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In vitro propagation of *Plumbago rosea* L.

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Abstract

In this present study a reproducible *in vitro* protocol has been standardized for direct micropropagation of a rare medicinal plant: *Plumbago rosea* L. using nodal explants. Eighty percentage of multiple shoot induction was achieved after 20 days in MS medium supplemented with 1.5mg/l BAP and 0.3mg/l NAA. After 30 days of establishment, the *in vitro* derived shoots were sub cultured in half strength MS medium supplemented with 0.4mg/l IBA and 0.4mg/l IAA. Seedlings were transformed into greenhouse condition after 45 days and 85 percentage of field survival was achieved.

Key words: *Plumbago rosea*, medicinal plant, direct organogenesis, *in vitro* propagation**.**

Abbreviations: BAP- 6-Benzylaminopurine; BA- Benzyl adenine; **NAA**- α-naphthalene acetic acid; IBA- Indole-3-Butyric Acid; IAA- Indole Acetic Acid; **MS**- Murashige and Skoog's (1962) medium; **PGR**- Plant Growth Regulator. 2-iP- 2-isopentinyle adenine; Kin- Kinetin.

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1. Introduction

In view of the tremendously growing world population, increasing anthropogenic activities, rapidly eroding natural ecosystem, etc. the natural habitat for a great number of herbs and trees are dwindling, many of them are facing extinction. In India, the medicinal plant sector has traditionally occupied an important position in the social, cultural, spiritual and the medicinal arena of rural and traditional healers. In recent years the growing demand for herbal products has led to a quantum jump in volume of plant material traded within and outside the country. Very small proportions of the medicinal plants are lichens, ferns, algae, etc.; the majority of the medicinal plants is higher plants. Though India has one of the 17 mega biodiversity countries of the world, the growing demand is putting a heavy strain on the existing resources, causing a number of species to be either threatened or endangered category. About 90% of medicinal plants used by industries are collected from the wild. While over 800 species are used in production by industry, less than 20 species of plants are under commercial cultivation. Over 70% of the plant collections involve destructive harvesting because of the use of parts like roots, bark, wood, stem and the whole plant in case of herbs. This poses a definite threat to the genetic stocks and to the diversity of medicinal plants. Thus the development of new *in vitro* regeneration strategies for such rare and important medicinal plants become the need of the hour.

In recent years, tissue culture has emerged as a promising technique to obtain genetically pure elite populations under *in vitro* conditions rather than have indifferent populations. *In vitro* propagation has proven as a potential tool for large scale production of medicinal plant species, products thereof and germplasm conservation (Hassan and Khatun, 2010). *In vitro* conservation through micropropagation, which allows making numerous clones of rare plants by exploring the totipotency of suitable tissues isolated from the mother plant (Kanungo and Sahoo, 2011).

Plumbago rosea L. is one among such rare medicinal planted belongs to the family of Plumbaginaceae. This plant has been reported to have the major active principles such as plumbagin; the leaves contain apigethe nin, cuteolin, 7-0-genc sides, 2 and β amyrin, palmistic acid and β - sitosrtrol. Flowers contain azaleation, capensiridue 3- rhamnoside. Plumbagin is an important naphthoguinnoe that shows anticancer (Parimala and Sachawand, 1993), antimicrobial (Didry *et al.*, 1994) and anti-fertility activities (Bhargava, 1984). Roots are used as styptic in scrofula in treating black water fever and as anti-emetics. The powdered roots are smeared to move warts. But, in natural habitat, this plant is under severe threat due to the non-availability of proper cultivation system and exploitation of local communities for traditional medical purposes. Hence, in this present study an attempt was made to evolve a rapid, reproducible in vitro regeneration protocol for the direct micropropagation of *Plumbago rosea* L. using nodal segments.

2. Materials and Methods

Explants of *P. rosea* was collected from Papanasam hills in the Western Ghats, potted and established in St. Xavier's College herbal garden for routine use. Voucher specimens were deposited in St. Xavier's College herbarium (XCH – 25687) and the identity of the specimens were confirmed with reference flora (Gamble, 1956; Matthew, 1983). Nodal explants were collected from the young shoots, segments were washed with running tap water for 5 min followed by Bavastin (1%), Tween 20 treatments for 5-10 minutes and then surface sterilized in 0.1 (w/v) $HgCl_2$ solution for 3 minutes and then thoroughly rinsed with sterile distilled water for 3 times. Surface sterilized explants were finally trimmed (0.5-1.0cm) and aseptically implanted on solid Murashige and Skoog (1962) medium containing 3% sucrose, 0.6% agar and BAP (0.5 to 3.0 mg/l) + NAA (0.1 to 0.6 mg/l) for the induction of multiple shoots and roots. *In vitro* derived shoots were transferred to ½ MS medium supplemented with IBA and IAA (0.2 - 1.0 mg/l). The cultures were maintained at 25±2°C under white fluorescence light (2000 Lux for 16 h/d) photoperiod). For acclimatization, the platelets with well developed roots were removed from culture tubes, washed in sterile distilled water to remove the remnants of agar and platelets were planted separately onto 10cm diameter poly cup filled with potting mixture of sand, garden soil and backyard manure (1:1:1). Plants were kept in a mist chamber with relative humidity of 70%, irrigated with 10x MS media for 2 weeks and establishment rate was recorded. Statistical evaluation and comparison of results were carried out using Statistical Analysis System (SAS). Differences between means were assessed for significance at $p < 0.05$ by Duncan's multiple range test (DMRT).

