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ORIGINAL ARTICLE

In vitro Induction and Proliferation of Adventitious roots and Estimation of Plumbagin content from *Plumbago auriculata Lam.*

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ABSTRACT
Plumbago is one of the most important Indian medicinal plants belonging to the family Plumbaginaceae due to the presence of Plumbagin, a secondary metabolite. There is a growing demand for plumbago leaves and roots in pharmaceutical trades as it shows therapeutic properties like anti-malarial, anti-microbial, anticancer, anti-inflammatory, and anti oxidant activities. In the present studies, in-vitro root cultures were established by using leaves, nodal segments and apical shoots of Plumbago auriculata as explants. Murashige and Skoog media supplemented with different combinations of auxins and cytokinins were used to study the in vitro root induction. Growth hormones like Indole-3-butyric acid (IBA) and 1-naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA) and 1-naphthalene acetic acid (NAA) along with 6-benzylaminopurine (BAP) and 1-naphthalene acetic acid (NAA) were used independently and in combination to study the root growth from inoculated explants. The best results in terms of root induction were observed by using media supplied with 1.5mg/l IBA. Of the combinations used, best rooting was indicated when two auxins IAA and NAA used in the concentration of 1.5mg/l and 0.1mg/l respectively. The explants were sub-cultured in liquid MS basal media and newly designed G2 media for root elongation. TLC and UV-Visible spectrophotometric analysis of plumbagin was carried out from in vivo root, leaf and stem and in vitro roots. The results showed that concentration of plumbagin in in vitro root samples were higher than that of in vivo plant materials. In vitro generation of roots may give a commercially viable method for plumbagin production for medicinal purposes.
Keywords: *In-vitro root culture; Plumbago auriculata; Plumbagin; spectrophotometric analysis.*

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Abbreviations: MS, Murashige and Skoog; IBA, Indole-3-butyric acid; NAA, 1-naphthalene acetic acid; BAP, 6-benzylaminopurine; NAA, 1-naphthalene acetic acid; TLC, Thin Layer Chromatography.

INTRODUCTION

Medicinal plants are gaining great interest in pharmaceutical industries for the production of high valued secondary compounds [1]. Different extracts from traditional medicinal plants have been successfully tested for their pharmaceutical properties. Many reports show the effectiveness of traditional herbs against microorganisms, as a result, plants have become one of the bases of modern medicine [2]. *Plumbago* is a shrub belonging to the family Plumbaginaceae, vernacularly known as chitraka, chitrakmula, kodiveli etc. [3]. Roots and leaves of plant have potential therapeutic properties like anti-anthrogenic, carditoxic, hepatoprotective, neuroprotective, anti-atherogenic, cardiotonic, hepatoprotective and neuroprotective properties [4]. In Indian traditional medicine, *Plumbago zeylanica* L. has been assigned medicinal properties and is used in formulations of a number of ayurvedic compounds [5].

Roots are the main source of plumbagin, a naphthoquinone derivative (5-hydroxy-2-methyl-1, 4-naphthoquinone, chemical formula-$C_{11}H_8O_3$) of commercial interest for its pharmacological properties such as anticancer [6], antioxidant [4], antimicrobial [7], and antifertility [8], antiallergic [9], antiplasmodial [10] etc.

Interventions of modern biotechnological approaches are necessary to meet with the increasing demands. Multiple use of this plant species have necessitated its large scale collection as raw material to the pharmaceutical industry, leading to over exploitation and disappearing the wild population. The therapeutic use of *Plumbago* is limited due to its insufficient supply from the natural sources as the plants grows slowly and take several years to produce quality roots [11]. There is a need to multiply the root system which has potential bioactive compounds which would be beneficial for the sustainable utilization of this medicinal plant for its bioactive ingredients, thereby provides an alternative method rather than destroying whole plants. Plumbagin is mainly distributed in the cells of secondary cortex and medullary rays of its roots [12]. Adventitious roots are natural, grown vigorously in phytohormone supplemented medium and have shown tremendous potential of accumulation of valuable secondary metabolites [13]. I*n vitro* culture of *Plumbago* roots may give a promising source for plumbagin production. The present studies are carried out to investigate the effect of different explants, plant growth regulators and media on induction and establishment of *in-vitro* root cultures of *P. auriculata,* and to compare the presence of plumbagin concentration from the *in vitro* roots and *in vivo* plant parts. The main aim of the present study was optimization of media using various combinations of growth regulators for different explants, along with designing of subculture media for *in-vitro* roots. Qualitative and quantitative analysis of plumbagin contents were carried out from *in-vivo* and *in-vitro* samples.

