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In vitro Regeneration of the Medicinal Plant, *Plumbago zeylanica* L. with Reference to a Unique Population in Maruthamalai, the Western Ghats, India

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Abstract

The *in vitro* regeneration of *Plubago zeylanica* exhibited that the callus was initiated in the basal medium containing BAP, NAA, 2, 4-D, and IBA. The high amount (90%) of organic calli was induced in the basal medium supplemented with 2, 4-D, alone at 2.0 mg/l. In the subculture the adventitious shoot formation was prominently higher (83%) in the basal medium containing BAP, and NAA at 3.5 and 0.3 mg/l, respectively. IAA (1.0 mg/l) effectively produced higher percentage (90) of roots and root growth. After sequential hardening, survivability rate was observed to be significantly higher (80%) in the hardening medium containing garden soil, sand and vermicompost in the ratio of 1 : 1 : 1 by volume under greenhouse condition.

Introduction

Tissue culture technology offers an alternative method for the conservation of germplasm as well as micropropagation of medicinally important plant resources. Presently there a is great demand for the use of plant based medicaments in place of synthetic drugs. As a result of non-scientific exploitation, most of the medicinal plant resources are being threatened and are on the verge of extinction. Therefore, application of this technology provides materials required for the isolation of drugs by the pharmaceutical industries without depleting natural plant resources.

Plumbago zeylanica L., a member of Plumbaginaceae, is a rambling subscandent perennial herb or undershrub. The roots are used extensively in China and other Asian countries for the treatment of cancer, rheumatoid arthritis, dysmenorrhea, and contusion of extremities (Atta-ur-Rahman 1988). Extract of the root is given internally or applied to the ostium uteri, causes abortion (Premakumari et al. 1977 and Bharghava 1984). The roots contain an alkaloid - plumbagin, a natural napthaquinone, possessing various pharmacological

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activities such as antimalarial, (Didry et al. 1994), anticancer, cardiotonic, antifertility action, antibiotic and antineoplastic (Kirtikar and Basu 1975, Modi 1961, Krishnaswamy and Purushottamam 1980, Pillai et al. 1981). The root stimulates the secretion of sweat urine and bile and has a stimulant action on the nervous system. Coconut oil is processed with the root to a straw yellow colour and is used as a hair tonic, stimulating hair growth.

Propagation through seed is very difficult due to poor seed quality, lower germination rate and less seedling survivability as under natural field conditions (Chaplot et al. 2005). Many protocol have been developed for *in vitro* regeneration of this plant. However, it was not notably a success for a particular population growing in the glens of Maruthamalai, the Western Ghats, Coimbatore, India. In this habitat, due to the existence of adjoining steep rocky slope, the availability of sunlight is known to be around 5 hrs only in a day. This micro-climatic condition results in the growth of this species at above normal height (> 6 feet) with a bushy appearance. Hence, in the present study, a standard protocol was developed through *in vitro* culture by using leaf explants of this particular population of *P. zeylanica*.

Materials and Methods

Leaf segments from the young and healthy branches of *Plumbago zeylanica* present in the glens of Maruthamalai, the Western Ghats were used as explants. The collected immature leaves were washed with tap water twice and then treated with 5% Tween-20 solution for 5 min for surface sterilization and rinsed with tap water. To eliminate the fungal contamination, explants were further treated with 5% antibiotics (Ampicillin and Rifampicin) for 30 min followed by three rinses in sterile double distilled water. Furthermore, surface sterilization was carried out by dipping the explants in a 0.1% $HgCl_2$ for 3 min followed by three - four rinses in sterilized double distilled water. MS basal medium containing 3% sucrose solidified with 1% agar (tissue culture grade, Himedia, India) was used. The pH of the medium was adjusted to 5.6 - 5.8 prior to the addition of agar. The explants were transferred to culture bottles containing 25 ml MS basal medium supplemented with different concentrations of BAP, 2, 4-D and IBA for callus induction. Cultures were incubated at 25 ± 2°C under 16 hrs photoperiods from cool white fluorescent tubes giving 2000 lux at culture level. Callus from these primary cultures were transferred to MS containing different concentrations of BAP, NAA, Kn and IAA and incubated in light for shoot differentiation. Shoot buds that originated from leaf callus after 30 days were subcultured. Data on shoot proliferation efficiency were recorded after six weeks of culture. Proliferated shoots were transferred to MS fortified with various concentrations of NAA, IAA and IBA for adventitious root formation. For

hardening, pot culture experiment was conducted by using a mixture of garden soil, sand and vermicompost (1 : 1 : 1); survivability rates were determined after 40 days of hardening. Three replicates were maintained for all experiments.

