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Shoot multiplication and direct organogenesis of an important medicinal plant *Plumbago zeylanica* L. (Plumbaginaceae).

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ABSTRACT:

Efficient micropropagation protocol was developed for *Plumbago zeylanica* L., a species threatened due to over exploitation for medicinal purposes and habitat destruction in Southern Peninsular India. Multiple shoot induction was more successful using nodes as explants on Murashige and Skoog's (MS) medium supplemented with 1mg/L benzyl amino purine (BAP) . Shoots, when transferred to MS medium containing 0.2 – 0.5 mg/L gibberellic acid (GA3) showed variable elongation. Further, MS medium fortified with 1.5 mg/L BAP induced highest frequency of shoots through adventitious *de novo* organogenesis. Shoots developed were rooted on full strength MS medium with either α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA). Optimum shoots and root multiplication were obtained within 8 weeks. *In vitro* derived plantlets were successfully weaned and transferred to soil and showed 90 % survival rate.

Keywords:
Plumbago zeylanica L., medicinal plant, caulogenesis, rhizogenesis, *In vitro* rooting, *de novo* organogenesis.

Abbreviations:
Murashige and Skoog's (MS), Benzyl Amino Purine (BAP), Gibberellic Acid (GA3), α-Naphthalene Acetic Acid (NAA), Indole-3-Butyric Acid (IBA), Indole-3-Acetic Acid (IAA), Mercuric Chloride (HgCl2).

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INTRODUCTION

Plumbago, reported to comprise 10-20 species (family Plumbaginaceae), is native to warm temperate to tropical regions of the World. It can be propagated by seeds or cuttings. *Plumbago zeylanica* L. is a perennial rambling subscandent under shrub distributed throughout India. Flowers are white with conspicuous glandular persistent calyx. The root is a rich source of alkaloid plumbagin, a naturally occurring naphthoquinone, which is reported to have wide pharmaceutical applications such as antibacterial, antifungal, anti-fertility and anti-tumor properties (Kini *et al,* 1996).

The roots are used in many Ayurvedic preparations for the treatment of diseases like diarrhoea, dyspepsia, rheumatism, anasarca and piles. It is also efficiently used as a diuretic, caustic and expectorant. The root extract is made into a paste and applied externally in leprosy and other skin diseases. The increasing demand of herbal medicine is due to their fewer side effects in comparison to synthetic drugs and antibiotics (Krishnaswamy and Purushothaman, 1980). The gradual decline in the population of this species demands the launching of conservation efforts so as to ensure continuous and ample supply by establishing a balanced cycle of harvest and renewal. Such conservation strategies would ensure immense availability of this valuable medicinal herb which is in great demand by the pharmaceutical industry. Only a small percentage of medicinal plants, used in the industry are cultivated. Most of them are collected from the wild, very often in a destructive and unsustainable manner. Keeping the above facts in mind, the present study was undertaken to develop a suitable protocol in the species for the rapid propagation through shoot multiplication and *de novo* organogenesis.

MATERIALS AND METHODS

Plant material

Nodal segments and leaf segments of *Plumbago zeylanica* L. (Plumbaginaceae), collected from the green house, Department of Botany, University of Kerala, Kariavattom, were washed in running tap water followed by soaking and washing with 10% labolene solution (a commercial neutral detergent, Qualigens, India) for 15 min. The explants after washing with sterile distilled water, were surface disinfected with HgCl2 (0.1% w/v for 8 min) . After repeated washes with sterile double distilled water to remove traces of HgCl2, the explants were trimmed to appropriate sizes and inoculated.

Medium and culture conditions

The explants were cultured in Murashige and Skoog's (1962) medium (MS) containing various growth regulators (BAP, Kinetin, NAA, IAA, IBA) at different concentrations either alone or in combinations. The dispensed media, after adjustment of the pH to 5.7 ± 1, were autoclaved at 121°C and 15 lbs pressure for 20 min. All the cultures were maintained at 24 ± 2°C under 12h photoperiod with 3000 lux light intensity using fluorescent lights at 60- 70% relative humidity. Ten cultures were raised for each treatment and all experiments were repeated thrice.

