Plant Tissue Cult. & Biotech. **19**(1): 79-87, 2009 (June)

PTC&B

# Plant Regeneration from Leaf Explants of *Plumbago rosea* L.

**M. Gopalakrishnan, B. Janarthananm, G. Lakshmi Sai and T. Sekar***

*Plant Tissue culture Laboratory, P.G. & Research Department of Botany, Pachaiyappa's College, University of Madras, Chennai-600 030, Tamil Nadu, India*

*Key words: In vitro* culture, Leaf explants, Mass propagation, *Plumbago rosea*

## Abstract

Leaf explants of *Plumbago rosea* L. an important medicinal plant inoculated on MS supplemented with 6.66 μM BAP and 2.69 μM NAA produced numerous (105 ± 0.3) shootlets with an average length of 3.1 ± 0.0 cm. Small shootlets were transferred to shoot elongation medium supplemented with 1.11 μM BAP plus 1.44 μM $GA_3$. The elongated shootlets transferred to half strength MS basal medium without any plant growth regulator produced 4.3 ± 0.2 rootlets per plants with average root length of 4.0 ± 0.0 cm after 25 days of culture. Rooted plantlets were transferred for hardening showed 80 - 90 per cent of plantlets success-fully established in the field. Potentially more than 50,000 plantlets could be produced within five subcultures from without callus phase obtained from leaf explant. Maximum root differentiation from leaf explants was obtained on MS supplemented with 5.38 μM IBA. The roots developed by the above method is an alternative for the controlled production of secondary metabolites.

## Introduction

*Plumbago rosea* L. a valuable medicinal plant belongs to Plumbaginaceace (Chetia and Handique 2000) distributed in the tropical regions of India (The Wealth of India 1989) and is used in the treatment of dyspepsia, piles, diarrhoea and skin disease (Kritikar and Basu 1975). The root contains an alkaloid - plumbagin, a natural naphthaquinone, possessing various pharmacological activities such as antimalarial, antimicrobial (Didry et al. 1994) and used as anticancer drugs (Krishnaswamy and Purushottaman 1980, Jayaraman 1987). Earlier *in vitro* propagation of *Plumbago rosea* was attempted through organogenesis from different explants such as leaves, shoot tips, axillary buds and callus (Rout 2002, Satheesh Kumar and Seeni 2003, Satheesh Kumar and Bhavanandan 1988) and somatic embryo (Das and Rout 2002). The present study was aimed at

*Corresponding author: < tsekar_2005@yahoo.com>.

developing a simple, rapid and an efficient protocol of plant regeneration from leaf explants of *Plumbago rosea.* In order to meet the demand of this species an *in vitro* system has been investigated to generate roots in bulk, which is known to accumulate more secondary metabolites.

## Materials and Methods

The healthy plants of *Plumbago rosea* (Syn. *P. indica*) were collected from Krishi Vijanan Kendra, Thelliyoor, Kerala and raised in pots containing soil and farm yard manure (1 : 1) in greenhouse in the Department of Botany, Pachaiyappa's College, Chennai-600 030, India. Leaf explants were surface sterilized by cleaning thoroughly under running tap water and washed with a solution of Tween 20 (Two drops in 100 ml water) for 5 min, and again washed with sterile distilled water. The cleaned explants were finally treated with $HgCl_2$ (0.1%) for 3 min under aseptic conditions and washed six times with sterile distilled water to remove traces of $HgCl_2$.

After surface sterilization, leaf explants were trimmed and inoculated on MS basal medium supplemented with different concentrations and combinations of BA (0.56 - 22.20 μM) and NAA (0.54 - 5.36 μM) for shoot induction. For shoot elongation, small shoots were transferred to MS basal medium supplemented with BA (1.11 - 4.44 μM) + $GA_3$ (0.29 - 2.89 μM).