3. Results and Discussion

Tissue culture of medicinal plants holds tremendous potential for the production of high quality plant based medicines. In this present study, *in vitro* morphogenetic potential of nodal segments of *P. rosea* was evaluated to devise a rapid, reproducible protocol for clonal propagation. Among various concentrations and combinations of PGRs tested, MS media supplemented with BAP (0.5 - 3.0 mg/l) in combination with NAA (0.1 - 0.6 mg/l) found to be

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suitable to produce high number of shoots after twenty days of inoculation.

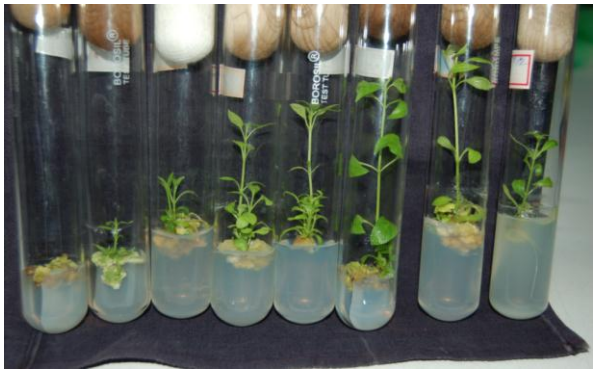

Fig- 1: MS media augmented with 1.5 mg/l BAP and 0.3 mg/l NAA for maximum number of Shoots induction and ½ MS media augmented with 0.4 mg/l IBA for maximum number of roots induction.

Table 1: Effect PGRs on shoot induction of *P. rosea* after 30 days

No. of explants	MS medium with PGR (mg/l)		% of response	Maximum no. of Shoots / explants
	BAP	NAA		
10	0.5	0.1	20	06.44 ± 1.2
10	1.0	0.2	60	06.62 ± 0.9
10	1.5	0.3	80	13.62 ± 0.8
10	2.0	0.4	70	07.67 ± 0.4
10	2.5	0.5	30	05.76 ± 0.3
10	3.0	0.6	10	01.78 ± 0.6

*all value s are average of 10 sets of inoculated plants

Table 2: Effect of PGRs on root induction from in vitro micro shoots of *P. rosea* after 45 days

½ MS Medium with PGR	½ MS Medium with PGR (mg/l)	% of response	Maximum No. of Roots / Shoots
IAA	0.0	30	01.66 ± 0.1
	0.2	40	02.08 ± 1.3
	0.4	60	09.35 ± 1.1
	0.6	50	07.67 ± 0.5
	0.8	30	04.55 ± 0.0
	1.0	20	00.67 ± 0.1
IBA	0.0	40	01.11 ± 0.1
	0.2	60	08.66 ± 0.5
	0.4	90	11.76 ± 0.3
	0.6	70	04.68 ± 0.1
	0.8	40	02.64 ± 0.5
	1.0	20	01.54 ± 0.0

The pivotal role of cytokinins in plant tissue culture has been well documented (George *et al.,* 2004). Growth and multiplication of shoot buds and adventitious shoots either from already existing shoot primordium (Sivasubramanian *et al.,* 2002) have been the significant role played by a variety of cytokinin like substances supplemented in culture medium. This includes BA, BAP, 2-iP and Kin. Shoot bud and axillary bud dormancy has been revoked and multiple shoot growth has been achieved in several medicinal plants by amending ideal concentration of cytokinins (John De Britto and Mahesh, 2009; Mahesh *et al.,* 2010; 2011; 2012; Maridass *et al.,* 2010a; 2010b; 2012). The level of cytokinin from 0.5 µM to 50 µM has been widely used to induce various morphogenetic responses *in vitro*.. But in, this present study, we could obtain an average of 13.62±1.6 shoots from nodal explants cultured on MS media augmented with 1.5 mg/l BAP and 0.3 mg/l NAA (Table-1 & Fig-1). Although both cytokinin and IAA can be produced in roots and shoots the production of these major hormonal signals does not occur randomly but is regulated by the location of synthesizing cells in the plant body and their developmental stage, and is influenced by environmental conditions. It is observed from the results of this present study, nodal explants of *P. rosea* has enough kinetic potential to coordinate with the lower level of cytokinin and auxins and to elicit the shoot induction process at maximum level (Fig-1). This elevated and cumulative response may be due to the presence of indigenous cytokinins and auxins in the healthy explants selected for this present study.

