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Direct Shoot Regeneration of *Plumbago zeylanica Linn.* through Tissue Culture Technology

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Chitrak is scientifically known as Plumbago zeylanica Linn. belongs to Plumbaginaceae. Root shows medicinally valuable drug in pharmacological enterprise as the humanity is certainly prone to numerous contagious ailments due to ecological imbalance triggered by the current technology development. One of its remarkable phytoconstituents is plumbagin, a naphthoquinone molecule used to treat skin conditions, tumours, and tenacious chronic rheumatoid arthritis. The objective was to standardize the procedure of Plumbago zeylanica direct-organogenesis micropropagation. The solution contained various concentrations and combinations of auxin and cytokinins, and the nodal explant was used for quick micropropagation. The maximum multiple shoots (18.24 ± 0.51) per explant with shoot length 4. 2cm ± 0.20 cm was produced as the MS medium supplemented with B5 vitamins together with 2mg/l 6- Benzyleaminopurine (BAP) and 1mg/l Indole-3- acetic acid (IAA). The regenerated shoots were rooted in half strength MS medium with B5 vitamins that contained indole-3-butyric acid (IBA). The highest rooting frequency (100%) was seen in the 1.5 mg/l IBA-containing rooting media. The extreme roots were 9.2±0.14 per explant. The average root length was 4.2± 0.10 cm. The in vitro rooted Shoots were then transplanted in the field showing a 100 percentage survival rate.

Keywords: Direct Shoot Regeneration; Nodal segment; Plumbago Zeylanica; Plumbagin; Shoot multiplication.

Day by day the demand and usage of medicinal plants are ever growing due to its medicinal activity without causing any side effects[1]. The World Health Organization (WHO) holds the views that close to eighty percentage of the folks consume herbal medicine through various forms either directly or the extract of them[2]. As stated by Mandavkar[3], the genus Plumbago has three species: *Plumbago indica, Plumbago capensis, and Plumbago zeylenica*. *P. zeylanica* which is commonly known as Ceylon Leadwort is one of the medicinal plants used extensively by rural and tribal people more than hundred years[4] as traditional medicine. Various extracts of the solvents showed abundant phytochemicals viz: phenol, tannins, protein, flavonoids and carbohydrates[5]. Moreover, the root part contains more plumbagin, a naphthoquinone[6] bioactive component associated with pharmacological activities such as anticancer, antimicrobial, wound healing, antidotes for snake bite, mental disorder and body pain. The leaf extract is utilized for skin disease[7] whereas

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the root is to cure headache, cough and cold in Madhya Pradesh. The community known as *Thottianaickans* mixes the root powder with the goat milk to prevent recurrent urination[8]. Of the six compounds characterized from Plumbago zylencia, Phenol, 2,4, bis (1,1-dimethyl ethyl) -(7311) under *in silico* showed better interaction with matrix metalloproteins in oral squamous cell carcinoma[9]. The large usage of the plant parts leads to greater exploitation. Due to poor seed laying in natural field settings, it is difficult to propagate chitrak by seed germination[10]. Therefore, a modern technique is required for the rapid proliferation of *Plumbago zeylanica*. *In vitro* culture method is used widely both to propagate medicinal plants and to conserve them. Numerous explants, including shoot tips[11], auxiliary buds[12], leaf discs[13] and nodal segments[14] were to be used successfully to produce regenerable cultures either through somatic embryogenesis or organogenesis with varying degree of success.

Several research work had been carried out using MS medium with the combination of Auxin and Cytokinins along with additives. However not much research has been conducted across the world for mass propagation of *Plumbago zeylanica* by means of MS medium enriched with B_5 vitamins. Therefore, the current study is to develop a very efficient micropropagation protocol from nodal segments of *Plumbago zeylanica* using the MS medium supplemented with B5 vitamins. The protocol also was standardized to reintroduce the regenerated plantlets to the natural habitat.

MATERIALS AND METHODS

Plant resource and preparation of explant

The viable and healthy nodal explants were collected from one-year-old plant at herbal garden Loyola College, Chennai-34. The explants were cut into 4-6cm and thoroughly rinsed for 15 minutes under running water. They were carefully cleaned with sterile distilled water after being washed with 1 percentage of Tween -20 solution. The explants were immersed in 0.2% (w/v) bavistin for 10 minutes following this sterile distilled water used thrice to wash. The sterilized nodal segments were exposed to 0.1% (w/v) HgCl2 for three-minute before being properly rinsed three times using double distilled sterile water (DDW). The double distilled sterile H_2O was used to rinse the explants and placed them on the whatman filter paper for air dry. After a few minutes, the nodal segments were excised into small segments and used as explant for micropropagation.