After 25 days, the fully developed shootlets were transferred to half strength MS basal medium without plant growth regulators for root induction. The cultures containing the rooted plantlets were transferred to the greenhouse at 60 - 70 per cent humidity. For root differentiation from leaf explants, after surface sterilization the leaf explants were transferred to MS supplemented with IBA (0.49 - 4.92 μM), NAA (0.5 - 5.37 μM) and IAA (0.57 - 5. 71 μM). The number of roots and their length were measured after 25 days of culture. The hardened plantlets were fertigated with dilute MS basal media. After 15 days the fully acclimatized plantlets were transferred to pots (6 cm diam) containing red soil, vermiculite and farmyard manure in 1 : 1 : 1 ratio. Established plantlets were then transferred to bigger pots (14 cm diam).

For all the above studies, MS basal medium containing 3% (w/v) sucrose was used. All the plant growth regulators were filter-sterilized using 0.2 μm filter (Minisart®, Sartorius) prior to addition to culture medium. The media were adjusted to pH 5.6 ± 0.2, and were solidified with 0.9% (w/v) agar (Hi media) and autoclaved at 121°C for 15 min under 1.16 kg/$cm^2$ pressure and transferred to culture room. Cultures were maintained at 25 ± 2°C under 16/8 light and dark cycle, 45 μ mol/$m^2$/5 irradiance level provided by white cool fluorescent tubes with 55 - 60% relative humidity. Each experiment was repeated three times and

each treatment had six replicates. The data were subjected to ANOVA and means were performed by the DMRT using SPSS (SPSS ver. 16.0).

## Results and Discussion

Leaf explants were inoculated on MS supplemented with different concentration and combinations of BA (1.11 - 8.88 μM) and NAA (0.54 - 5.36 μM) showed differential response according to the hormonal concentration used (Table 1). In the present study, BA along with NAA seems to influence the induction of shoots in *Plumbago rosea.* Leaf segments transferred to MS containing BA alone (1.11 - 8.88 μM) grew well and also developed micro shoot (Fig. 1A). BA 4.44 μM produced three - four small shoots and an average length of 2.3 cm after 35 days, whereas the higher concentration of BA (8.88 μM) produced dark green callus and compact structures which ultimately turned brown and failed to develop into normal shoots. BA has been considered to be one of the most active cytokinins for organogenic differentiation in plant tissue culture (Gupta et al. 1996, Gururaj et al. 2007, Echeverrigaray and Fracaro 2004, Baskaran and Jayabalan 2005).

The combined effect of BA and IAA in varying concentrations was studied for shoot multiplication of *Plumbago rosea.* Leaf explants were inoculated on MS supplemented with BA (1.11 - 8.88 μM) and NAA (0.54 - 5.36 μM). The leaf explants transferred on MS basal media containing 6.66 μM BA and 2.69 μM NAA showed better growth response and produced 105 ± 0.3 shoots with an average length of 3.1 ± 0.3 cm after 35 days of culture (Fig. 1B,C). At lower concentration BA produced less number of shoots, whereas higher concentration of BA explants with NAA produced green callus with small shoots, when exposed to other concentrations of BA along with NAA the callus swelled, turned brown and failed to initiate shoot. In the present study the development of shoots from leaf explants and the use of 6.66 μM BA and 2.69 μM NAA produced maximum number of shoots 105 ± 0.3 in first subculture (Fig. 1D).

However, an earlier report available on the production of shoot buds from semi-mature leaf of *P. rosea* on MS supplemented with BA, IAA with 50 mg/l of AdS showed the development of maximum shoots (Rout 2002). This may be due to the use of already differentiated leaf segment as explant for *in vitro* regeneration. On the other hand, the use of BA and NAA for shoot development from leaf observed in the present study is in agreement with earlier reports on organogenesis of dicot plants (Echeverrigaray et al. 2004, Prajapati et al. 2003, Alagumanian et al. 2004, Shahanaz et al. 2007). This synergistic combination of auxin and cytokinin on organogenic differentiation has been well explained in several systems (Pereira et al. 2000, Pretto and Santarem 2000, Xie and Hong 2001).