Results and Discussion

The callus induction in the leafy explants was noted two - three weeks after inoculation. The response of explants for callus formation was appreciable when cultured on MS supplemented with BAP, NAA, 2, 4-D and IBA in various concentrations. However, a higher percentage of (90) leaf segments responded well for callusing in the basal medium containing the 2, 4-D alone at 2.0 mg/l (Table 1 and Fig.1a). The varied response of explants for callus formation in various combinations and concentrations of growth regulators could be due to

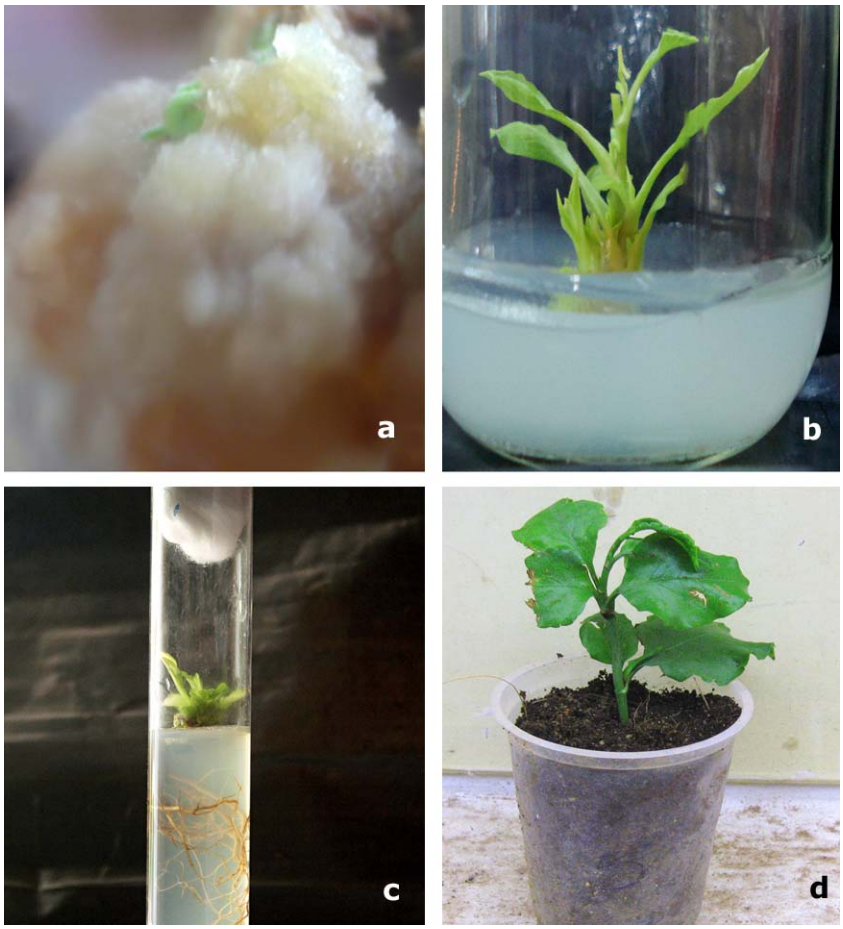

Fig. 1. Stages in *in vitro* regeneration through leaf explant. (a) Effective callusing in basal medium. (b) Successful shooting by subculturing of leaf derived callus. (c) High amount of rooting during the subculturing of shoots. (d) A plantlet derived from leaf explants during hardening.

Table 1. Effective callusing and shooting from leaf explant of *Plumbago zeylanica* in the MS fortified with different concentrations of growth regulators.