Medium: preparation and its conditions

The MS medium with B_5 vitamins was used throughout the experiments. The medium with different concentrations of plant growth hormones had 3% (w/v) sucrose. The medium that had no plant growth regulators was considered as a control. The medium's pH was changed to 5.7. with 0.1 N of NaOH solution or 0.1N and 1N of HCl solution beforehand incorporating agar at 0.8% (w/v). Each test tube (Borosil) was filled with molten media (10 mL), capped with cotton-plugs, then autoclaved for 15 minutes at 121ÚC. White fluorescent tube lights were used as source of light for the cultures with a 16-hour photoperiod of light with the temperature of 24 ±2ÚC. The level of light was 50 µmol m^{-2} $s^{-1.}$

Direct shoot proliferation

The excised nodal segments of *P. zeylanica* were inoculated individually on the MS medium with B_5 vitamins supplemented with different cytokinins alone, BAP (0.5 - 4.0mg/l) and with the combinations of auxin, IAA (0.25 – 1.5mg/l). The primary shoot initiation occurred after 6 days of the inoculation. Multiple shoots proliferated as the primary shoots were sub cultured. The experiment was repeated thrice at two weeks' intervals onto their appropriate medium. The number of shoots and the percentage of the shoot induction were reported subsequently after first subculture.

Root induction

Each proliferated shoot (2-3 cm length) was separated from shoot mass bunch. Each one was inoculated to induce root. The medium contained full strength MS medium with B5 vitamins with 3 percentage of sucrose (w/v) and 0.8 percentage (w/v) agar. The MS medium had been augmented with dissimilar auxin concentrations Indole-3-butyric acid, indole-3-acetic acid and 1-Naphthalene Acetic Acid (0.5 - 4.0 microgram per liter) and in combinations of auxin IBA with NAA and IAA (0.25- 2.0mg/l). The rooted plantlets were then washed carefully with distilled and were kept in liquid medium for a week. After twenty-one days of inoculation, the total number of roots along with its length for per shoot were measured.

Acclimatization

The plantlets that developed roots were implanted in medium sized pot comprising the mixture of sterile soil, coco pit, vermicomposting and red sand (1:1:1:1). The pots were moistened with 1/4th strength MS medium with B_5 vitamins without sucrose and plant growth hormones for a month till the emerging of new leaves. The regenerated plantlets were planted in bigger size pots and kept them in green house three weeks followed by transplantation in the field.

Data: Collection and Analysis

Each experiment was performed three times with the minimum 25 explants per experiment in all tryouts. The average percentage of explants that responded, the average total of shoots with its length per explant, and the average amount of roots and its length were all noted. SPSS v 20.0 was used for a statistical analysis on the data, and the experiment's mean data were represented as mean standard error (SE).

RESULT AND DISCUSSION

Direct shoot proliferation

The nodal explants of *P. zeylanica* were proliferated in a short span of time under *in vitro* condition. Nodal segments (36 - 96%) exhibited good response to direct shoot proliferation (Table1). The type and concentration of cytokinins with the combination of auxin particularly IAA had a significant effect on the abundant proliferation of shoots per explant and the mean length than BAP and NAA combination. The MS medium with B5 vitamins supplemented with BAP 2 mg/l and IAA 1 mg/l exhibited the highest response (96%) and produced maximum shoots 18.24 ± 0.51 shoots and 4.22 cm in length per explant (fig. 1E & H, Table 1). The current investigation was in consistent with the earlier study of shoot propagation in *Saussurea involucrata*[15] and *Coleus blumei*[16]. The propagation of shoots in Jojoba (*Simmondsia chinensis* (Link) Schn.) was facilitated well as the MS medium is supplemented with B_5 vitamins[17].