**Table 1. Effect of different concentrations of BA and NAA on MS on *in vitro* shoot multiplication from leaf explant of *Plumbago rosea*.**

| Plant growth regulator (μM) | | Shoot induction (%) | Number of shoot per explant | Shoot length (cm) |
|---|---|---|---|---|
| BA | NAA | | | |
| 0.44 | | - | - | - |
| 2.22 | | - | - | - |
| 4.44 | | 20.0 ± 0.0$^{h}$ | 2.3 ± 0.53$^{j}$ | 1.1 ± 0.5$^{e}$ |
| 8.88 | | 50.2 ± 5.3$^{de}$ | 3.3 ± 0.20$^{i}$ | 1.3 ± 0.1$^{e}$ |
| 11.10 | | 38.3 ± 2.8$^{g}$ | 2.6 ± 0.17$^{j}$ | 0.9 ± 0.5$^{e}$ |
| | 0.54 | 10.0 ± 0.0$^{i}$ | 1.6 ± 2.0$^{k}$ | 1.3 ± 0.25$^{e}$ |
| | 2.69 | 10.0 ± 0.0$^{i}$ | 2.0 ± 0.5$^{jk}$ | 1.2 ± 0.45$^{e}$ |
| | 5.38 | - | - | - |
| | 8.07 | - | - | - |
| 4.44 | 0.54 | 40.0 ± 0.0$^{fg}$ | 5.0 ± 0.50$^{h}$ | 1.8 ± 0.1$^{d}$ |
| | 2.69 | 48.0 ± 5.7$^{e}$ | 7.0 ± 0.20$^{f}$ | 2.3 ± 0.2$^{bcd}$ |
| | 5.38 | 40.0 ± 0.0$^{fg}$ | 6.3 ± 0.10$^{g}$ | 2.1 ± 0.1$^{cd}$ |
| | 8.07 | 35.0 ± 8.7$^{g}$ | 5.3 ± 0.60$^{h}$ | 1.9 ± 0.5$^{d}$ |
| 6.66 | 0.54 | 45.0 ± 0.0$^{ef}$ | 16.0 ± 0.4$^{c}$ | 2.3 ± 0.25$^{bcd}$ |
| | 2.69 | 80.0 ± 6.0$^{a}$ | 105.0 ± 0.3$^{a}$ | 3.1 ± 0.30$^{a}$ |
| | 5.38 | 60.0 ± 5.0$^{c}$ | 21.6 ±0.1$^{b}$ | 2.0 ± 0.1$^{d}$ |
| | 8.07 | 40.0 ± 5.0$^{fg}$ | 12.0 ± 0.1$^{e}$ | 2.0 ± 0.12$^{d}$ |
| 8.88 | 0.54 | 48.5± 5.1$^{e}$ | 7.0 ± 0.20$^{f}$ | 2.6 ± 0.10$^{b}$ |
| | 2.69 | 56.7± 6.9$^{c}$ | 13.3 ± 0.20$^{d}$ | 2.5 ± 0.20$^{bc}$ |
| | 5.38 | 71.6 ± 2.9$^{b}$ | 16.3 ± 0.14$^{c}$ | 2.3 ± 0.10$^{bcd}$ |
| | 8.07 | 45.0 ± 8.7$^{ef}$ | 12.3 ± 0.14$^{e}$ | 1.9 ± 0.12$^{d}$ |
| 11.10 | 0.54 | 40.0± 5.0$^{fg}$ | 5.3 ± 0.60$^{h}$ | 1.9 ± 0.50$^{d}$ |
| | 2.69 | 55.0 ± 6.0$^{cd}$ | 6.3 ± 0.60$^{g}$ | 2.6 ± 0.10$^{b}$ |
| | 5.38 | 41.6 ± 5.7$^{fg}$ | 6.0 ± 0.20$^{g}$ | 2.2 ± 0.20$^{bcd}$ |
| | 8.07 | 35.0 ± 5.0$^{g}$ | 6.0 ± 0.10$^{g}$ | 2.2 ± 0.10$^{bcd}$ |

Explants were cultured on MS basal media supplemented with BAP and NAA. Data were recorded after 35 days of culture. Results represent mean ± SD of six replicated experiments. Values denoted by different letters differ significantly at $p < 0.05$.