MATERIAL AND METHODS

For plant tissue culture standard materials and methods were followed [14].

Plant Material

The plant material of *P. auriculata* was obtained from the premises of Rise n' Shine Biotech Pvt. Ltd. located at Pune District of Maharashtra, India. The plant was authenticated by comparing the morphological characters described in the literature [15]. Leaf, stem and root samples were collected for *in-vivo* studies. For *in vitro* studies, shoot apex, leaves, internodes were used as explants.

Chemicals and reagents

All chemicals and reagents used were of analytical grade. Purified analytical grade plumbagin was procured from Sigma-Aldrich (P7262 Sigma). Standard Stock solution of plumbagin 1mg/ml was prepared in absolute alcohol.

Sterilization of plant material

The explants were cut 1-2 cm and immersed in 0.5 per cent labolene detergent solution and treated with 5% a fungicidal solution for 30 minutes followed by 0.1 per cent $HgCl_2$ (mercuric chloride) for 5 minutes and washed three to four times with sterile distilled water.

Inoculation and Culture Condition

The explants were then inoculated aseptically and incubated at 25±1ºC at 16 hours light period and 8 hours dark, for a period of 30 days and were observed daily for any noticeable changes.

Media: Manipulation of culture media is an important area given attention for increasing secondary metabolite accumulation [16]. MS Media fortified with various growth hormones like NAA (α-Naphthalene acetic acid), IAA (Indole-3-acetic acid), IBA (Indole-3-butyric acid) and 2,4-D (2,4-Dichlorophenoxyacetic acid) and BAP (6- Benzyl amino purine) were checked independently (Table 1) and in combination (Table 2) for *in vitro* root induction. *In vitro* generated roots were subcultured aseptically on subculture media (Table 3).

Extraction

For extraction of plumbagin, method by Arunachalam et al. [17] was followed with some modifications. Dried and powdered *in vivo* leaf, stem, roots and *in vitro* cultured roots were extracted with 70% methanol in 1:10 (w/v) ratio. The solvent was evaporated and dried extract was dissolved in 70% methanol and equal amount of chloroform and water to get a three layer separation. Chloroform was employed as solvent for these extractions, as it is reported earlier as the solvent for naphthoquinone extraction [18 & 19]. Chloroform layer which contained dissolved plumbagin was separated and water soluble impurities were removed. Extract kept for evaporation and 500µl methanol (100%) was added to use as sample for plumbagin analysis.

TLC Analysis

Samples from leaves, stems, roots extracts and *in vitro* generated root extracts were loaded on thin layer of silica gel prepared on glass plate. Solvent system used was methanol: water (9:1). Standard plumbagin (Sigma Aldrich) was used to analyze the presence of plumbagin by comparing Rf values.

Spectrophotometric Analysis

Spectrophotometric Analysis was carried out using Beckman Coulter DU 800 UV-Visible Spectrophotometer following method given by Shalini et al. [20] with required modifications.

Wavelength of maximum absorbance was determined using standard stock solution (1mg/ml) of plumbagin. The absorbance of the colored solution was scanned in the range of 400 to 800 nm against reagent blank. Standard graph was plotted against the concentration of plumbagin. *In vivo* root, stem and leaves sample and *in vitro* root sample were measured for absorbance at 520nm. The concentration of unknown samples was computed from calibration graph or from the regression equation.

RESULTS AND DISCUSSION

Establishment of an efficient and reproducible organogenic system in medicinally important plant species is a pre-requisite for basic research and commercial exploitation. Root cultures can be used in many ways including studies of carbohydrate metabolism, mineral nutrient requirements, essentiality of vitamins and other growth regulators, differentiation of the root apex and gravitropism [21].