Table 1. Effects of plant growth regulators (PGRs) on direct shoot induction of *P. zeylanica*

Plant growth regulators (mg/l)			Shoot induction (%)	No. of shoots/ explants Mean	Mean shoot Length (cm)
Control	-				
BAP	NAA	IAA			
0.5	0	0	24	3.2±0.22	1.74
1.0	0	0	32	3.68±0.11	1.86
1.5	0	0	36	3.08±0.17	1.56
2.0	0	0	32	4.68±0.17	2.02
2.5	0	0	28	4.56±0.13	2.76
3	0	0	20	3.48±0.1	1.36
4	0	0	16	6.84±0.16	2.06
0.5	0	0.25	36	8±0.24	2.76
1.0	0	0.50	48	3.8±0.15	1.76
1.5	0	0.75	60	7.8±0.17	2.54
2.0	0	1.0	96	18.24±0.51	4.22
2.5	0	1.25	84	5.8±0.17	2.48
3	0	1.50	72	7.72±0.2	2.58
4	0	2.0	48	10.28±0.26	2.74
0.5	0.25	0	20	5±0.15	1.48
1.0	0.50	0	24	3±0.14	1.44
1.5	0.75	0	32	7.56±0.1	1.73
2.0	1.0	0	40	3.56±0.1	1.52
2.5	1.25	0	28	1.56±0.1	1.16
3	1.50	0	20	4.16±0.15	1.48
4	2.0	0	12	1.68±0.1	0.82

Table 2. Effects of plant growth regulators (PGRs) on root induction of *P. zeylanica*

Plant growth regulators (mg/l)			Root induction (%)	No. of roots/ shoot Mean	Mean root Length (cm)
Control	-				
IBA	NAA	IAA			
0.5	0	0	40	1.68±0.1	1.82
1.0	0	0	60	0.52±0.1	0.48
1.5	0	0	92	2±0.16	1.52
2.0	0	0	68	2.08±0.15	2.10
2.5	0	0	48	3.12±0.19	1.64
3	0	0	32	3±0.17	1.60
4	0	0	16	9.2±0.14	4.24
0.5	0	0.25	32	2.32±0.1	2.00
1.0	0	0.50	40	3.24±0.27	2.00
1.5	0	0.75	36	2.56±0.1	2.58
2.0	0	1.0	44	3.04±0.17	3.08
2.5	0	1.25	32	4.16±0.16	2.52
3	0	1.50	24	2.28±0.15	1.96
4	0	2.0	20	4.28±0.16	3.04
0.5	0.25	0	28	3.32±0.22	2.16
1.0	0.50	0	36	1.4±0.1	2.40
1.5	0.75	0	32	2.04±0.16	2.56
2.0	1.0	0	28	3.12±0.18	1.60
2.5	1.25	0	20	0.72±0.09	0.48
3	1.50	0	20	1.4±0.22	1.12
4	2.0	0	12	1.4±0.1	1.44