The microshoots were transferred to MS (elongation) supplemented with BA (1.11 - 4.44 μM) and $GA_3$ (0.29 - 2.89 μM). The microshoots transferred on elongation media containing 2.22 μM BA and 0.5 mg/l $GA_3$ showed better growth response and shoot elongation with an average length of 2.0 ± 0.0 cm after 25 days culture (Table 2, Fig. 1E,F). Similar results are reported by Golegaonkar and Kantharajah (2006) on microshoot elongation from leaf explants of *Capsicúm*

*annuum* on MS supplemented with 4.44 μM BA + 2.9 μM $GA_3$ was optimum for shoot elongation.

**Table 2. Effect of different concentrations of BA and $GA_3$ on MS on shoot elongation from *in vitro* microshoot.**

| Plant growth regulator (μM) | | Inoculated micro-shoot (3.1 cm) | Shoot elongation (%) | Shoot length (cm) |
|---|---|---|---|---|
| BA | GA | | | |
| 0.56 | 0.29 | | - | - |
| | 0.72 | | - | - |
| | 1.44 | | - | - |
| | 2.89 | | - | - |
| 1.11 | 0.29 | | $21.7 \pm 2.9^{c}$ | $0.5 \pm 0.2^{c}$ |
| | 0.72 | | $33.3 \pm 7.6^{b}$ | $0.8 \pm 0.3^{b}$ |
| | 1.44 | | $65.0 \pm 5.0^{a}$ | $2.0 \pm 0.0^{a}$ |
| | 2.89 | | $15.0 \pm 0.0^{de}$ | $0.9 \pm 0.2^{b}$ |
| 2.22 | 0.29 | | $20.0 \pm 0.0^{cd}$ | $1.0 \pm 0.0^{b}$ |
| | 0.72 | | $21.7 \pm 5.7^{c}$ | $1.0 \pm 0.0^{b}$ |
| | 1.44 | | $15.0 \pm 5.0^{de}$ | $0.5 \pm 0.3^{c}$ |
| | 2.89 | | $10.0 \pm 0.0^{e}$ | $0.5 \pm 0.2^{c}$ |
| 4.44 | 0.29 | | - | - |
| | 0.72 | | - | - |
| | 1.44 | | - | - |
| | 2.89 | | | - |

Data were recorded after 25 days of culture. Results represent mean ± SD of six replicated experiments. Values denoted by different letters differ significantly at $p < 0.05$.

Individual shoots from a multiple shoot complex originated from the leaf explant separated after 25 days of shootlets were transferred to half strength MS without plant growth regulator. The response of root initiation and proliferation was found to be higher. The number of roots was 4.3 ± 0.2 with 80% response and average root length of 5.1 ± 0.0 cm after 30 days (Table 3, Fig. 1G). In all the media the first root appeared after 15 days of culture and the root system was well developed. In the present study rooting was achieved in media without plant growth regulators, whereas the earlier reports (Rout 2002, Satheeshkumar and Seeni 2003, Satheesh Kumar and Bhavanandan 1988) showed the root induction at higher concentrations of IBA. The observation on the reduction of MS salts strength to half enhances the rooting frequency which is in confirmity with earlier finding of (Paridas and Thirunavoukkarasu 2005, Pawar et al. 2002, Laskar 2005).

More than 90 per cent of plantlets survival was observed on hardening in red soil, vermiculite and farmyard manure (1 : 1 : 1) for one week. However, the rate of survival decreased to 10 per cent after two - three weeks of acclimatization. It was observed that gradual acclimatization of *in vitro* grown plants to external environ-ment is most essential for *P. rosea.* More than 80 per cent of the plants transferred to pots survived and resumed growth (Fig.1H).