Caulogenesis

The nodes of 0.5-1.0 cm were inoculated on MS medium containing BAP and KIN alone at concentrations (0.5-3 mg/L) were used for multiplication of shoots whereas BAP or KIN alone (1-4 mg/L) or in combination with IAA (0.1-0.2 mg/L) were used for direct regeneration from nodes and leaves. Within 10 days, rapid growth has been observed and the explants were sub cultured after every 28-30 days.

In vitro rooting

Two to three centimeters of well-developed shoots were excised and inoculated on MS medium with 3% sucrose containing individual concentration of (0.5-2 mg/L) IAA, IBA, and NAA. The combination of two different auxins, NAA+IBA (data not presented) were also studied.

Hardening and Acclimatization

Micro shoots with well-developed root systems were transferred directly to small pots containing sterile vermiculite and coco peat in (1:1) ratio and nourished with half strength MS basal liquid medium. Survival rate of the plantlets and the plantlets established in the field were recorded.

Experimental design and statistical analysis

All experiments were carried out in a randomized design, ten replicates were raised for each treatment and experiments were repeated thrice. The data were analyzed statistically using one way analysis of variance (ANOVA), and the data, mean ± SD of at least three different experiments were represented and compared using Tukey-Kramer multiple comparisons test with the level of significant $P < 0.05$.

RESULTS AND DISCUSSION

The morphogenetic responses of nodal explants to various cytokinins (BAP, Kinetin) are represented in the table (**Table 1**). Nodal explants cultured on growth regulator free MS medium

Table 1. Effect of different concentrations of cytokinins on MS medium for shoot multiplication in *Plumbago zeylanica*

Cytokinin concentration	Cytokinin concentration	Mean number of shoots after 30 days * (mg/L)
BAP	BAP	
0.5	0	9.0 ± 0.25
1.0	0	20.2 ± 0.32
1.5	0	15.7 ± 0.12
2.0	0	8.0 ± 0.84
2.5	0	5.7 ± 0.12
3.0	0	4.5 ± 0.12
0	0.5	6.4 ± 0.54
0	1.0	12.8 ± 0.32
0	1.5	8.5 ± 0.18
0	2.0	6.2 ± 0.74
0	2.5	4.8 ± 0.25
0	3.0	3.7 ± 0.25

***Values represent mean ± SD of ten replicates and the experiments were repeated thrice. The level of significance at 5% probability level.**

showed no sign of proliferation even after two weeks. Addition of a cytokinin was essential to induce multiple shoot formation from the explants. Of the two cytokinins tested, BAP was more effective than KIN. The nodal segments responded by an initial enlargement of the dormant axillary buds followed by bud break within a week, and multiple shoot induction and proliferation within four weeks of culture. The frequency of axillary shoot proliferation and the number of shoots per explant increased with increasing concentration of BAP up to some extent (Table 1)**.** BAP 1mg/L showed the highest shoot multiplication (20.2 ± 0.32) ability ($P < 0.05$) (**Fig. 1**). The results were found significant at 5% level. Thus, the results strongly suggest that the BAP is the most effective plant growth regulator for inducing multiple shoots in *P. zeylanica*. These results were in consonant with the multiple shoot induction reported in *Ceropegia intermedia* (Karuppusamy *et al.,* 2009), *C. bulbosa* (John Britto *et al.,* 2003), *C. candelabrum* (Beena *et al.*, 2003) and *Centella asiatica* (Karthikeyan *et al.,* 2009).

