A study on *Ex-situ* conservation of endemic and rare medicinal plant; *Plumbago rosea* L. through *in-vitro* propagation.

Dr. Ashok Paltati, Naveena Durga T, Prof. Ratna Kumar P. K,
Research scholar, Student, Professor
Center for Advanced studies, Department of Botany,
Andhra University, Visakhapatnam, Andhra Pradesh, India-530 003.

ABSTRACT

Plumbago rosea L. a critically endangered medicinal plant in Andhra Pradesh. An improved protocol for *in-vitro* direct organogenesis of *Plumbago rosea* L. from nodal segments was developed. Nodal segments of field grown *Plumbago rosea* L. plant were cultured on CleriGel solidified MS media supplemented with different concentrations individually and in combinations of 6BA, KN, GA_3 and NAA for multiple shooting and IAA, and IBA for root proliferation of cultured shoots. The nodal segments produced number of multiple shoots depending up on nature and combination of plant growth regulators (PGR). The elongated shoot were excised and rooted on rooting media and were acclimatized in field with 90% survivability.

Key words: Direct organogenesis, synergistic effect, PGR's, *Plumbago rosea* L.

Abbreviations: PGR (plant growth regulators), 6BA (6 Benzyladenine), KN (Kinetin), GA_3 (Gibberellic Acid 3), IAA (Indole 3 acetic acid), IBA (Indole 3 butyric acid), NAA (Naphthalene acetic acid),

INTRODUCTION

Medicinal plants are important source of traditional and synthetic medicines containing different types of organic compounds having therapeutic properties. Approximately 80% of people in developing countries still rely on traditional medicines for their primary health care. This usually involves the use of plant extracts (Vieira and Skorupa 1993). Many medicinal plant species are disappearing at an alarming rate, as a result of rapid agricultural and urban development, deforestation and indiscriminate collection and usage of these plants. The tissue culture technique has been proved very efficient in rapid mass propagation and conservation of these rare and endangered medicinal plants.(Fay 1992, Sagore et al. 2000, Lakshmi and Mythili 2003).

Plumbago rosea L. (synonym is *Plumbago indica* L.) which belongs to the family Plumbaginaceae, this family constitute other *Plumbago* species like *Plumbago zeylanica, P. europaea, P. capensis and P. auriculata*. It is vernacularly called as Laal chitrak, Yeera chitramoolam, laal cheeta etc, and in English it is called as Rose coloured leadwort plant. *P. rosea* L. is an important rare medicinal herb distributed in Southeast Asia and sparsely in some parts of India, especially in southern part of India, like Kerala and Andhra Pradesh. Andhra Pradesh accommodates more than 1% of this plant population, while the status of *P. rosea* L. in Andhra

Pradesh is under endangered condition given by CAMP - IUCN (K.N. Reddy and C. Sudhkar Reddy 2008). *P. rosea* L. is mostly used for its tuberous roots, root bark and milky juice of whole plant by tribal people, as it contain two important alkaloids, napthaquinone and plumbagin (Ghani 1998). These alkaloids have antibacterial, antifungal, antimalarial, cardiotonic, antimutagenic and insecticidal activities. In Ayurveda these roots are used in preparations for the treatment of leucoderma, secondary syphilis, edema, piles, intestinal worms, rheumatism and skin diseases etc. This species has being rare in India and become endangered in some parts of the country like Andhra Pradesh, needs to be propagated rapidly to meet up the medicinal demand and also for conservation purposes. *In-vitro* propagation could become a very essential tool to propagate these plants rapidly at higher rate. Many important medicinal plants have been successfully propagated *in-vitro* either by organogenesis (Erdei et al. 1981, Shoyama et al. 1983, Hatano et al. 1986, Matsumoto et al. 1986, Hiraoka and Oyanagi 1988, Nishioka 1988, Tsay et al. 1989, Huang et al. 2000, Chen et al. 2001, Chueh et al. 2001) or by somatic embryogenesis (Hiraoka et al. 1986, Kitamura et al. 1989, Tsay and Huang 1998, Sagare et al. 2000). The present work was therefore undertaken with a view to establishing an efficient protocol for *in vitro* rapid propagation of this medicinal plant through direct organogenesis from the nodal segments of the field grown plant.