**Table 3. Effect of IBA, IAA and NAA on root differentiation from leaf explants.**

| Plant growth regulator (μM) | Root differentiation (%) | Fresh weight (g/l) | Dry weight (g/l) |
|---|---|---|---|
| IBA | | | |
| 0.49 | - | - | - |
| 0.98 | 35.0 ± 0.0$^{c}$ | 3.8 ± 0.03$^{d}$ | 0.7 ± 0.0$^{d}$ |
| 2.46 | 80.0 ± 5.0$^{a}$ | 27.4 ± 0.02$^{b}$ | 4.3 ± 0.0$^{a}$ |
| 4.90 | 41.6 ± 5.7$^{bc}$ | 4.3 ± 0.03$^{c}$ | 0.8 ± 0.0$^{c}$ |
| IAA | | | |
| 0.57 | - | - | - |
| 1.14 | - | - | - |
| 2.85 | 45.0 ± 8.7$^{b}$ | 28.1 ± 0.01$^{a}$ | 3.8 ± 0.0$^{b}$ |
| 5.71 | 21.7 ± 5.7$^{d}$ | 3.4 ± 0.02$^{e}$ | 0.2 ± 0.0$^{e}$ |
| NAA | | | |
| 0.54 | - | - | - |
| 1.07 | - | - | - |
| 2.69 | - | - | - |
| 5.37 | - | - | - |

Data were recorded after 25 days of culture. Results represent mean ± SD of six replicated experiments. Values denoted by different letters differ significantly at $p < 0.05$.

The leaf explants were transferred to MS supplemented with IBA (0.49 - 4.92 μM), NAA (0.54 - 5.37 μM) and IAA (0.57 - 5. 71 μM) for root differentiation. The maximum root differentiation from leaf explants was achieved on MS supplemented with 2.46 μM IBA. The fresh weight of differentiated roots and their development was maximum with 2.85 μM IAA (28.1 g/l) followed by 2.46 μM IBA (27.4 g/l) after 25 day of cultures, whereas dry weight of differentiated root was maximum in IBA (4.3 g/l) than IAA (3.8 g/l) (Table 3, Fig. 1 I-L). However, an earlier report available on the root differentiation of *Rauwolfia serpentina* from leaf, node and stem cuttings of derived calli was obtained on MS supplemented with NAA (2.0 mg/l) and BAP (1.5 mg/l) (Sehrawat et al. 2002). Another report indicates the formation of root differentiation from *Decalepis*

*arayalpathra* in MS containing 0.2 mg/l IBA and 0.1 mg/l NAA (Sudha and Seeni 2001) to support the present findings.

