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In vitro conservation and adventitious shoot regeneration of *Plumbago indica* L. - a genetic resource of India

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Abstract

An efficient protocol is described for the rapid *in vitro* multiplication of *Plumbago indica* L., an important genetic resource, from shoot tip. The explants cultured on to MS medium containing different concentrations of BAP, Kn and NAA either alone or in combination for investigation of morphogenetic response. The best shoot proliferation (11.3± 0.01) was observed MS media supplemented with 3.0 mg/l BAP along with 0.5 mg/l NAA. The highest shoot elongation observed MS medium containing 1.0 mg/l BAP in combination with 0.6 mg/l GA_3 (11.5± 2.5). The highest rooting frequency (80%) obtained from MS medium in combination 1.0 mg/l IBA. Maximum 90% response was achieved using 2% sorbitol and 2% mannitol in combination with MS medium at 10℃ temperature up to 6 months by *in vitro* slow growth technique. Regenerated plantlets were transferred to greenhouse conditions and observed 85% survival frequencies. The diploid status (2n= 14) of regenerated plantlets as well as mother plant was determined using chromosome counts of root- tips. The regenerated plants were cytologically, morphologically are stable which offers an exclusive opportunity to obtained true-to-type plants of the source plant.

Keywords: *Plumbago indica*, micropropagation, *in vitro* conservation, somatic chromosome

1. Introduction

Plumbago indica L. commonly famous as Lalchitra be appropriate to the plumbaginaceae family is an appreciated indigenous medicinal plant extensively used in the treatments for leprosy, dyspepsia, diarrhea, skin diseases etc. This plant originated in India and now regularly cultivated in South India (Reddy *et al*., 2009). The principal source of plumbagin production is from *Plumbago* species principally *P. indica* (Mallavadani *et al*. 2002). The roots of this plant are the main source of an alkaloid, plumbagin which has been used as an anticancer drug and considered it an important

genetic resource. There is an increased demand of the pharmaceutical industry but its limited supply is a major problem. This is normally propagated through seed or asexual propagation but germination rate is very leisurely and low in nature. Therefore, in this study, the experiments were conducted to normalize the source of explants and culture media for multiple proliferation of shoot resulting in mass propagation of homogenous elite plantlets of these plantlets through tissue culture technique (Holobiuc *et al*. 2009). Regeneration and efficacious proliferation of genetically stable plants from cultures are advantage in any *in vitro* conservation exertion [29]. Assessment of phytochemical content through HPLC technique is very standard in herbal medicine and pharmaceutical industry due to informal learns and is not partial by the volatility or stability of the sample compound [16]. The cytological consistency of micropropagated plants requirements to be tested before using this protocol at profitable level [10, 11, 12, 13].

In imperative to the flow of defeat of biodiversity, an effort to conserve through *in vitro* practice of *P. indica*, has been ready in this revision. Shoot tips were familiarized for creating *in vitro* culture, prompted to multiply. The goal of the contemporaneous study based on to improve a simple, effectual protocol via shoot tip culture for large scale uniform plant manufacture, *in vitro* conservation consuming slow growth performance of *P. indica* to define the ideal method for the production of a higher number of plants and also appraised genetic stability of regenerates through somatic chromosome study. In association of this study, also recognized plumbagin content assessment technique through HPLC technique. This protocol will oblige in the future as a base to establish an *in vitro* genetic bank for germplasm conservation, to progress the enhancing value of these species.

2. Materials and Methods

A. Plant material

Plumbago indica L. were serene from several localities in West Bengal, India, were grown in the trial garden at RKMVC College campus under indistinguishable environmental conditions. Collected specimen was established by the reference number from BSI- CNH/2014/Tech.II/78/200.

B. Surface sterilization of explants

2% bavistin solution (w/v, a systemic fungicide) for 20; 5% Tween- 20 (v/v, a liquid soap solution) for 10 minutes; Finally, surface disinfection done with 0.1% (w/v) mercuric chloride solution for 5 minutes and washed systematically with hygienic distill water beforehand inoculation.

C. Culture media and Culture Condition

Excised shoot tips (8-12 mm) were inoculated on to MS [**18**] medium alone as control and MS medium with altered concentrations and combinations of cytokinins (BAP and Kn) either separately

or with auxin (NAA). Multiple shoots were sub- cultured on to MS medium complemented with BAP (1.0 mg/l) and varying concentrations of gibberellic acid (GA_3) (0.2, 0.4, 0.6, 0.8, and 1.0 mg/l) for elongation of shoots. For rooting, *in vitro* grown micro shoots (3-4 cm) were eliminated and inserted on to MS basal media with different concentrations of IBA and IAA (0.2, 0.5 and 1.0 mg/l). All the cultures were incubated at 24± 2 °C temperature and 55± 5% relative humidity under 16/8 h photoperiod.

