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In vitro mass propagation of *Plumbago zeylanica* L., through direct organogenesis

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Abstract

An efficient protocol was developed for *in vitro* mass propagation of an important medicinal shrub, *Plumbago zeylanica* L., (Plumbaginaceae) through direct organogenesis using shoot tip and nodal explants. Best shoot induction was observed on MS basal medium supplemented with 0.5 mg/l BAP, in which 86.4% of nodal explants responded to produce maximum number (12.4 ± 0.66) of shoots per culture. *In vitro* raised shoots rooted on half strength MS medium with 0.5 mg/l IAA. For acclimatization and transplantation, the plantlets in the rooted culture tubes were kept in normal room temperature for 7 days before transplanting in pots where plantlets were reared for three weeks. The survival rate of regenerated plantlets was 85%.

Key words : *Plumbago zeylanica*, Medicinal plant, Organogenesis, Shoot proliferation, Mass propagation, Acclimatization

Introduction

Plumbago zeylanica L. commonly known as 'Chitra' belongs to the family - Plumbaginaceae, an undershrub with somewhat woody stem, strangling branches, thin ovate leaves, white flowers in elongate spikes and angled seed capsules (Fig.1a), grow in wastlands and graveyards in almost all areas of Bangladesh (Ghani, 2003). The roots are used extensively in China and other Asian countries for the treatment of cancer, rheumatoid arthritis, diarrhoea and extrem constipation (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 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 paste is applied in order to open abscesses; a paste prepared with milk, vinegar or salt and water is used as an external application in leprosy and other skin disease of an obstinate character (Anonymous, 1989). Paste of equal proportion of root and bark is applied locally to haemorrhoids and in skin disease, whereas root bark decoction is taken orally two to three times daily to treat diarrhoea (Jaiswal, 2010). 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 (Mallikadevi *et al.,* 2008).

Propagation through seed is very difficult due to poor quality, lower germination rate and less seedling survivability under natural field conditions (Chaplot *et al.,* 2005). In recent years, there has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants (Ajithkumar and Seeni 1998; Prakash *et al.* 1999). Commercial exploitation and elimination of natural habits consequent to urbanization has led to gradual extinction of several medicinal plants. Micropropagation is an effective approach to conserve such germplasm. *In vitro* propagation has proven as a potential technology for mass scale production of medicinal plant species (Hassan and Roy, 2005; Lui and Li, 2001; Martin 2002, 2003). It is important, therefore to develop an efficient micropropagation technique for *Plumbago zeylanica* L. for rapidly disseminate superior clones. There have been few reports to date on *in vitro* mass propagation of *Plumbago zeylanica* L. using shoot tip and nodal explants (Mallikadevi *et al.,* 2008; Sahoo and Debata, 1998; Seivakumar *et al.,* 2001). Mallikadevi *et al.* (2008) reported that the *in vitro*

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regeneration of *Plumbago zeylanica* L., exhibited that the callus was initiated in the basal medium containing BAP, NAA, 2,4-D and IBA and the high amount (90%) of organic calli was induced in the basal medium supplemented with 2.4-D alone at 2.0 mg/l and 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 and Sahoo and Debata (1998) reported that a rapid and highly effective method for plant micropropagation from vegetative shoot buds was established for this medicinal plant *Plumbago zeylanica* L.; multiple shoots were proliferated from nodal explants culture on MS basal medium supplemented with 0.25-1.0 mg/l Kn or 0.25-1.0 mg/l BAP and Seivakumar *et al.* (2001) also reported that rapid *in vitro* propagation using nodal explants cultured in MS basal medium supplimented with 10.0 mg/l ADS with 0.5 mg/l IBA. The present study was therefore undertaken to develop a protocol for *in vitro* mass propagation of this important medicinal shrub through direct organogenesis using shoot tip and nodal explants.

Materials and Methods

Plumbago zeylanica L., grown at Medicinal Plants Garden of Bangladesh Agricultural University, Mymensingh was used as a source of explants. Shoot tip and nodal explants with a single axillary bud were used for this purpose. The explants were washed thoroughly under running tap water, pre-soaked in liquid detergent for about 30 min, wiped with cotton

