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A Valued Medicinal Plant - Chitrak (*Plumbago zeylanica* Linn.) : Successful Plant Regeneration Through Various Explants and Field Performance

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Abstract

Protocols for plant propagation through axillary bud proliferation and organogenesis were established for Chitrak - *Plumbago zeylanica* Linn. (Plumbaginaceae). MS medium with 4.4 mg/l BA and 1.4 mg/l IAA elicited the maximum number of shoots (12 multiple shoots) from nodal explants. Leaf based callus differentiated into more than 30 shoots on MS with 160 mg/l adenine sulphate. The regenerated shoots were rooted on MS with 1.2 mg/l IBA within ten days. Almost, 96% of the rooted shoots survived hardening when transferred to the field. The regenerated plants did not show any morphological change and variation in levels of secondary metabolite when compared with the mother stock.

Introduction

Plumbago zeylanica Linn. (Chitrak) belongs to Plumbaginaceace. It is an important medicinal plant. It is grown as a perennial herb in most parts of India, but on larger scale in the plains of West Bengal and Southern India. The roots of *P. zeylanica*, *P. rosea* and *P. europaea* have been used extensively in China and other Asian countries for the treatment of cancer, rheumatoid arthritis, dysmenorrhea, and contusion of extremities (Atta-ur-Rahman 1988). Extracts of the root, when given internally or applied to the ostium uteri, causes abortion (Premakumari et al. 1977, Bharghava 1984). The root is pungent, diuretic, germicidal, astringent, vesicant. The roots contain an alkaloid - plumbagin, a natural napthaquinone, possessing various pharmacological activities such as antimalarial, antimicrobial (Didry et al. 1994), anticancer, cardiotonic, antifertility action, antibiotic and

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antineoplastic (Kirtikar and Basu 1975; Modi 1961, Krishnaswamy and Purushottamam 1980, Pillai et al. 1981). More than 32 patents involving plumbagin were obtained in the United States and many of these patents involve polymer scale prevention agents (US Patent and Trademark Office 1999). The root stimulates the secretion of sweat urine and bile and has a stimulant action on the nervous system. Roots are specially used in the treatment of rheumatism, skin disease, diarrhoea, piles, anasarca, ulcers, syphilis and carcinoma. It is also used as an appetizer. Milky juice is used as application in scabies and unhealthily ulcers. Its paste is applied externally in leprosy. Coconut oil is processed with the root to a straw yellow colour and is used as a hair tonic, which stimulates hair growth.

Propagation through seed is unreliable due to poor seed quality, erratic germination and seedling mortality as under natural field conditions. Due to the presence of natural nathaquinone, *P. zeylanica* is much sought after in western countries as *Chlorophytum borivilianum* for saponin content (Chaplot et al. 2005). Extensive and destructive harvesting of plants by the pharmaceutical industries for procurement of naturally occurring secondary metabolites (Plumbagin) from the plant and insufficient attempts to either allow its replenishment or its cultivation have led to the depletion of the natural plant population. Very few reports on cultivation, breeding and improvement programmes and *in vitro* studies of *P. zeylanica* are available despite its commercial importance. This paper deals with the standardization of a technique for micropropagation through multiple shoot formation. The protocol provides early bud-break with high frequency of shoot multiplication from axillary bud and leaf explants with comparatively a reduced requirement of plant growth hormones and successful acclimatization of plants in the soil. The performance of regenerated plants was also evaluated in the field.

Materials and Methods

The nodal explants and leaves of *P. zeylanica* from one-year-old plants were collected from the Botanical Garden of the Maharaja Sayajirao University of Baroda. They were washed first under running tap water (30 min) and treated with 0.2 % (v/v) aqueous surfactant Teepol (BDH, India) for 10 min followed by repeated rinsing with distilled water. Subsequently, explants were treated (20 min) with 0.1 % (w/v) carbendenzim (BASF, India). Further sterilization was done under aseptic conditions in a Laminar Airflow Hood (Lab Services, India). Explants were surface sterilized with 50 % (v/v) ethanol (1 min) and followed by 0.07 % (w/v) $HgCl_2$ (3 min). Finally, the explants were washed thoroughly (three - five times) with sterilized distilled water. The nodal explants were cut into appropriate size (0.8 cm) and young leaf lamina with mid rib (0.7 cm) was cut and cultured on MS medium.

Throughout the experiments full strength MS with 3 % (w/v) sucrose and gelled with 0.8 % (w/v) agar (Qualigens, India) was used. The pH of all media was adjusted to 5.8 prior to autoclaving (15 min). The cultures were incubated in a culture room with 25 ± 1°C and 16 hr photoperiod (50 μE/m²/s) provided by cool white fluorescent tubes (Phillips, India).