D. *In vitro* Conservation

Some typical slow growth procedures via minimal nutrient and long gap between subculture and as well as cold temperature storage approaches will be followed in this experiments. Mannitol and sorbitol (1.0 %–3.0 %, w/v) with 3% sucrose (w/v) and 0.8% (w/v) agar were used as slow growth regulators.

E. Acclimatization and field experiments

For hardening, tissue culture derived healthy rooted plantlets were indifferent from the agar medium and shifted to earthen pots having sterilized soilrite (Keltech Energies Ltd, Bangalore, India) and protected with transparent polythene bag for 30-35 days and lastly were moved to the trial field.

F. Mitotic chromosome study

Mitotic chromosome study of source plant as well as regenerated plants will be agreed out from root tips of young and healthy plant of *P. indica* using aceto-orcine staining method. Root tips were pretreated in saturated solution of *p*- dichlorobenzene for 6 hrs at 14°C and fixed with absolute alcohol and glacial acetic acid (3:1, v/v) for 24 hours at 4°C. Fixed root tips were stained with 2.0% aceto-orcein: 1(N) HCl (9:1, v/v) mixture followed by incubating for 2 h at room temperature. Chromosome plates were experiential in Leica DM750 microscope and photographed with Leica DFC 295 camera.

3. RESULTS AND DISCUSSION

The initial observable sign of green shoot bud (2-3) induction from nodal sectors was noticeable after 7 days of culture on MS medium augmented with BAP (0.5 mg/l). MS medium complemented with Kn and NAA in altered concentration and combinations were establish to be irrelevant responding. Among the concentrations of BAP alone ranging from 0.5 mg/l to 5.0 mg/l, offered the superior results than other two cytokinins. The higher concentrations (6.0 mg/l to 8.0 mg/l) of BAP and NAA exclusively made large amounts of white-greenish callus at the base of the nodal explants which outcomes in stunted shoots with mutilated rosette leaves. Moreover, among the concentrations of 3.0 mg/l BAP in combination with low amounts (0.5mg/l) of NAA was instigate to be the best in terms of amenable

a high number of healthy shoots (11.3± 0.01) with extreme shoot length was (5.06± 0.03) **(Table1) (Figure 1A & 1B)**.

The inhibitory effect of BAP in combination low amounts of auxin on shoot induction was also reported in *Plumbago zeylanica* (Chatterjee and Ghosh, 2015); *Tylophora indica* (Faisal *et al.*, 2005) *Morus alba* (Anis *et al*., 2003). Therefore, MS medium accompanied with 3.0 mg/l of BAP in combination with 0.5mg/l was particular as optimal shoot proliferation medium and used for additional study and by frequent sub- culturing of shoot tips from recently axenic shoots, a high-frequency rapid shoot culture practice was recognized.

Fig. 1A & 1B

Table-1: Influence of different cytokines on axillary bud proliferation and multiplication from nodal explants of *P. indica* (Data collected after 45 days of culture)

Growth regulators	Concentration (mgl^{-1})	% of explants forming axillary buds	Mean No. of Shoots/explants	Mean shoot length (cm)
BAP	0.5	60.0 ± 2.0^{c}	4.22 ± 0.33^{de}	2.86 ± 0.08^{f}
	1.0	63.0 ± 3.0^{b}	5.07 ± 0.43^{ab}	3.07 ± 0.03^{b}
	2.0	65.0 ± 1.1^{b}	5.28 ± 0.57^{a}	3.97 ± 0.03^{ab}
	3.0	72.0 ± 2.0^{a}	4.71 ± 0.50^{c}	4.43 ± 0.08^{d}
	5.0	67.0 ± 3.0^{ab}	3.67 ± 0.34^{hi}	3.06 ± 0.03^{gh}
Kinetin	0.5	50.0 ± 1.0^{bc}	4.34 ± 0.32^{fghi}	3.36 ± 0.06^{i}
	1.0	47.0 ± 1.2^{b}	3.76 ± 0.30^{d}	3.10 ± 0.03^{g}
	2.0	46.0 ± 0.5^{cd}	3.50 ± 0.35^{c}	2.33 ± 0.05^{d}