1) Root generation from auxins treatment

The production of adventitious roots in plant is controlled by growth substances and a key role in this process being played by auxins [22 & 23]. Of the auxins used, media supplemented with 1.5mg/l IBA exhibited best results for root initiation from stem explants (Fig1. Table1). Similar results were also reported by Sivansen et al. [24] in *plumbago zylanica*. IAA also showed callus mediated root formation at the concentration 1mg/l using stem explants (Fig 2 Table 1). Explants cultured on MS medium containing auxins induced roots from the cut ends and veins.

2) Root generation by using combination of auxins

Among the various combinations of auxins used, media with 1.5 mg/l IAA and 0.1 mg/l NAA showed the best results for rooting in stem and leaf explants (Fig.3 Table 2). Whereas in apical shoot explants the combination of IAA 1.0 mg/lit and NAA 1.0mg/lit indicated highest response. Chinnamadasamy [25] reported that optimum concentration of IAA and BA would be a good inducer of adventitious rooting in *P. zeylanica*. On the contrary, in Malus [26] IAA was effective for the production of roots and NAA strongly inhibited the root formation. Rooting response was also observed on medium containing 0.3 mg/l IBA and 0.6 mg/l NAA in leaves explants (Table-3) followed by stem and shoot apex. Panichayupakaranant [27] has also reported that the combination of 1.0 mg/lNAA and 0.1 mg/l kinetin was found to be best suited for growth promotion in *Plumbago rosea.* Callus induction was also observed in all the explants along with rooting. Various combinations of BAP and NAA indicated negligible response for root induction (Table 3). IAA was found to be the most effective at different concentrations tested for producing roots on the shoot cuttings. These results are in agreement with the previous reports [28 and 29].

3) Sub culturing of *in vitro* generated roots

Explants with root formation in *in-vitro* cultures were further subcultured to different combinations of media (Table 3). Among the media used, G2 media which is combination of BAP and NAA with the concentration 0.2mg/l BAP and 0.02 mg/l NAA exhibited the best response for rooting. The subcultured explants showed well grown root system as well as shoot growth. Root formation is observed on solid as well as liquid G2 media (Fig. 4a, 4b). Positive response was also observed by using liquid MS basal medium devoid of any plant growth regulators (Fig. 5). The stimulatory effect of basal medium on adventitious root induction and root quality has already been reported in *Eclipta alba* by Baskaran & Jayabalan [30].

4) TLC Analysis

The TLC analysis of *in vivo* samples showed that root had most matching profile to the standard plumbagin, followed stem and leaf. TLC with methanol: water solvent system in the ratio 9:1 gave R_f values of standard plumbagin, extracts of root, stem and leaf samples as 0.90, 0.88, 0.73 and 0.82 respectively. Whereas the R_f values of standard and *in-vitro* root samples were 0.82 and 0.87 respectively (Fig 6). TLC analysis of *in vitro* generated roots showed matching profile with the standard plumbagin indicated the presence of plumbagin content in *in-vitro* root sample.

4) Spectrophotometric Analysis for plumbagin content

The maximum absorbance for plumbagin was obtained at 520nm. The linear regression obtained is Y = 0.0122 x - 0.583. The correlation coefficient was found to be 0.9986, which indicated the best suitable standard graph for plumbagin estimation. Present studies indicated highest plumbagin content (0.09%) from root sample, followed by stem (0.022%) and leaf (0.014%) samples from *in vivo* plant samples. In case of *in vitro* generated roots the plumbagin content was found to be 0.38%. (Graph1). Present observation indicates that plumbagin content is higher in *in-vitro* generated roots than that of *in-vivo* stem, leaf and roots. These results are similar to that of Lenora et al. [31**]**, who reported that maximum content of plumbagin, in roots of tissue cultured field grown plants.

Present study reveals that for the induction and proliferation of adventitious *in vitro* root culture of *Plumbago auriculata* the media having the combination of 1.5 mg/l IAA and 0.1 mg/l NAA proved to be the best. Whereas for subculture, it was seen that G2 media having a concentration of 0.2 mg/l BAP and 0.02 mg/l NAA gave the best results for roots proliferation. Using the spectrophotometric methods for the comparison between *in vivo* plant parts and *in vitro* roots it was observed that a higher percentage (%) of plumbagin content can be obtained from *in vitro* roots. Development of an efficient and reproducible regeneration protocol from cells/tissues holds a tremendous potential for the production of high-quality plant based medicines [32]. Present investigation can be useful for the production of plumbagin on large scale. *In vitro* generation of *plumbago* roots will serve as a continuous supply with minimum space and efforts for medicinal purpose and will contribute to preserve the natural plant recourses. Further standardization of large scale cultures of *in-vitro* roots can give a commercially viable method for plumbagin production.