MATERIALS AND METHODS

Two months old rooted cuttings of *Plumbago rosea* L. were collected from the nursery at Addathegala in East Godavari Dist., Andhra Pradesh, and were established in garden pots of Department of Botany, Andhra University. Nodal segments of approximate 2 - 4 cm were used as explants. Surface sterilization of the explants was done by submerging them in 0.1% (w/v) HgCl2 solution mixed with 1% tween 20 for 5min with continuous agitation followed by a dip in 70% ethanol for 1 min. The segments were then washed thoroughly for three times with sterile distilled water and cultured on CleriGel { HiMedia Gelling agent made from culturing of *Sphingomonas elodea (formerly Pseudomonas elodea)* bacterium} solidified MS media supplemented with different concentrations and combinations of 6BA, KN, GA_3 and NAA for multiple shooting and IAA, and IBA for root proliferation of cultured shoots. All the media were solidified with 0.20% (w/v) CleriGel and pH was adjusted to 5.8 prior to autoclaving for 20 min at 121^0 C under 15 *lb* pressure. Culture vessels with inoculated explants were maintained under a regular cycle of 16 hour photoperiod and 8 hour dark period at 25 ± 2^0 C temperature.

Multiple shoot that developed from nodal explant on MS media supplemented with different shoot promoting PGRs individually and in combination to estimate synergistic effect and were rooted on transferring in rooting media. The MS media is supplemented with different root promoting PGR's like IAA and IBA

separately with different concentrations of sucrose and gelling agent *viz.* (i) full strength of MS + 3% (w/v) sucrose (ii) half strength of MS + 1.5% (w/v) sucrose, and (iii) one fourth strength of MS + 0.75% (w/v) sucrose. The well-rooted plantlets were finally transferred to pots with sandy-loam soil and humus at 2:1 ratio.

RESULTS AND DISCUSSION

Direct organogenesis (Shoot induction):

The multiple shoots from nodal explants *Plumbago rosea* L. were induced by inoculating the nodal segments on MS medium supplemented with different concentrations 6BA, KN and GA_3. The concentration of these PGR's added individually to the media were 1.0, 2.0, 3.0, and 4.0 mg L^{-1}. Initiation of auxiliary buds of nodal segments was observed after 10 days of inoculation and later these buds developed into multiple shoot lets after 2weeks and these shoots were sub cultured after 2weeks on the same media with same concentration of PGR. Shoots induction was observed in all the tubes with hormones. The best response was observed at 3.0 mg L^{-1} for all the three hormones tested. 6BA produced superior response over KN and GA_3 (Table 1), it produced the best response of shoot induction (88%), maximum no. of shoots (18) and lengthier shoots (4.2 cm). In this study 6BA, KN and GA_3 were used individually on shoot induction media. GA_3 produced more shoots when compared to KN but lesser than 6BA. 6BA is more superior and efficient than all other shoot promoting PGR's used for the study.

Table 1: Effect of 6BA, KN and GA_3 (Individually) on shoot induction (Direct organogenesis) from nodal segments of *Plumbago rosea* L. in terms of no. of shoots and length of shoots, after 4 weeks of incubation at ambient conditions

Concentration of cytokinins (mg L^{-1})		(%) of explants responding	Mean no. of shoots	Shoot length (cm)
6BA	1.0	42±2.76	12.74±0.66	2.7±0.11
	2.0	65±4.23	16.78±0.58	3.6±0.16
	3.0	88±3.91*	21.24±0.46*	4.2±0.23*
	4.0	38±3.15	13.82±0.37	2.3±0.17
KN	1.0	25±2.43	06.38±0.21	1.8±0.19
	2.0	43±4.34	10.54±0.67	2.0±0.13
	3.0	59±2.01	14.23±0.65	2.4±0.16
	4.0	21±1.76	03.87±0.74	1.3±0.18
GA_3	1.0	34±4.37	07.44±0.52	2.3±0.13
	2.0	51±1.94	12.18±0.41	2.7±0.22
	3.0	68±3.24	16.61±0.59	3.1±0.14
	4.0	29±4.39	08.22±0.77	1.7±0.22

Means followed by * in column are significantly different at $p \leq 0.1$

Synergistic effect of Hormones on Direct organogenesis (Shoot induction):