	3.0	43.0± 1.0^{c}	2.64± 0.58^{def}	2.10± 0.03^{f}
	5.0	40.0± 1.3^{bc}	2.10± 0.32^{hi}	1.40± 0.05^{g}
NAA	0.5	50.0± 0.5^{bc}	4.33± 0.44^{ef}	3.14± 0.14^{ghi}
	1.0	47.0± 1.2^{b}	4.12± 0.13^{gh}	3.10± 0.15^{i}
	2.0	45.0± 0.3^{cd}	3.72± 1.07^{ghi}	2.90± 0.20^{def}
	3.0	38.0± 1.0^{c}	3.02± 2.06^{h}	2.40± 0.02^{ifg}
	5.0	33.0± 1.7^{bc}	3.00± 0.04^{gh}	1.70± 0.52^{i}
BAP + Kn	0.5+0.5	53.0± 1.3^{c}	3.6± 0.23^{a}	3.68± 0.69^{g}
	1.0+0.5	44.0± 1.1^{cd}	4.2± 0.37^{ij}	4.50± 0.58^{cd}
	3.0+0.5	32.0± 1.6^{e}	3.20± 1.02^{c}	3.02± 0.51^{bc}
BAP + NAA	0.5+0.5	73.0± 1.2^{bc}	7.9± 1.20^{c}	3.97± 0.03^{a}
	1.0+0.5	78.0± 1.0^{a}	8.12± 0.40^{d}	4.0± 1.90^{cd}
	3.0+0.5	85.0±0.2^{a}	11.3± 0.01^{c}	5.06± 0.03^{gh}
Kn + NAA	0.5+0.5	49.0± 1.2^{fg}	4.10± 0.42^{cd}	2.84±1.2^{bc}
	2.0+0.5	43.0± 0.3^{g}	3.10± 0.30^{cd}	2.60± 0.2^{i}
	3.0+0.5	40.0± 1.0^{h}	2.7± 0.02^{cd}	1.30± 0.2^{i}

(Each value represents the mean ± SD of 10 replicates and each experiment was repeated thrice)

The result submits that small amounts of GA_3 (0.6 mg/l) in arrangement with 1.0 mg/l BAP were functioning in stimulating *P. indica* shoot elongation. Shoots reached a maximum height of 11.5± 2.5 cm during GA_3 treatments **(Table 2).** The same result displayed for shoot elongation for the effect of GA_3 in *Plumbago zeylanica* [24] and *Artemisia vulgaris* [25].

Table 2: Effect of GA_3 on shoot elongation of *P. indica* from regenerated shoots cultured on MS medium supplemented with GA_3 and BAP (1.0 mg/l). (Data collected after 30 days of culture.)

BAP (mg/l)	GA_3 (mg/l)	Shoot elongation response (%)	Mean Shoot (cm)
1.0	0.2	73.0± 1.0^{c}	6.2 ± 1.1^{c}
1.0	0.4	78.0± 2.2^{a}	8.3 ± 0.3^{b}
1.0	0.6	80.0± 1.3^{bc}	11.5 ± 2.5^{a}
1.0	0.8	70.0± 1.0^{c}	5.0 ± 1.4^{ab}
1.0	1.0	62.0± 0.3^{e}	4.6 ± 0.1^{ef}

(Each value represents the mean ± SD of 10 replicates and each experiment was repeated thrice).

To facilitate the induction of supreme number of roots within a small period of time, MS medium was added with different concentrations of IBA and IAA (0.2, 0.5 and 1.0 mg/l). The earliest and highest percentage (80%) of root initiation with maximum number (10.0± 0.25[a]) of roots per explants **(Figure 1C)** was attained on MS medium containing 1.0 mg/l of IBA (Table-3). The enhancing role of IBA among altered auxins on *in vitro* root initiation has been conveyed to be the most functioning in many previous experiments [Reddy *et al*., 2009; 26, 27, 28].

Fig. 1C

Table 3: Effect of different auxins on *in vitro* root induction of *P. indica*

Nutrient medium (3% Sucrose)	Concentrations of auxins (mg/l)	% of microshoots showing root induction	No. of roots induced micro shoot-1
MS	0	55.00± 1.1[c]	3.6± 0.12[b]
MS	IBA (0.2)	72.00± 0.5[fg]	3.1± 0.20[d]
MS	IBA (0.5)	75.00± 1.7[cd]	7.5± 0.11[a]
MS	IBA (1.0)	80.00± 3.0[a]	10.0± 0.25[a]
MS	IAA (0.2)	68.00± 0.2[b]	6.5± 0.32[a]

MS	IAA (0.5)	70.00± 0.5^{ef}	7.0± 0.33^{c}
MS	IAA (1.0)	73.00± 0.7^{c}	3.0± 0.35^{d}

(Each value represents the mean± SE 10 replicates and each experiment was conducted thrice.)

The cultures preserved in virtuous health conditions in measured medium (MS medium with 3% sucrose, without osmotic agents) with 100 % survival rate up to 2 to 3 months without subculture. But when cultures were continued in manitol and sorbitol survived up to 6 months lacking subculture.