Table 1: *In vitro* root generation in Media using Auxins IAA & IBA

		Degree of root formation in Explants		
Hormone	**Concentration (mg/l)**	**Shoot apex**	**Stem Internodes**	**Leaves**
IAA	0.50	**+**	**++**	**++**
	1.00	**++**	**+++**	**++**
	1.50	**+**	**+**	**++**
IBA	0.5	**++**	**++**	**+**
	1.0	**++**	**++**	**++**
	1.5	**++**	**++++**	**++**

- = no roots formed, + = low roots, ++ = good roots, +++ = very good roots, ++++ = excellent root.

Table 2: Effect of combinations of auxins and cytokinins and auxins on the *in vitro* root generation of different explants of *Plumbago auriculata.*

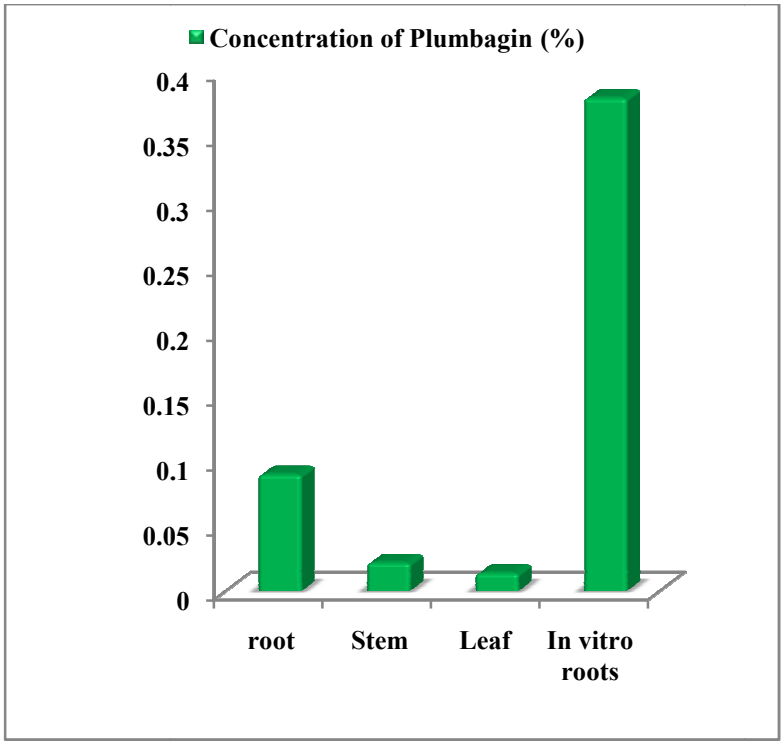
Hormone	**Concentration (mg/l)**	**Degree of root formation in Explants**		
		Apical shoot	**Stems Inter-nodes**	**Leaves**
IBA + NAA (mg/l)	0.3 + 0.6	**+**	**++**	**+++**
	1.0 + 1.5	**-**	**-**	**-**
	2.0 + 1.5	**-**	**-**	**-**
	1.5 + 1.0	**+**	**+**	**+**
	1.5 + 2.0	**+**	**++**	**++**
	0.5 + 1.0	**-**	**-**	**-**
IAA + NAA (mg/l)	0.2 + 0.2	**-**	**+**	**+**
	0.5 + 1.0	**-**	**+**	**+**
	1.0 + 1.0	**-**	**++**	**++**
	1.5 + 1.0	**+**	**+**	**+**
	1.0 + 1.5	**++**	**++**	**++**
	0.5 + 0.5	**-**	**-**	**-**
	1.5 + 0.1	**++**	**+++**	**++++**
BAP + NAA (mg/l)	0.5 + 1.75	**-**	**-**	**-**
	0.75 + 1.50	**-**	**-**	**-**
	1.0 + 1.25	**++**	**++**	**++**
	1.25 + 1.0	**-**	**-**	**-**
	1.50 + 0.75	**+**	**+**	**+**
	1.75 + 0.50	**-**	**-**	**-**

- = no roots formed, + = low roots, ++ = good roots, +++ = very good roots, ++++ = excellent root.