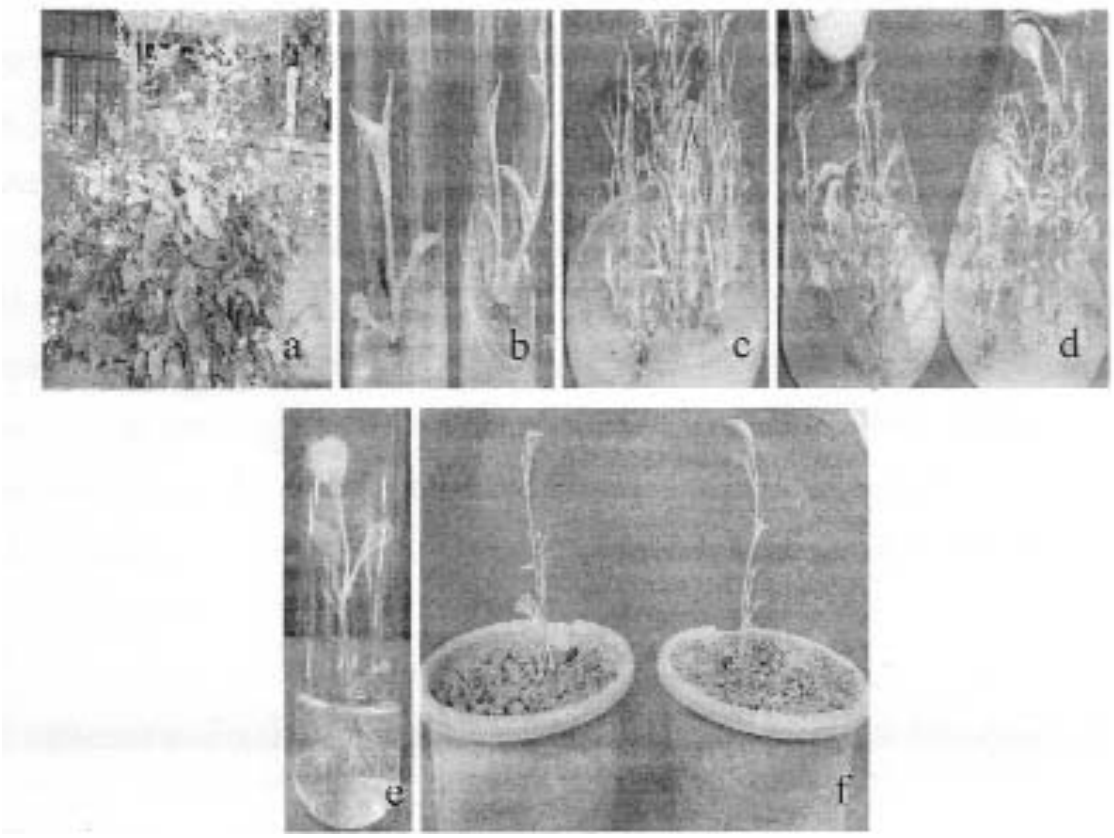

Fig 1: *In vitro* regeneration of *Plumbago zeylanica* L., from nodal explants.

(a) A mature plant from where explants were taken.
(b) Induction of shoots from nodal explants on MS + 0.5 mg/l BAP in three weeks of culture.
(c) Development and multiplication of shoots from nodal explants on MS + 0.5 mg/l BAP after six weeks of culture.
(d) Development and multiplication of shoots from nodal explants on MS + 0.5 mg/l BAP after nine weeks of culture.
(e) Rooting of *in vitro* regenerated shoots cultured on half strength MS + 0.5 mg/l IAA in third weeks of culture.
(f) Acclimatized regenerated plants of two months old.

and dipped in 70% (v/v) ethanol for 1 min. They were then surface-sterilized with 0.1% (w/v) mercuric chloride for 5 min, followed by five times rinse with sterile distilled water under laminar air flow cabinet. The surface-sterilized explants were sized to 1-1.5 cm length containing a single node with an axillary bud or a shoot tip with an apical bud. The explants were placed vertically on the culture medium. The new shoots induced from the *in vitro* cultures were further used as an explants for adventitious shoot regeneration.

MS (Murashige and Skoog, 1962) basal medium was used for shoot proliferation and adventitious shoot regeneration and half strength MS was used for *in vitro* root induction. All media were supplemented with 30 g/l sucrose, 7 g/l agar (Difco) and dispensed into 15x150 mm culture tubes and 250 ml conical flasks. The pH of the media was adjusted to 5.8 before autoclaving at 1.9 kg/cm^2 pressure at 121 °C for 20 min. The cultures were incubated for a 16 h photoperiod at 24 ± 2 ^{o}C under 1200 lux/m^2 fluorescent light.

Shoot proliferation from shoot tip and nodal explants was obtained in two separate sets of experiments. In the first experiment 0.5-2.0 mg/l BAP and 0.5-2.0 mg/l Kn were incorporated into MS media to select the best cytokinin for the response of shoot induction. In the second set, combination of BAP (0.5-2.0 mg/l) with NAA (0.1-0.5 mg/l) and BAP (0.5-2.0 mg/l) with IAA (0.1-0.5 mg/l) were assessed for shoot multiplication. Number of new shoot proliferation of each culture was recorded after every week of inoculation.

For *in vitro* rooting, individual shoots (3-5 cm) were excised from the proliferated shoot cultures and implanted onto half strength MS media with different concentrations and combinations of NAA, IBA and IAA.

The rooted plants were taken out from the culture tubes, washed to remove agar gel adhered to the roots and transplanted to plastic pots with soil and compost (1: 1) for hardening. The plantlets were kept in a polychamber at 80% relative humidity, 32 ± 2 ^{o}C temperatures for a 12 h photoperiod under 1500 lux/m^2 sun light for acclimation. Established plants were transplanted in earthen pots under natural conditions and the survival rate was recorded.