To study the synergistic effect of the hormones on shoot induction in *Plumbago rosea* L. cultures, four hormones viz. 6BA, KN, GA_3 and NAA were used in different concentrations and combinations in shoot proliferation media. The two concentrations 1.0 and 2.0 mg L^{-1} of NAA and KN added separately to all four concentrations 1.0, 2.0, 3.0 and 4.0 mg L^{-1} of 6BA, and GA_3 (Table 2). The synergistic effect of KN and NAA has improved the rate of shoot induction when compared to individual application of each hormone in the study. KN in combination of 6BA and GA_3 produced superior effect over NAA at all the four concentrations. Addition of 1 mg L^{-1} KN to 6BA produced more shoots (26.2) compared to the all other combination of KN and NAA with 6BA and GA_3. KN and NAA in combination of 6BA, and GA_3 also increased the length of shoots than individual application of PGR's. KN produced lengthier shoots compared to NAA in combination. Addition of two different hormones in the regeneration medium has augmented the shoot induction and produced more shoots than addition of individual hormones (Kantia and Kothari, 2002, Dimitrov *et al.*, 2003, Ewa and Halina, 2004). But at higher concentration of PGR's the rate of organogenesis (No. of shoots and length of shoots) has declined due to limiting effect of PGR's at higher concentration in *Plumbago rosea* L *in-vitro* direct organogenesis.

Table 2: The effect of 6BA and GA_3 in combination with KN and NAA on Multiple shoot (No. of shoots and shoot length) induction in *Plumbago rosea* L. after 4 weeks of incubation at ambient conditions.

PGRs conc. and combinations (mg L^{-1})	Number of shoot (Mean ± S.E)*
1.0 BA + 1.0 KN	15.17±0.21
2.0 BA + 1.0 KN	19.68±0.32
3.0 BA + 1.0 KN	25.24±0.16*
4.0 BA + 1.0 KN	16.31±0.19
1.0 BA + 2.0 KN	15.67±0.46
2.0 BA + 2.0 KN	20.14±0.61
3.0 BA + 2.0 KN	18.24±0.22
4.0 BA + 2.0 KN	13.72±0.51
1.0 BA + 1.0 NAA	13.54±0.29
2.0 BA + 1.0 NAA	17.45±0.11
3.0 BA + 1.0 NAA	21.41±0.71
4.0 BA + 1.0 NAA	14.56±0.28
1.0 BA + 2.0 NAA	13.14±0.61
2.0 BA + 2.0 NAA	16.42±0.15
3.0 BA + 2.0 NAA	17.11±0.26
4.0 BA + 2.0 NAA	12.09±0.11
1.0 GA_3+ 1.0 KN	12.66±0.41
2.0 GA_3+ 1.0 KN	14.34±0.73
3.0 GA_3+ 1.0 KN	17.09±0.31
4.0 GA_3+ 1.0 KN	11.14±0.77
1.0 GA_3 + 2.0 KN	11.18±0.21

2.0 GA_3 + 2.0 KN	12.67±0.29
3.0 GA_3 + 2.0 KN	16.78±0.37
4.0 GA_3 + 2.0 KN	10.08±0.13
1.0 GA_3 + 1.0 NAA	10.91±0.61
2.0 GA_3 + 1.0 NAA	13.11±0.76
3.0 GA_3 + 1.0 NAA	16.24±0.33
4.0 GA_3 + 1.0 NAA	10.32±0.49
1.0 GA_3 + 2.0 NAA	09.75±0.83
2.0 GA_3 + 2.0 NAA	12.24±0.19
3.0 GA_3 + 2.0 NAA	15.91±0.22
4.0 GA_3 + 2.0 NAA	08.89±0.11

Means followed by * in column are significantly different at $p \leq 0.1$

Direct organogenesis (Root induction):

Well-elongated shoots from nodal segments on shoot induction medium were excised and transferred to the root induction medium. The root induction medium consists of different concentrations of IAA and IBA. The concentrations fortified in the study for better proliferation were 0.5, 1.0 and 1.5 mg L^{-1} along with different concentrations of sucrose and gelling agent *viz.* (i) full strength of MS + 3% (w/v) sucrose (ii) half strength of MS + 1.5% (w/v) sucrose, and (iii) one fourth strength of MS + 0.75% (w/v) sucrose (Table 3). One fourth strength of MS supplemented with 0.75% (w/v) sucrose proved to be efficient for induction of strong and stout root system. This finding revealed that nutritional stress along with IAA was propitious for sprouting of roots. Similar finding was also reported by Hossain and Bhadra (2002 and 2009). Of the two hormones IAA and IBA tested for rooting, the best results were observed in IAA at all three concentrations. The best results were observed in medium fortified with 1.0 mg L^{-1} IAA. The most no. of roots (10.5) and lengthier roots (9.2) cm were obtained on One fourth strength of MS supplemented with 0.75% (w/v) sucrose + 1.0 mg L^{-1}. From this study it is apparent that one fourth strength MS media is superior over full and half strength MS medium for efficient rooting *in-vitro* in *Plumbago rosea* L.