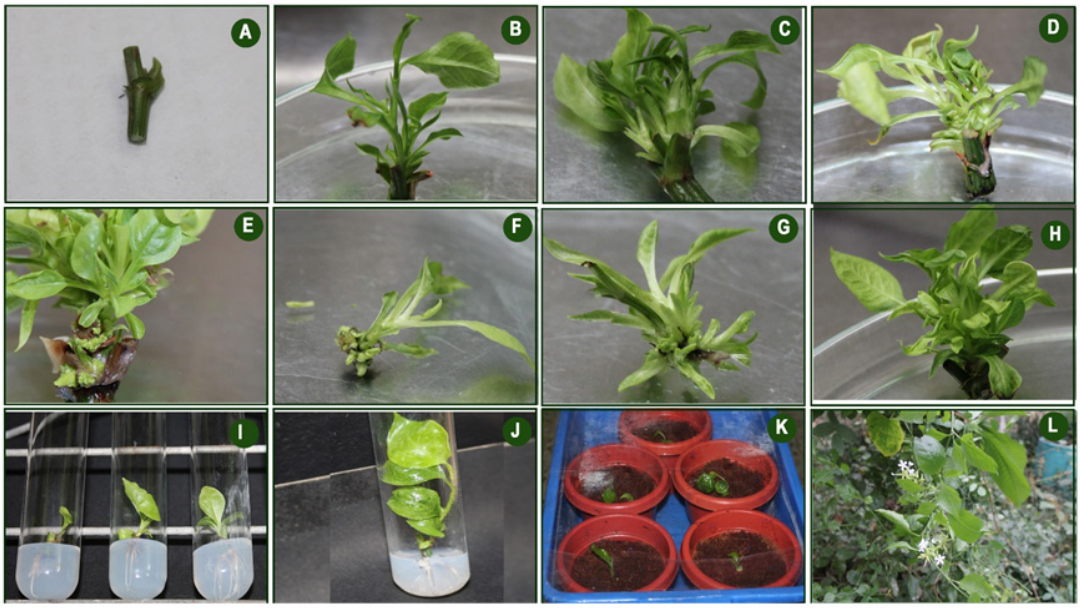

Fig. 1. Stages of plant regeneration of *Plumbago zeylanica*. A. Nodal explant; B – E. Different developmental stages of direct shoot proliferation from the cultured nodal explant after 20 days; F -G. Initiation of multiple minute shoot regeneration from the node after 30 days of the inoculation and development of them; H. Multiple shoots from the single nodal explant; I. Root initiation and development of *Plumbago zeylanica;* J. Regeneration of whole plant; K. Adaptation of regenerated plants; and L. Flowering of regenerated plant showing in the field

Root induction

For the root induction of the shoots, half-strength MS medium having B5 vitamins in addition various auxin concentrations, either alone or in combination, has been described (Table 2). Once the shoots were inoculated on the half-strength Murashige and Skoog medium with B5 vitamins supplemented with IBA 1.5mg/l alone compared to the absence of PGR tubes, more roots were formed. Among three auxin (IBA, NAA and IAA) IBA was most effective to induce root than IAA and NAA in Table 2. MS (half volume) medium with B5 vitamins complemented with IBA (1.5 mg/l) prompted 9.2± 0.14 roots per shoot