The basal medium was supplemented with BA (0.0 - 8.8 mg/l) and IAA (0.0 - 2.88 mg/l) at different concentrations, either alone or in combinations. Initiation of callus formation from the base of leaf lamina was observed on MS supplemented with BA, IAA and AdS. Root induction on shoots was achieved on full strength MS with IAA/IBA at different concentrations. Well developed rooted shoots were removed from the culture vessels, washed gently under running tap water and planted in plastic bags containing a potting mixture of sand, soil and farmyard manure in the ratio of 1 : 1 : 1. The plantlets were kept in the net house for acclimation (two - three weeks) before their subsequent transfer to the field. Humidity was maintained by sprinkling water regularly throughout the day (Jasrai et al. 1999). Plants were gradually exposed to the normal conditions and transferred to the Medicinal Garden of GSFC Science Foundation.

The experiments were set up in a completely randomized design. Ten cultures were raised for each treatment and all experiments were repeated thrice. Qualitative analysis was carried out through thin layer chromatography. The shade-dried roots of *in vitro* raised plants and mother plants were crushed into powder form and were subjected to phytochemical analysis (Harborne 1964).

Results and Discussion

Bud break on the nodal segments was achieved on MS with 6.7 mg/l BA and 1.4 mg/l IAA (Fig. 1A). When MS supplemented with different concentrations of BA and IAA was used, multiple shoots emerged from the nodal explants within two weeks of incubation (Fig. 1B). Among different concentrations of growth hormones tested, 4.4 mg/l BA and 1.4 mg/l IAA elicited the maximum number of shoots (12 multiple shoots) from nodal explants (Table 1). Direct shoot regeneration from nodal explants have been reported earlier (Selvakumar et al. 2001) on MS medium with 27.2 mg/l AdS + 2.46 mg/l IBA. Similarly, Verma et al. (2002) reported rapid propagation of *P. zeylanica* with maximum of four multiple shoots per nodal segment with 8.87 mg/l BA and 0.49 mg/l IBA. The present study exemplifies a positive modification of shoot induction efficacy on MS with low concentrations of auxin and cytokinin. Excision and culture of the nodal segments from *in vitro* derived shoots facilitated the development of increased number of shoots. The elongation of shoots (4 - 5 cm) was observed on the same proliferation medium within two weeks of incubation (Fig. 1D). On an average within three subcultures, single node explant generated 36 shoots in

presence of 4.4 mg/l BA and 1.44 mg/l IAA. The shoot multiplication at this enhanced pace was also achieved in subsequent cultures up to six - eight cycles (Data not presented).

