) with V_4 media composition.

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Table 6 Comparative data of PGR combinations and response from earlier studies in *Plumbago* spp.

Reference	Media composition	Response and time taken	% Survival
Satheesh Kumar and	Callus - 2.5 mgl^{-1} BA and 1.5 mgl^{-1} NAA	Nodular callus 4 weeks	95-98%
Seeni 2003	Shooting - 2-3 mgl^{-1} BA	23-76 shoots in 18-23 weeks	
P. rosea	Rooting - ½ MS+0.1 mgl^{-1} IBA	4 weeks	
Rout 2002	Shooting - 0.5 mgl^{-1} BAP, 0.25 mgl^{-1} IAA, 50 mgl^{-1} adenine sulfate	23-45 shoots in 4 weeks	84-92%
P. rosea and *P. zeylanica*	Rooting - ½ MS + 0.25 mgl^{-1} IBA	4 weeks	
Das and Rout 2002	Callus - 0.25 mgl^{-1} kinetin and 2.0 mgl^{-1} 1-naphthaleneacetic acid (NAA)	Somatic embryos	28%
P. rosea	Shooting - 1.0 mgl^{-1} Kn, 0.5 mgl^{-1} GA_3 and 0.1 mgl^{-1} NAA	50-60 plantlets in 4 weeks	
	Rooting - ½ MS+0.01 – 0.25 mgl^{-1} Kn	4 weeks	
Rout *et al* .1999	Callus - 2.32µMKinetin,10.74µM NAA	Compact green calli	90%
P. zeylanica	Shooting - 4.44µM BA, 1.42µM IAA	26-42 shoots in 4 weeks	
	Rooting - 0.57µM IAA	2 weeks	
Satheesh Kumar and	Callus - 2,4-D (2.5 mgl^{-1}) and kinetin (1.5 mgl^{-1})	Compact green calli	60%
Bhavanandan 1988	Shooting – 2 mgl^{-1} BA+1 mgl^{-1} NAA	No information on number of shoots	
P. rosea	Rooting - MS+1.5 mgl^{-1} IBA	2 weeks	
Present study	Callus - 6.0 mgl^{-1} BAP and 0.5 mgl^{-1} IBA	Nodular and friable green calli	100%
	Shooting - 0.25 mgl^{-1} BAP+0.25 mgl^{-1} Kinetin+50 mgl^{-1} Ascorbic acid	25-30 shoots 4 weeks	
	Rooting - ½ MS+0.2 mgl^{-1} IBA	2 weeks	

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