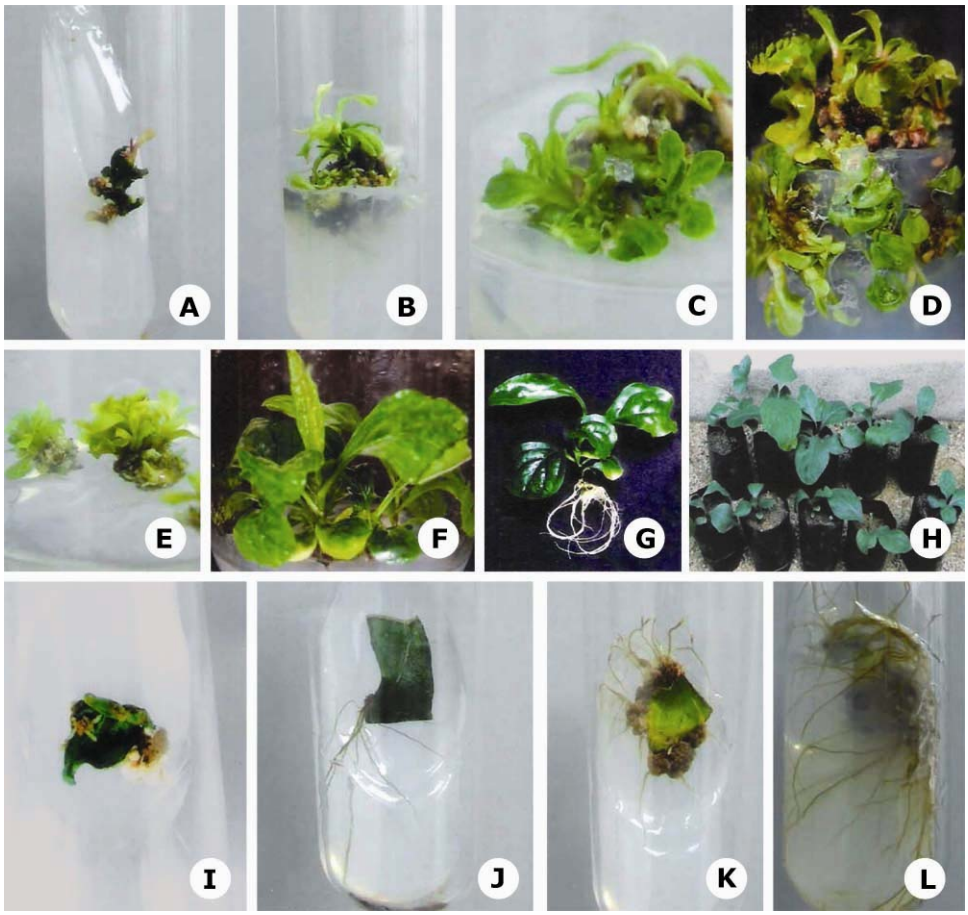


Fig. 1. *In vitro* regeneration of *Plumbaga rosea*. A. Initiation of shoot from leaf explants. B. Initiation of multiple shoots from leaf explants. C,D. Multiple shoot proliferation. E. Shoot elongation. F. Well developed shoots. G. A well developed shoot with rootlets. H. Hardened and acclimatized plant. I. Root initiation from leaf explants. J,K. Root development from the leaf explants. L. Multiple root proliferation.

The results showed the ability of the leaf explants to produce higher number of shootlets without intervening callus phase, where all the plantlets were uniform in height and growth. This study establishes a simple, rapid, high frequency micropropagation method for *P. rosea.* from leaf explants and also this present system of normal root culture would be beneficial for the sustainable utilization of this rare endemic medicinal plant for its bioactive ingredients, thereby providing an alternative method rather than destroying whole plants that are not under cultivation.

## Acknowledgement

The authors thank the Principal and Dr. C.R. Bojan, Head of the Department of Botany for providing facilities and encouragement for this work. They gratefully acknowledge the National Medical Plants Board, Department of AYUSH, Ministry of Health and Family Welfare, Government of India for financial support.

## Reference

**Alagumanian S, Saravana PV, Balachandar R, Rameshkannan K** and **Rao MV** (2004) Plant regeneration from leaf and stem explants of *Solanum trilobatum*. Curr. Sci. **86**: 1478 -1480.

**The wealth of India** (1989) A Dictionary of India Raw materials and Industrial Products. Publication and information Directorate, CSIR, New Delhi, Vol. **III:** 163-165.

**Baskaran P** and **Jayabalan N** (2005) An efficient micropropagation system for *Eclipta alba* - a valuable medicinal herb. In Vitro Cellular and Developmental Biology - Plant. **41:** 532-539.

**Chetia S** and **Handique PJ** (2000) High frequency *in vitro* shoots multiplication of *Plumbago indica,* a rare medicinal plant. Curr. Sci. **78**: 1187-1188.

**Das G** and **Rout CR** (2002) Plant regeneration through somatic embryogenesis in leaf derived callus of *Plumbago rosea.* Biolgi. Planta. 299-302.

**Didry N, Dubrevil L** and **Pinkas, M** (1994) Activity of anthraquinonic and naphthoquinonic compounds on oral bacteria, Die Pharmazi. **49**: 681-683.

**Echeverrigaray S, Fracaro F, Andrade LB, Biasio S** and **Atti-Serafini L** (2004) *In vitro* shoot regeneration from leaf explants of *Matricaria chamomile*. Plant Cell Tiss. Org Cult. **60**(1): 1-4.

**Golegaonkar PG** and **Kantharajah AS** (2006) High-frequency adventitious shoot bud induction and shoot elongation of Chile pepper (*Capsicúm annuum* L.). Biomedical and Life Sci. **42**(4): 341-344.