Table 3: The effect of PGR's (IAA and IBA) along with variation in % concentration of sucrose and strength of MS media on root induction in *Plumbago rosea* L. after 4 weeks of incubation at ambient conditions.

(RP) PGR	PGR conc. + %(w/v) of Sucrose + Strength of MS media	No. of roots	Root length
IAA	0.5 IAA + 3% + Full strength of MS	06.85±0.22	4.8±0.21
	0.5 IAA + 1.5% + 1/2 strength of MS	09.41±0.12	5.3±0.11
	0.5 IAA + 0.75% + 1/4 strength of MS	11.19±0.38	7.7±0.25
	1.0 IAA + 3% + Full strength of MS	11.89±0.18	6.3±0.13
	1.0 IAA + 1.5% + 1/2 strength of MS	16.51±0.11	8.7±0.14
	1.0 IAA + 0.75% + 1/4 strength of MS	24.75±0.25*	9.2±0.18*
	1.5 IAA + 3% + Full strength of MS	09.47±0.41	4.1±0.20
	1.5 IAA + 1.5% + 1/2 strength of MS	13.66±0.09	5.7±0.21
	1.5 IAA + 0.75% + 1/4 strength of MS	17.11±0.21	7.5±0.10

IBA	0.5 IBA + 3% + Full strength of MS	06.27±0.08	3.9±0.11
	0.5 IBA + 1.5% + 1/2 strength of MS	08.15±0.27	5.1±0.15
	0.5 IBA + 0.75% + 1/4 strength of MS	10.79±0.03	6.9±0.23
	1.0 IBA + 3% + Full strength of MS	11.10±0.31	5.8±0.08
	1.0 IBA + 1.5% + 1/2 strength of MS	14.87±0.68	7.6±0.12
	1.0 IBA + 0.75% + 1/4 strength of MS	18.08±0.24	9.0±0.10
	1.5 IBA + 3% + Full strength of MS	08.23±0.06	3.7±0.17
	1.5 IBA + 1.5% + 1/2 strength of MS	11.91±0.55	4.9±0.11
	1.5 IBA + 0.75% + 1/4 strength of MS	15.36±0.67	6.7±0.28

Means followed by * in column are significantly different at $p \leq 0.1$


A

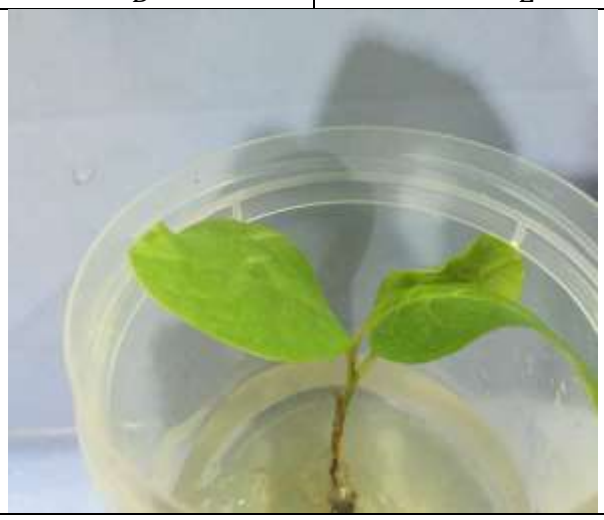

B


C


D


E


F


G


H

Fig.1. **A**. *Plumbago rosea* L. Nodal segment inoculated on shoot induction media. **B, C, D**. Stages of Initiation and development of multiple shoots from nodal segments. **E, F**. Sub-culturing of growth Initiated nodal segments for more multiple shoots. **G.** Induction of strong and stout root system in individual plant. H. *In-vitro* grown plantlet of *Plumbago rosea* L. is acclimatized and growing in polythene bags in outside environment.

Acclimatization and transplanting:

The rooted plantlets were carefully removed from the culture tubes and cleaned with distilled water to remove the media. The plants were then placed on a sterilized mixture of soil + sand + compost (1:1:1 ratio) in culture bottles, to maintain high humidity the bottles mouth are covered by autoclaved aluminium foil and kept in culture room. After two weeks of incubation the plants were transferred to pots containing vermicompost and sand in a ratio of 50:50 and maintained under green before transferring to the field with 90% survival rate.