Results and Discussion

Shoot tip and nodal explants of *Plumbago zeylanica* L. were cultured on MS media supplemented with various concentration of BAP alone and with NAA or IAA for multiple shoot regeneration. The explants were found to be swollen and they produced two to three shoots within three weeks after inoculation (Fig. 1b) on MS media containing BAP alone but the number of shoots increased up to 12.4 ± 0.66 when the explants were cultured in MS with 0.5 mg/l BAP (Table I, Fig. 1c). Both the explants responded in the same medium

Table I: Effect of growth regulators in MS on multiple shoot regeneration of *Plumbago zeylanica* L. shoot tips and nodal explants

Growth regulators (mg/l)			shoot tips		nodal explants	
BAP	NAA	IAA	% of explants forming shoots	Mean No. of Shoot/explant	% of explants forming shoots	Mean No. of Shoot/explant
0.5			74.2±2.58	9.4± 0.65	86.4±1.70	12.4± 0.66
1.0			63.4±1.57	7.6± 0.45	71.4±2.38	11.6± 0.72
1.5			57.6±2.16	6.4± 0.72	67.6±2.16	10.4± 1.18
2.0			34.8±2.58	5.8±0.76	33.6±1.84	09.4±0.82
0.5	0.1		61.4±2.87	6.2± 0.59	68.6±1.70	10.6± 1.14
1.0	0.2		42.6±0.87	5.6±0.77	43.6±0.51	09.6±0.91
1.5	0.5		28.2±1.66	4.0± 0.63	41.2±2.47	08.4± 0.66
2.0	0.5		22.2±1.96	3.4± 0.45	32.2±0.66	07.8± 0.95
0.5		0.1	48.8±1.77	5.0± 0.39	56.8±2.14	09.2± 0.76
1.0		0.2	26.6±1.66	4.2± 0.65	47.6±2.10	08.2± 0.51
1.5		0.5	21.0±1.14	3.4± 0.45	32.6±1.63	07.4± 0.91
2.0		0.5	16.2±0.86	2.4± 0.59	18.4±0.93	06.4± 0.66

Results are mean ± SE of three experiments with 15 replications.

but highest numbers of micro shoots were observed to be induced from nodal explants (Fig.1d). Combinations of BAP with NAA or IAA were not found to be suitable than BAP alone for shoot induction (Table I) and combinations of Kn with NAA or IAA were also not found to be suitable for shoot induction (Data were not shown). Sahoo and Debata (1998) reported that a rapid and highly effective method for plant micropropagation from vegetative shoot buds was established for this medicinal plant *Plumbago zeylanica* L. ; multiple shoots were proliferated from nodal explants culture on MS basal medium supplemented with 0.25-1.0 mg/l Kn or 0.25-1.0 mg/l BAP. A similar phenomenon was observed in *Plumbago zeylanica* L. by other researchers (Gbadamosi and Egunyomi, 2010; Mallikadevi *et al.,* 2008; Selvakumar *et al.,* 2001; Rout *et al.,* 1999; Verma *et al.,* 2002).

86.2% regenerated shoots rooted (Fig.1e) when cultured individually on root induction medium consisted of half-strength MS medium with 0.5 mg/l IAA (Table II). Use of auxins singly or in combination for rooting was also reported by different authors (Bhadra *et al.*, 2009; Gopalakrishnan *et al.,* 2009; Hassan and Khatun, 2010; Kumar and Bhavanandan, 1988; Sivakumar and Krishnamurthy 2000; Yogananth and Basu, 2009).

Table II: Effect of auxin(s) on root induction in regenerated shoots of *Plumbago zeylanica* L., on half strength MS

Growth regulators (mg/l)			% of shoots producing roots	No. of roots/shoot
IAA	IBA	IAA		
0.5			86.2±0.71	14.8±0.76
0.75			67.2±1.53	11.8±0.65
1.0			63.2±1.46	09.2±0.76
1.5			61.0±0.10	07.6±0.72
	0.5		77.8±1.85	06.2±0.76
	0.75		64.2±1.53	05.0±0.63
	1.0		62.0±0.71	04.8±0.59
	1.5		59.4±1.08	03.2±0.71
		0.5	65.2±1.16	05.8±0.65
		0.75	61.4±0.75	04.6±0.96
		1.0	62.6±0.93	03.6±0.72
		1.5	56.2±1.85	02.2±0.76

Data were recorded after four weeks of culture. Results are mean ± SE of 15 replications.

After four weeks the rooted shoots were transferred to pots. None of the plantlets were survived when directly transferred from rooting medium to the pot under natural conditions. About 85 percent of the transplanted plants of *Plumbago zeylanica* L., survived if the plants in the rooting culture tubes were kept in normal room temperature for seven days before transplantation in pots and reared for three weeks. The plantlets were reared under semi-controlled temperature (30±2^{o}C) and light (1500 lux) in a chamber with 80 percent humidity. During this period of acclimation shoots elongated, leaves expanded and turned deep green and healthier (Fig.1f).

After three weeks, plants were transferred to an open place and gradually acclimatized to outdoor conditions, where 85 percent plants were survived. The technique described here appears to be readily adaptable for large scale clonal propagation and plantation for sustainable use.

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