Multiple shoots obtained on MS medium containing 1mg/L BAP after two weeks of culture when transferred to medium supplemented with 0.2 -0.5mg/L GA3 showed variable rate of elongation. Maximum elongation was observed when the medium fortified with 0.5mg/L GA3 (**Fig. 2**). The results were found significant at 5% level. The stimulatory effect of GA3 in the elongation of shoot bud is because it promotes cell division and elongation in the sub apical zone of the shoots (John *et al*., 1997). The role of GA3 on shoot differentiation and elongation has been reported in *Withania somnifera* (Sivanesan *et al.,* 2007).

Adventitious *de novo* organogenesis was achieved from leaf explants after 12 days of incubation. Among the various combinations of plant growth regulators used, cytokinins alone were very effective in inducing direct morphogenesis. The results are in corroboration with the organogenesis obtained in other plants (Faisal and Anis, 2006; Shinde *et al.,* 2009). Presence of BAP (1.5 mg/L) alone in the medium induced the initiation of about fifteen shoots directly from the explants after four weeks of culture (Fig. 3). Further increase in concentration of BAP or KIN resulted in the development of lesser number of shoots (Table **2**). Higher levels of cytokinin may be supra optimal

Table 2. Effect of different concentrations of growth regulators on MS medium for Direct organogenesis in *Plumbago zeylanica*

Hormones mg/L			Mean number of shoots after 30 days * (mg/L)
BA	KIN	IAA	
1	0	0	8.6 ± 0.12
1.5	0	0	15.2 ± 0.25
2	0	0	9.3 ± 0.74
3	0	0	5.6 ± 0.29
4	0	0	3.8 ± 0.35
0	1	0	6.8 ± 0.18
0	1.5	0	10.3 ± 0.25
0	2	0	7.0 ± 0.31
0	3	0	4.6 ± 0.54
0	4	0	3.2 ± 0.32
1	0	0.1	6.3 ± 0.23
1.5	0	0.1	10.3 ± 0.20
1.5	0	0.2	7.0 ± 0.31
2	0	0.1	5.8 ± 0.51
3	0	0.1	4.1 ± 0.12
4	0	0.2	3.0 ± 0.35
0	1	0.1	5.6 ± 0.31
0	1.5	0.1	8.8 ± 0.28
0	1.5	0.2	7.6 ± 0.12
0	2	0.1	4.9 ± 0.43
0	3	0.1	3.8 ± 0.18
0	4	0.2	2.7 ± 0.25

***Values represent mean ± SD of ten replicates and the experiments were repeated thrice. The level of significance at 5% probability level.**

for shoot regeneration. Of the two cytokinins used, BAP was found to be most effective for producing maximum number of shoots. Superiority of BAP over KIN on *de novo* organogenesis was reported earlier in *Eryngium foetidum* (Gayathri *et al.,* 2006) and *Phyllanthus niruri* (Karthikeyan *et al.*, 2007). The synergistic effect of cytokinins and auxins also favored direct regeneration of shoots. BAP (1.5mg/L) with 0.1mg/L IAA induced maximum shoot regeneration. Combination of BAP and IAA favored shoot initiation was noted in the case of *Capsicum annuum* (Sobhanakumari and Lalithakumari,, 2003). The results were found significant at 5% level.

Individual concentration of IAA, IBA, NAA and combination of two different auxins were used for *in vitro* rooting. IBA 1.5mg/L induced maximum number of roots. The present result coincides with the observation obtained by others (Abri and Staden, 2001; Vadawale *et al* ., 2004). The roots induced in NAA containing medium were long but less in number. However, the combination of two different auxins IBA + NAA had the maximum impact on the elongation of roots.

The well developed plantlets were transferred to plastic cups containing autoclaved vermiculite and coco peat in 1:1 ratio. The plantlets were acclimatized in a mist chamber. The humidity was maintained at 95 % in the initial days. Later on, the percent of humidity was decreased by pricking the plastic cover with a needle. On the 30th day, the plants were transferred to the pots. The survival rate of plantlets was 90% (**Fig. 4**). The results were found significant at 5% level. The plantlets were successfully adapted to the natural environment and exhibited their similarity with that of mother plants. The present study therefore projects the successful micropropagation protocol established that can be employed in the propagation of *Plumbago zeylanica* L. for its conservation and domestication.