CONCLUSION:

The protocol developed for production of *Plumbago rosea* L. plantlets *in-vitro* can be used reliably for propagation in a commercial scale and *ex-situ* conservation of this very important medicinal plant species. From the present study it is apparent that, the use of 6BA in combination of KN has increased the rate of shoot induction in *Plumbago rosea* L. than all other combination of PGR's reported in the study.

REFERRENCES:

Chen CC, Chen SJ, Sagare AP and Tsay HS. 2001. Adventitious shoot regeneration from stem internode explants of *Adenophora triphylla* (Thunb.) A. DC (Campanulaceae) – An important medicinal herb. *Botanical Bulletin of Academia Sinica*.

Erdei I, Kiss Z, Maliga P. 1981. Rapid clonal multiplication of *Digitalis lanata* in tissue culture. *Plant Cell Reports*.

Fay MF. 1992. Conservation of rare and endangered plants using *in vitro* methods. *In vitro Cellular and Developmental Biology*.

Hatano K, Shoyama Y, Nishioka I. 1986. Multiplication of *Pinellia ternate* by callus culture of leaf segment. Shoyakugaku Zasshi.

Hiraoka N, Oyanagi M. 1988. *In vitro* propagation of *Glehnia littoralis* from shoot tips. *Plant Cell Reports*, 7.

Hossain MM, Bhadra SK. 2002. Mass scale propagation of Chrysanthemum (*Chrysanthemum morifolium* Ramat.) through tissue culture. *The Chittagong Univ. J. Sci.* 26 (1&2).

Hossain MM, Bhadra SK. 2009. In-vitro micropropagation of *Plumbago rosea* L. through induction of direct and indirect organogenesis. *Plant tissue culture and biotech*. 19 (2).

Huang CL, Hsieh MT, Hsieh WC, Sagare AP and Tsay HS. 2000. *In vitro* propagation of *Limonium wrightii* (Hance) Ktze. (Plumbaginaceae), an ethno-medicinal plant, from shoot-tip, leaf- and inflorescence-node explants. *In vitro Cellular and Developmental Biology* - Plant 36.

Lakshmi M, Mythili S. 2003. Somatic embryogenesis and regeneration of callus cultures of *Kaempferia galang* – medicinal plant. *J. Medicinal and Aromatic Plants*, 25.

Matsumoto M, Nagano M, Shoyama Y and Nishioka I. 1986. New vegetative propagation method of *Rehmannia glutinosa.* Shoyakugaku Zasshi 40.

Nair SV, Gaurav B, Maitrayee C, Arun S, Anil Kumar V, Chinchu B, Asoke B, Raja B. 2016. Antimicrobial activity of plumbagin, a naturally occuring napthoquinone from *Plumbago rosea*, against *Staphylococcus aureus* and *Candida indica. Int J Med Microbiol*, 306 (4), 237-248.

Nishioka I. 1988. Clonal multiplication of medicinal plants by tissue culture. *Shoyakugaku Zasshi* 42.

Pharkphoom Panichayupakaranant and Tewtrakul Supinya. 2002. Plumbagin production by root cultures of *Plumbago rosea. Electronic Journal of Biotechnology*, 5(3), 228-232.

Reddy KN, Sudhakar Reddy C. 2008. First Red List of Medicinal Plants of Andhra Pradesh, India - Conservation Assessment and Management Planning. *Ethnobotanical Leaflets,* 12.

Sagare AP, Lee YL, Lin TC, Chen CC and Tsay HS. 2000. Cytokinin-induced somatic embryogenesis and plant regeneration in *Corydalis yanhusuo* (Fumariaceae) - A medicinal plant. *Plant Science*, 160.

Shoyama Y, Hatano K and Nishioka I. 1983. Clonal multiplication of *Pinellia ternate* by tissue culture. *Planta Medica,* 49.

Skoog F and Miller RM (1957) Chemical regulation of growth and organ formation in plant tissue culture *in vitro*. Sym. Soc. Exp. Biol. 11.

Tsay HS, Huang HL. 1998 Somatic embryo formation and germination from immature embryo-derived suspension-cultured cells of *Angelica sinensis* (Olive) Diels. *Plant Cell Reports,* 17.

Tsay HS, Gau TG, Chen CC. 1989. Rapid clonal propagation of *Pinellia ternate* by tissue culture. *Plant Cell Reports,* 8.

Vieira RF, Skorupa LA. 1993. Brazilian medicinal plants gene bank. *Acta Hortculturae*, 330.