Growth regulators (mg/l)			Callus formation (%)	Shoot proliferation (%)	Number of shoots/callus	Shoot length (cm)
2,4-D	BAP	NAA				
0.1	0.0	0.0	19.67 ^{bc} ± 1.53	-	-	-
0.2	0.0	0.0	35.67 ^{e} ± 2.08	-	-	-
0.5	0.0	0.0	47.67 ^{g} ± 1.53	-	-	-
1.0	0.0	0.0	70.33 ^{l} ± 3.21	-	-	-
1.5	0.0	0.0	75.33 ^{m} ± 2.52	-	-	-
2.0	0.0	0.0	90.00 ^{n} ± 1.00	-	-	-
2.5	0.0	0.0	70.33 ^{l} ± 2.08	-	-	-
3.0	0.0	0.0	74.67 ^{m} ± 1.53	-	-	-
0.0	0.5	0.3	-	17.00 ^{efg} ± 2.00	4.33 $^{a-e}$ ± 2.52	1.00 ^{abc} ± 1.00
0.0	1.0	0.3	-	27.33 ^{ij} ± 2.52	5.33 $^{a-f}$ ± 2.52	2.00 $^{a-e}$ ± 1.00
0.0	1.5	0.3	-	36.00 ^{l} ± 2.00	8.00 $^{c-i}$ ± 2.00	2.00 $^{a-e}$ ± 2.00
0.0	2.0	0.3	-	41.67 ^{m} ± 3.06	12.67 $^{i-n}$ ± 3.06	3.00 $^{b-h}$ ± 1.00
0.0	2.5	0.3	-	65.67 ^{q} ± 2.52	15.33 ^{mn} ± 2.52	3.00 $^{b-h}$ ± 2.00
0.0	3.0	0.3	-	75.33 ^{s} ± 2.52	15.33 ^{mn} ± 1.53	5.33 ^{hi} ± 1.53
0.0	3.5	0.3	-	83.00 ^{t} ± 3.00	17.00 ^{n} ± 3.00	8.67 ^{k} ± 2.08
0.0	4.0	0.3	-	81.00 ^{t} ± 3.00	15.33 ^{mn} ± 2.08	7.67 ^{jk} ± 2.08

Means followed by common letters in column(s) are not significantly different at 5% level by DMRT.

Table 2. Effect of different concentrations of growth regulators in the MS on rooting percentage, root number and root length after subculturing of leaf calli derived shoots of *Plumbago zeylanica*.

Growth regulator (mg/l)			Shoots rooted (%)	Number of roots/shoot	Root length (cm)
IAA	IBA	NAA			
0.3	0.0	0.0	10.00 ^{a} ± 2.00	2.00 ^{a} ± 1.00	0.63 ^{ab} ± 0.35
0.6	0.0	0.0	25.67 ^{d} ± 2.08	3.00 ^{a} ± 1.00	0.93 $^{a-d}$ ± 0.35
0.9	0.0	0.0	39.67 ^{fg} ± 3.06	3.33 ^{a} ± 2.52	1.50 ^{de} ± 0.30
1.2	0.0	0.0	46.33 ^{hi} ± 2.52	4.00 ^{ab} ± 1.00	2.17 ^{fg} ± 0.40
1.5	0.0	0.0	57.00 ^{j} ± 3.00	5.33 $^{a-d}$ ± 1.53	2.40 ^{g} ± 0.30
1.8	0.0	0.0	71.00 ^{l} ± 2.00	8.00 ^{cde} ± 2.00	3.17 ^{h} ± 0.31
2.2	0.0	0.0	85.00 ^{n} ± 3.00	9.67 ^{def} ± 2.08	3.87 ^{i} ± 0.45
3.0	0.0	0.0	80.00 ^{m} ± 3.00	10.67 ^{efg} ± 2.08	4.50 ^{jk} ± 0.40
0.0	0.3	0.0	8.33 ^{a} ± 2.52	2.33 ^{a} ± 0.58	0.43 ^{a} ± 0.25
0.0	0.6	0.0	11.67 ^{ab} ± 2.08	2.00 ^{a} ± 2.00	0.70 ^{abc} ± 0.20
0.0	0.9	0.0	17.33 ^{c} ± 1.53	3.00 ^{a} ± 2.00	0.97 $^{a-d}$ ± 0.40
0.0	1.2	0.0	28.33 ^{d} ± 2.52	5.00 ^{abc} ± 2.65	1.30 ^{cde} ± 0.40
0.0	1.5	0.0	36.00 ^{ef} ± 2.00	5.33 $^{a-d}$ ± 2.52	1.70 ^{ef} ± 0.30
0.0	1.8	0.0	43.33 ^{gh} ± 3.06	7.67 $^{b-c}$ ± 2.08	3.13 ^{h} ± 0.25
0.0	2.2	0.0	59.67 ^{j} ± 3.06	7.33 $^{b-e}$ ± 3.06	4.47 ^{jk} ± 0.35
0.0	3.0	0.0	67.00 ^{k} ± 3.00	9.00 ^{def} ± 1.00	4.60 ^{k} ± 0.30
0.0	0.0	0.3	15.33 ^{bc} ± 1.53	5.00 ^{abc} ± 2.65	1.17 $^{b-e}$ ± 0.25
0.0	0.0	0.6	28.00 ^{d} ± 2.00	5.33 $^{a-d}$ ± 1.53	1.43 ^{de} ± 0.35
0.0	0.0	0.9	34.00 ^{e} ± 3.00	7.67 $^{b-e}$ ± 3.06	1.77 ^{ef} ± 0.21
0.0	0.0	1.2	49.33 ^{i} ± 1.53	11.00 ^{efg} ± 2.65	2.40 ^{g} ± 0.30
0.0	0.0	1.5	58.67 ^{j} ± 2.08	10.67 ^{efg} ± 2.52	3.97 ^{ij} ± 0.40
0.0	0.0	1.8	71.67 ^{l} ± 2.08	12.00 ^{fg} ± 2.00	6.70 ^{l} ± 0.56
0.0	0.0	2.2	87.67 ^{o} ± 1.53	11.33 ^{efg} ± 3.06	8.03 ^{m} ± 0.15
0.0	0.0	3.0	80.67 m ± 2.52	13.00 ^{g} ± 2.65	8.43 ^{m} ± 0.35