At low temperature system (4°C), all the cultures exposed pitiable presentation and culture did not continue as in case of control at 25°C. In adequate temperature system (10^{o}C) the cultures grew well with condensed growth in appraisals to control. This experiment advises that cultures growth could be abridged at 18°C, but storage period could not be amplified to maintain healthy cultures. The effects of MS medium in arrangement with different concentration of sorbitol and mannitol, on growth of *P. indica* cultures were calculated. The adding of osmotic agents 2% sorbitol (w/v) and 2% mannitol (w/v) in arrangement with MS medium has augmented survival rate 90% at 10°C. Growth overpowering had positively abridged the labor during culture maintenance in the tissue culture laboratory and also encouraged symmetry of growth among the rehabilitated plantlets. No marks of shoot or root growth was perceived during storing. Adding sucrose to the media has prohibited dehydration in storing but did not progress shelf-life of germplasm. All the cultures in storing condition were able to form roots during re-growth and effectively adapted in soil rite. Similar reports have predictable post–storage advantageous effect in *Withania somnifera* [30] and *Plumbago zeylanica* [24]. Our studies delivered an active protocol for conservation of this medicinal plant under slow growth conditions. Germplasm can be stored efficiently for 6 months without any subcultures.

The efficaciously rooted plantlets were assignment to a small earthen pot having soilrite and covered with transferent polytheen bags for hardening. Finally, the plantlets were shifted to Poly House and continued 3- 4 weeks and then it transferred to the trial field condition (Figure 1D) and there was 85% survival rate. There was no perceptible difference among the acclimatized plants with respect to morphological and growth individualities. All the micropropagated plants were free from peripheral disease. Numbers of plants producing healthy fruits were perceived and the viability of the seeds was also tested. (Fig. 1D & 1E)


Fig. 1D & 1E

Root tips from the mother plant presented 2n=14 chromosomes; cytological preparations from the *in vitro* derived plantlets (25 root tips and 50 metaphase/ root tip) presented mostly diploid number (2n= 14) of chromosomes. All regenerates so far studied do not show any visible cytological variations (Figure). The chromosome number (2n=14) remains the same without any noticeable structural changes (Fig-1F). Thus the somatic chromosome pair of *in vitro* generated *P. indica* plants continued stable even after passing through three cycles of multiplication. It indicates that reduced growth conditions useful in our studies along with the inherent genotypic potentiality and stability of the species facilitated to maintain germplasm over such a short period of time in preserved and chromosomal equality among the regenerates. This present finding is agreed with some other studies where regenerants are initiate cytological stable [Ghosh and Sen, 1994; Chatterjee and Ghosh, 2015].

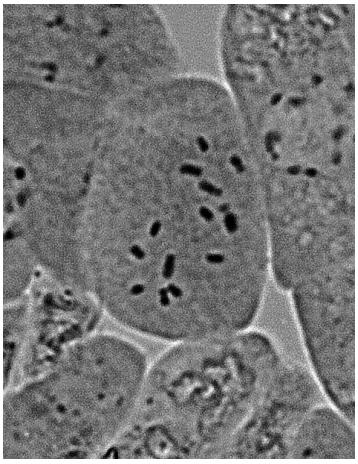

Fig. 1F

In association with this study, a simple and precise HPLC technique has been advanced for the quantification of plumbagin from the root of *P. indica* was useful in crude sample extracts, by associating the retention time with that of the standard sample. A sharp and symmetric peak of plumbagin was

achieved with good baseline resolution and minimal tailing, thus facilitating the accurate measurement of peak area. Satisfactory retention times and good determination of plumbagin was completed using C18 column with mobile phase methanol: water (90:10, v/v). The HPLC analysis was accepted in isocratic condition and detected at a wavelength of 260 nm was got for standard plumbagin. It was saw that concentration of plumbagin crude extract was 1.7% of conventional grown plant and 1.5% of tissue culture raised plant. The differentiation is very minor. Regenerated plants are stable through the evaluation of plumbagin content. In this present study, the suggested phytochemical analysis procedure may be used as an alternative method for quantification and standardization of plumbagin from the stem of *P. indica*. The comparable practice reported by Sivakumar and Dhana Rajan [35], Balajee *et al.*, [36]. (Panichayupakaranant and Tewtrakul 2002; Jaisi et al. 2013).

Our studies provide a simple and operative micropropagation protocol for mass propagation. We also recognized a simple process for keep of culture in the mode of slow growth that plays a significant role for *in vitro* conservation determination. The method also put forward here has the advantages of both simplicity and competence. In connection with this study we also documented a simple protocol to prove the stability of regenerated plants through chromosomal analysis, and plumbagin content evaluation to make it well apposite for commercial propagation and to produce uniform planting materials in large scale for use in pharmaceutical industries.

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