Fig. (1-4). *In vitro* shoot multiplication and direct shoot regeneration of *Plumbago zeylanica L.:*

1. **Multiple shoot induction on MS meium + 1mg/L BAP.**
2. **Elongation of multiple shoots on MS medium + 0.5 mg/L GA_3.**
3. **Adventitious shoots on MS medium + 1.5 mg/L BAP.**
4. **Acclimatized plantlet.**

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REFERENCES

Abri AL, Van Staden J. 2001. Micropropagation of the endangered *Aloe polyphylla, Plant Growth Regulat.*, 33:19-23.

Beena MR, Martin KP, Kirti B, Hariharan M. 2003. Rapid in vitro propagation of medicinally important *Ceropegia candelabrum*, *Plant Cell Tissue Organ Cult*., 72:285-289.

Faisal M, Anis M. 2006. Thidiazuron induced high frequency axillary shoot multiplication in *Psoralea corylifolia*. *Biol Plant.*., 50:437-440.

Gayathri MC, Madhu M, Kavyashree R, Dhananjayan, SP. 2006. A protocol for in vitro regeneration of Eryngium foetidum L.*, Indian J Biotechnol.,* 5:249-251.

John Britto S, Natrajan E, Arockiasamy DI. 2003. In vitro flowering and shoot multiplication from nodal explants of *Ceropegia bulbosa* Roxb. Var. bulbosa. *Taiwania.* 48:106-111.

John S, Soniya EV, Valsala K, Nair GM. 1997. In vitro adventitious shoot formation from mature leaves and leaf derived calli of *Naregamia aleata* W & A. *Indian J Exp Biol.,* 35:1249-1251.

Karthikeyan K, Chandran C, Kulothungan S. 2009. Rapid clonal multiplication through in vitro axillary shoot proliferation of *Centella asiatica* L., *Indian J Biotechnol.,* 8:232-235.

Karthikeyan K, Chandran C, Kulothungan S. 2007. Rapid regeneration of *Phyllanthus niruri* L. from shoot tip and nodal explants*, Indian J Appl Pure Biol.,* 22:337-342.

Karuppusamy S, Kiranmai C, Aruna V, Pullaiah T. 2009 In vitro conservation of *Ceropegia intermedia* – an endemic plant of south India, *African Journal of Biotechnology.*, 8 (17):4052-4057.

Kini DP, Pandey S, Shenoy BD, Singh UV, Uduppa N. 1996. Antitumor and antifertility activities of plumbagin scontrolled release formulations, *Indian J Exp Biol.,* 35:374-379.

Krishnaswamy M, Purushothaman, KK. 1980. Plumbagin a study of its anticancer, antibacterial and antifungal properties *Indian J Exp Biol.,* 18:876 -877.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant..,* 15:473-479.

Shinde AN, Malpathak N, Fulzele DP. 2009. Induced high frequency shoot regeneration and enhanced isoflavones production in *Psoralea corylifolia*, *Rec Nat Prod.*, 3:38-45.

Sivanesan I. 2007. Direct regeneration from apical bud explants of *Withania somnifera* Dunal. *Indian J Biotechnol.,* 6:125-127.

Sobhakumari VP, Lalithakumari D. 2003. Direct plant regeneration from shoot tip cultures of *Capsicum annuum* L. cv PLR-1, *Phytomorphology* 53:235-242.

Vadawale AV, Mehta-Bhatt P, Dave AM. 2004. Rapid in vitro propagation of Aswagandha *(Withania somnifera*) through axillary bud multiplication and indirect organogenesis. *Phytomorphology* 54:59-64.