The supplementation of cytokinin in shoot induction, multiplication and shoot elongation media is indispensable. Occasionally, low level of cytokinin is required in differentiation, maturation and germination of somatic embryos in cultured conditions. Cell and protoplast cultures require the combination of cytokinin and auxin to induce cell wall growth, cell division, mass multiplication, differentiation and regeneration of whole plants. The effects of auxins and cytokinins on shoot multiplication of *Plumbago rosea* has been evaluated under different culture systems (Das and Rout, 2002; Sivanesan and Jeong, 2009). In all previous reports, supplementation of cytokinin (BAP) in the range of 1.0-25µM and the co-supplementation of auxin (NAA/IAA) between 0.5-5.0µM was found to elicit shoot proliferation at the maximum level. Similar observations were made in other species of Plumbogo such as Plumbaga zeylanica (Rout *et al*., 1999; Selvakumar *et al*., 2001; Das and Rout, 2002; Sivanesan and Jeong, 2009).
Micro-propagated plantlets found to have similar morphology and growth pattern (Fig-1) during the acclimation and field transfer. It indicates that the platelets have a satisfactory level of genetic integrity

as required for the success of regeneration protocol. Genetic integrity microprogated plantlets of *Plamboga zeylanica* has been evaluated through RAPD markers and found monomorphic (Rout and Das, 2002). These observations support the findings of this present study. *In vitro* morphogenetic potential of axillary bud and nodal segments were evaluated with the supplementation of plant growth regulators in several important medicinal plants such as *Piper longum* (Sonia and Das, 2002), *Janakia arayalpathra* (Sudha *et al.,* 2005; Thangavel, 2008), *Eclipta alba* (Husain and Anis, 2006), *Decalepis hamiltonii* (Thangavel, 2008) *Leucojum aestivum* and *Lilium rhodopaeum* (Marina *et al.*,1994). In all these reports, supplementation of MS media with BAP and co-supplementation of either NAA or IAA was highlighted as a prime requisite for direct organogenesis. Further, instead of increasing the concentration of cytokinins into multifold to achieve higher rate of shoot proliferation co-supplementation of an auxin in lower concentration was also found to be significant. The findings of this present study corroborate with these reports as the high frequency of shoot multiplication has been obtained when the nodal segments were cultured on MS medium supplemented with BAP (1.5mg/l) and co-supplemented with NAA (0.3mg/l) (Fig-1&Table-1). Propagation from existing meristems (shoot tips and axillary buds) produces plants that are genetically identical with the donor plants (Sharma *et al.,* 2010). Plant regeneration from shoot and stem meristems yielded encouraging results in medicinal plants like *Catharanthus roseus, Cinchona ledgeriana, Digitalis sp., Rehmannia glutinosa, Ravulfia serpentine, Isoplexis canariensis* (Roy *et al.,* 1994; Tripathi and Tripathi, 2003).

For root induction, *in vitro* derived shoots were transferred to ½ MS media augmented with 0.2 to 1.0 mg/l of IBA and IAA respectively. Maximum number of roots (11.76±0.3) were achieved on ½ MS media augmented with 0.4 mg/l IBA (Fig-1& Table-2). When the medium was supplemented with IBA and IAA separately, root induction was obtained. However, IBA was found to be more effective and induced higher root initiation and establishment (Table-2 & Fig-1). Root induction was accomplished by adding IBA, at different concentrations. Roots were developed after 10 days from the date of transfer of shoots into rooting medium. Enhanced *in vitro* rooting was achieved in *Plambago rosea* by supplementing the MS medium with 0.49µM IBA. In case of *plambago zeylanica*, high efficiency of root induction was achieved when half strength MS medium was supplemented with 0.5mg/l of NAA (Sivanesan and Jeong, 2009). Similar patterns of root induction was achieved when the *in vitro* developed shoots were transferred into MS medium fortified with 4.92mg/l of IBA (Selvakumar *et al*., 2001). The results of this present study find good agreement with these earlier reports in *Plumbago sp*. Maximum root induction capacity was observed in medium supplemented with 0.4mg/l IBA and 0.4mg/l IAA. Sujata *et al*., (2002) reported a higher percentage of root induction in *Salvadora persica, in* ½ MS media supplemented with 3.0 mg/l IBA. Similarly, MS medium fortified with 0.5 mg/l IBA, 0.1 mg/l NAA and 0.1 mg/l IAA was found to be suitable for higher root induction in in *Spilanthes paniculata* (Mahendran *et al.,* 2006). Hence, the results of this present study could provide a vital piece of information for the establishment of a rapid protocol for large scale multiplication of medicinally important plant *P. rosea* with good level of genetic fidelity in a short period.

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