**Gupta N, Jain SK** and **Srivastava** (1996) *In vitro* Micropropagation of multipurpose leguminous tree *Delonix regia*. Phytomorphology **46**: 267-275.

**Gururaj HB, Giridhar P** and **Ravishankar GA** (2007) Micropropagation of *Tinospora cordifolia* (wild) Miers ex Hook. F & Thoms - a multipurpose medicinal plant. Curr. Sci. **92**: 23-26.

**Jayaraman KS** (1987) India seeks scientific basis of traditional remedies. Nature. **326**: 323.

**Kirtikar KR** and **Basu BD** (1975) Indian Medicinal Plants (Eds). Blatter, B. Caius, J.F. and Mhaskar, K.S. M/s Bishensingh Mahendra Pal Singh, Dehra Dun. **2**: 1469.

**Krishnaswamy M** and **Purushottamam KK** (1980) Plumbagin, a study of its anticancer, antibacterial and antifungal properties. Ind. J. Exp. Biol. **18**: 876-877.

**Lasker MA, Lyngdo HJP, Buam JJ** and **Syiem D** (2005) Plantlet regeneration via adventitious shoot bud proliferation from leaf explants in *Potentilla fulgens*. Indian J. Biotechnol. **4**: 257-260.

**Paridas AB** and **Thirunavoukkarasu M** (2005) Regeneration of plantlets from leaf and internode explants of *Pogostemon cablin*. J. Trop. Med. Plants **6**: 253-257.

**Pawar PK, Pawar CS, Narkhede BA, Teli NP, Bhalsing SR** and **Maheswari VL** (2002) A technique for rapid micropropagation of *Solanum surattense* Burn. F. I. J. Biotech. **1**: 201-204.

**Pereira AM, Bertoni BW, Appezzato-da-glória B, Araujo, ARB, Januário AH, Lourenco, M.V** and **Franca SC** (2000) Micropropagation of *Pothomorphe umbellate* via direct organogenesis from leaf explants. Plant Cell Tiss. Org. Cult. **60**: 47-53.

**Prajapati HA, Patel D, Mehta SR,** and **Subramanian RB** (2003) *In vitro* regeneration of *Curculigo orchioides* Gaertn., an endangered anticarcinogenic herb. Curr. Sci. **84**: 747 -749.

**Pretto FR** and **Santarém ER** (2000) Callus formation and plant Regeneration from *Hypericum perforatum* leaves. Plant Cell Tiss. Org. Cult. **62**: 107-113.

**Rout GR** (2002) Direct plant regeneration from leaf explants of *Plumbago* species and its genetic fidelity through RAPD markers. Ann. Appl. Biol. **104**: 305-313.

**Satheesh Kumar K** and **Seeni S** (2003) *In vitro* mass multiplication and production of roots in *Plumbago rosea*. Plant Med. **69**: 83-86.

**Satheesh Kumar K** and **Bhavanandan KV** (1988) Micropropagation of *Plumbago rosea* Linn. Plant Cell Tiss. Org. Cult. **15**: 275-278.

**Sehrawat AR, Sanjogta U** and **Chodhury JB** (2002) Establishment of plantlets and evaluation of differentiated roots for alkaloids in *Rauwolfia serpentine*. J. Plant Biochemist. and Biotechnol. **11**: 105-108.

**Shahanaz BA, Kottackal PM**, **Chun-Lai Z**, **Nishitha IK, Ligimol AS** and **Madhusoodanan PV** (2007) Organogenesis from leaf and internode explants of *Ophiorrhiza prostrata,* an anticancer drug (camptothecin) producing plant. Curr. Sci. **10:** 15.

**Sudha CG** and **Seeni S** (2001) Establishment and analysis of Fast - growing normal root culture of *Decalepis arayalpathra*, a rare endemic plant, Curr. Sci. **81**(4): 371-374.

**Xie DY** and **Hong Y** (2001) Regeneration of *Acacia mangium* through somatic embryogenesis. Plant Cell Report **20**: 34-40.