their polarity and due also to the endogenous and exogenous concentrations of growth regulators and their respective ratios (Samantaray et al. 1995, Rout et al. 1999b, Naika and Krishna 2008).

Subculturing experiments were conducted by using callus as secondary explants for shoot proliferation and root induction on MS with various combinations and concentrations of BAP, NAA, Kn and IAA. The shoot formation was more pronounced (83%) in the basal medium containing BAP and NAA (3.5 and 0.3 mg/l, respectively) followed by 81% in BAP and NAA (at 4.0 and 0.3 mg/l) and 75% at BAP and NAA (at 3.0 and 0.3 mg/l, respectively) (Table 1 and Fig. 1b). The results indicate that shooting was more pronounced on MS augmented with a high amount of BAP and a lower quantity of auxin, NAA. Earlier Thomas and Gangaprasad (2005) and Rout (2004) reported the requirement of a greater quantity of BAP with a least amount of auxin for effective shoot formation in *Enicostema axillare* and *Clitoria ternatea,* respctively. In addition, the number of shoots was found to be higher (17/callus) with a greater shoot length (8.67 cm) in the basal medium containing BAP and NAA at 3.5 and 0.3 mg/l, respectively (Table 1).

Similar results were reported by Shrivastava and Manerjee (2008) in *Jatropha curcas*. The initiation and the growth of the adventitious roots were appreciable in the basal medium fortified with various concentrations and combinations of IBA, IAA and NAA. A higher percentage of (87.67) shoots were found to effectively produce roots on MS fortified with NAA alone at 2.2 mg/l (Table 2). On the other hand, the number of roots produced and root lengths were higher (13/shoot and 8.43 cm, respectively) in the basal medium containing auxin, NAA alone at 3.0 mg/l. (Table 2 and Fig. 1c). The supplements of basal medium with the other auxins, IAA and IBA also enhanced the rooting attributes responses considerably indicating the essentiality of auxin in the root formation of this species. Earlier Pande et al. (2000), Karuppusamy and Pullaiah (2007) reported the importance of auxin in the root formation during the subculturing of secondary explants in many wild species.

Hardening is a crucial step prior to transplantation of plants to soil. The well developed plantlets were hardened in various potting media. After sequential hardening, survivability rate of plantlets was significantly greater (80%) in the hardening medium as described elsewhere in the article (Fig. 1d). The protocol reported here is reproducible, only for the populations of *P. zeylanica* distributed in the glens of Maruthamalai, the Western Ghats, Coimbatore. It has the potential to conserve the germplasm allowing at the same time a large scale micropropagation of this important regional medicinal plant.

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