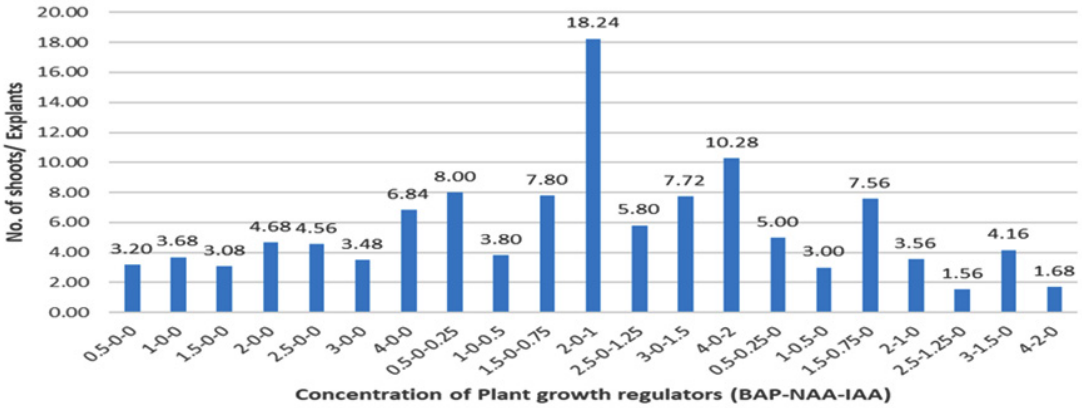

Fig. 2. Number of shoots / explants of *Plumbago zeylanica*

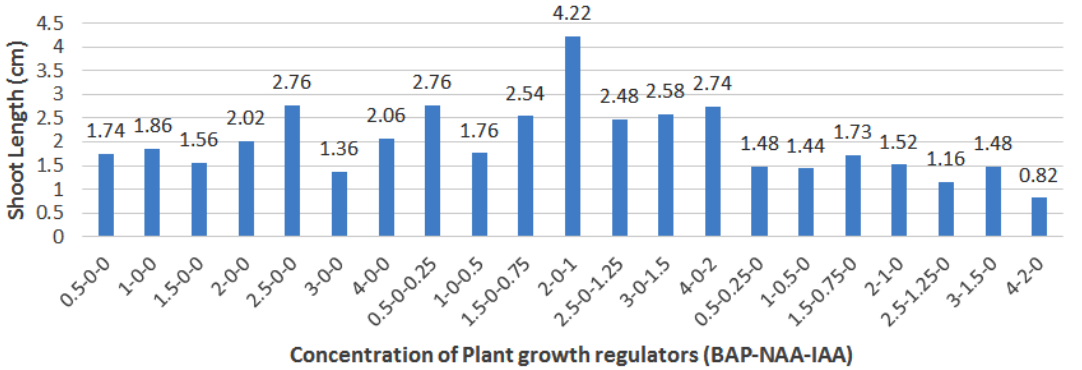

Fig. 3. The shoot length (cm) of the propagated plants of *Plumbago zeylanica*

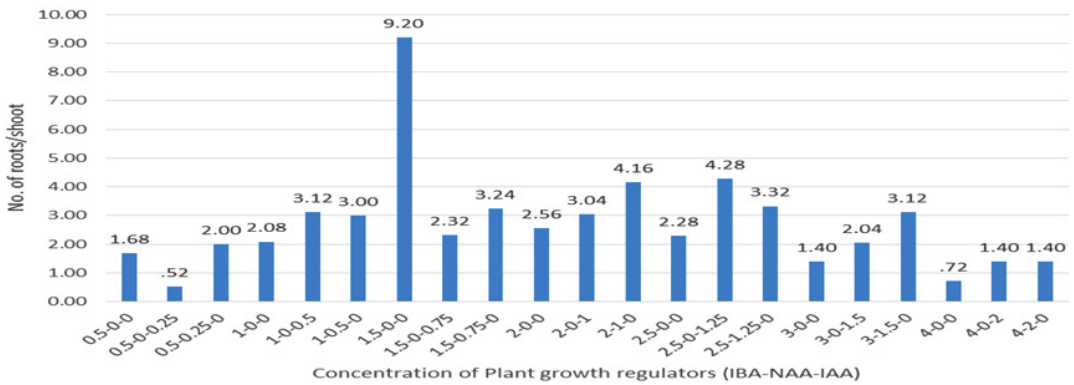

Fig. 4. Number of roots / explants of *Plumbago zeylanica*

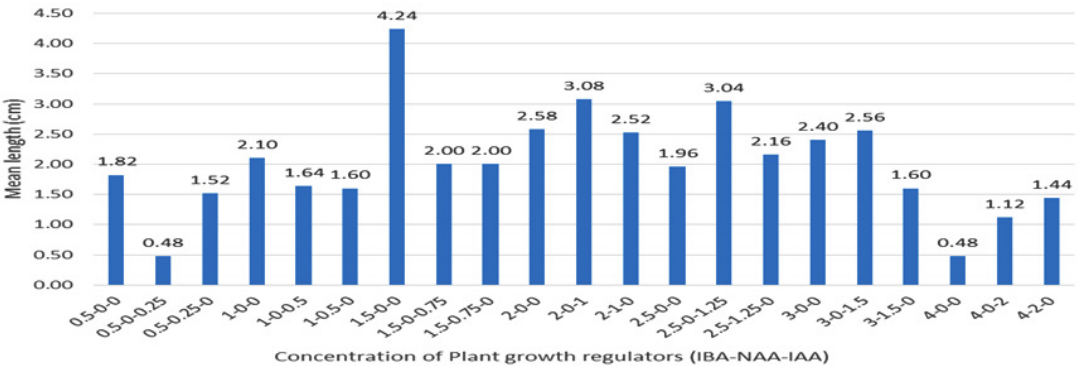

Fig. 5. The Root length (cm) of the propagated plants of *Plumbago zeylanica*

with mean root size of 4.2 cm (Table 2 & fig. 4&5). Similarly, Ceaser[18] (*Bacopa monnieri*) and Srivastava[19] (*Portulaca grandiflora*) reported the effective root induction by IBA.

Acclimatization

The rooted plants were planted in pots having combination of sterile soil, coco pit, vermicomposting and red sand with equal proportion (fig. 1K). The pots were well-looked-after in green house nearly a several days and planted in the field with 100% survivability. The redeveloped plants were as identical as the mother plant in morphology (fig.1L).

CONCLUSION

For the direct shoot-regeneration from the nodal explants (*Plumbago zeylanica),* an effective technique has been designed. The combination of BAP (2mg/1) and IAA (1mg/1) was found more successful to induce highest number of shoots. The whole saplings were efficaciously developed in the field settings with a 100% survivability using half-strength MS media with B5 vitamins. The conservation of the medicinal plant of *Plumbago zeylanica* would be accomplished using the tissue culture procedure and also to produce more secondary metabolites especially plumbagin for the therapeutics reasons for the future generations.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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