Table 3: Sub-culturing of *in vitro* generated roots on subculture media

Hormone (mg/l)	Concentration	Root formation
MS liquid basal media without plant growth -hormones.	----	+++
S7 (IAA+NAA)	1.5 + 0.1	++
S5 (IAA+NAA)	1.0 + 1.5	++
G2 (BAP+NAA)	0.2 + 0.02	++++

- = no roots formed, + = low roots, ++ = good roots, +++ = very good roots, ++++ = excellent root.



Graph 1: Estimation of Plumbagin in different samples using spectrophotometric analysis.

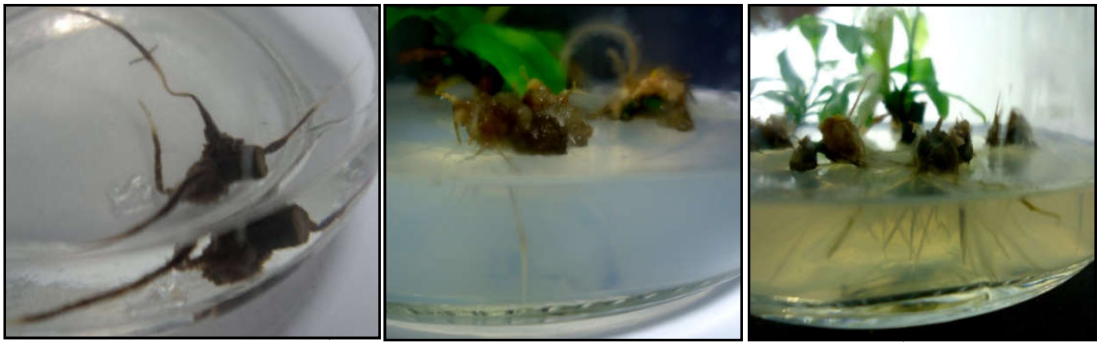


Fig1 **Fig 2** **Fig 3**

Fig1. Root culture on media supplemented with 1.5 mg/l IBA using stem explants.
Fig2: Callus mediated development of roots using media IAA 1mg/l using stem explants.
Fig3: Root formation using the media combination IAA 1.5 mg/l and NAA 0.1 mg/l using leaf explant.

Fig 4 (a)

Fig 4 (b)

Fig 5.

Fig4: Sub culturing of explants on G2 media a) solid b) liquid.
Fig 5: Sub culturing of explants on liquid MS Basal media devoid of any growth regulators.

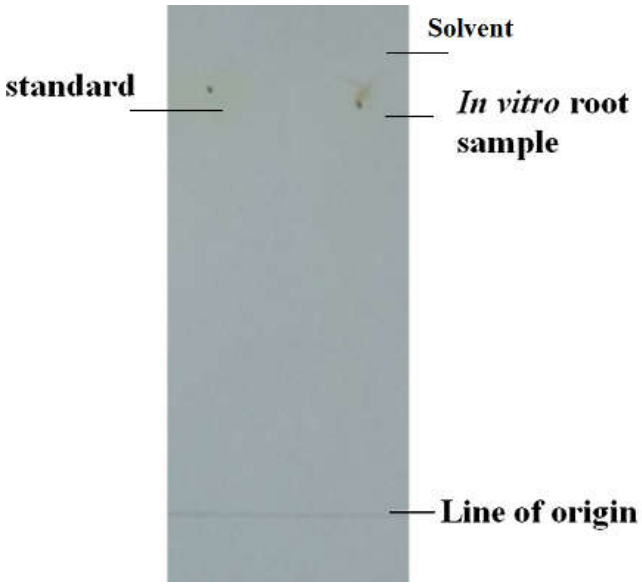


Fig 6: TLC analysis of Standard plumbagin and *in vitro* root sample.

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