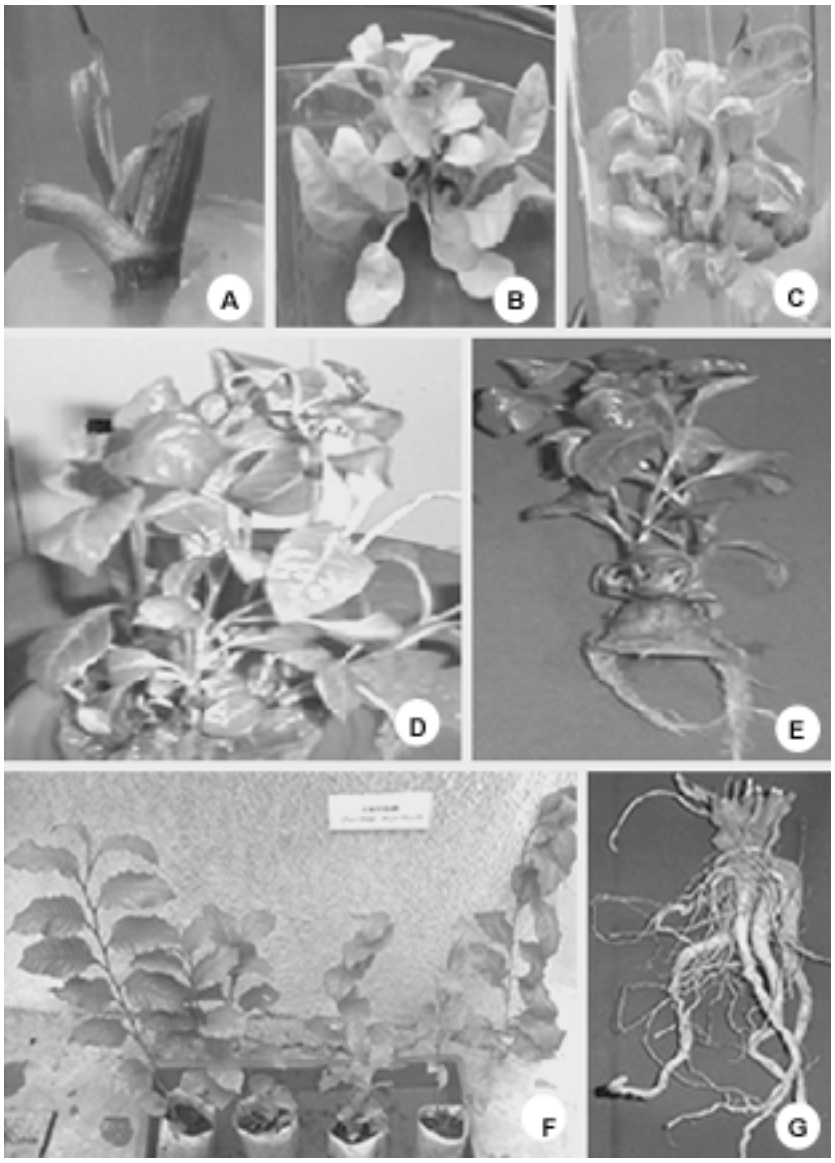

Fig. 1. Micropropagation through axillary bud proliferation and leaf callus of *P. zeylanica.* (A) Bud break from nodal explants of *P. zeylanica.* (B) Multiple shoots formation on MS containing BA (4.4 mg/l) and IAA (1.4 mg/l). (C) Shoot formation from callus on MS with BA (4.4 mg/l), IAA (1.4 mg/l) and AdS (160 mg/l). (D) Elongation growth of shoots. (E) Induction of roots on regenerated shoots on MS containing IBA (1.2 mg/l). (F) Hardened plants ready for transplantation to field in potting mixture in sand, soil and FYM in the ratio of 1:1:1. (G) The harvested roots of *in vitro* raised plants in the process of drying.

Callus initiation was observed from young leaves on MS medium supplemented with BA (0.0 - 8.8 mg/l), IAA (0.0 - 2.88 mg/l) and AdS (160 mg/l). Callus formation from the leaf explants of *P. zeylanica* is in agreement with results obtained by Rout et al. (1999) in the same species however varied in hormonal combinations. Best callus (nodular) formation was observed on MS medium containing 6.7 mg/l BA, 1.42 mg/l IAA and 160 mg/l AdS.

Table 1. Effect of different combinations of BA and IAA in MS on shoot formation through nodal explants of *P. zeylanica.*

Concentrations (mg/l)		Response (%)	Number of shoots/node* (Mean ± SD)
BA	IAA		
0.0	0.0	0.0	0.0
2.2	0.5	10	1.5 ± 0.26
4.4	0.5	45	4.5 ± 0.16
4.4	**1.4**	**85**	**12.1 ± 1.34**
4.4	2.8	69	5.5 ± 0.21
6.7	1.4	58	4.8 ± 2.26
8.8	1.4	55	4.1 ± 1.20

*Values are of three repetitions; ten cultures per replicate; scored after three weeks.

Leaf callus developed on MS with 6.7 mg/l BA, 1.42 mg/l IAA and 160 mg/l AdS underwent organogenesis (Fig. 1C) after three weeks of incubation onto various regeneration media containing different concentrations of BA, IAA and AdS. The highest number of shoots from the leaf callus was observed on MS with 4.4 mg/l BA, 1.42 mg/l IAA and 160 mg/l AdS (Table 2). On an average 30 shoots were recorded in callus cultures through organogenesis. Subsequent

Table 2. Effect of different combinations of growth regulators in MS with 160 mg/l AdS on shoot bud regeneration from leaf callus of *P. zeylanica.*

Concentration (μM)		Response (%)	Number of shoots/culture* (Mean ± SD)
BA	IAA		
0.0	0.0	-	-
2.2	1.4	-	-
2.2	2.8	25	2.08 ± 1.75
4.4	1.4	93	30.16 ± 1.43
4.4	2.8	85	22.02 ± 1.02
6.7	1.4	75	15.09 ± 1.02
6.7	2.8	67	9.54 ± 1.12
8.8	1.4	60	6.36 ± 1.43

*Values are of three replicates; ten cultures per replicate; scored after three weeks.

subcultures (up to six cycles) of organogenic callus resulted in an extensive proliferation and an enhanced rate of caulogenesis with more than 35 shoots.

Present results are consistent with the earlier report on Ashwagandha indicating that cytokinin and auxin influenced shoot bud regeneration (Verdia et al. 2006).

Well-developed shoots (4 - 5 cm with three nodes) generated through axillary bud proliferation and leaf callus were excised and cultured on MS medium with different concentrations of auxins for root induction. Root induction was found to be more prominent in the medium containing IAA (0.57 mg/l) and IBA (1.2 mg/l) alone. Roots elongated up to 12 - 13 cm within 15 days of incubation period (Fig. 1E). Earlier workers (Rout et al. 1999, Selvakumar et al. 2001, Verma et al. 2002) had reported smaller number of roots (4 - 5 roots) on half strength MS containing 0.57 mg/l IAA, 4.92 mg/l IBA and 0.49 mg/l IBA respectively. While profuse rooting was observed on full strength MS supplemented with IAA and IBA alone (Table 3), the best result (15 roots) was obtained on MS with IBA (1.2 mg/l) within 10 days.

Table 3. Effect of different auxins in MS medium on root induction from generated shoots.

Concentration (μM)		Number of roots/shoot* (Mean ± SD)	Root length (cm)
IAA	0.0		
	0.57	12.54 ± 1.12	10.2 ± 0.45
	1.42	6.36 ± 1.43	5.36 ± 0.28
IBA	0.0	-	-
	0.49	3.9 ± 0.25	5.05 ± 0.02
	1.2	15.04 ± 1.12	13.41 ± 0.25
	2.46	8.36 ± 1.43	6.12 ± 0.45

*Values are of three replicates; ten cultures per replicate; scored after two weeks.

The potency of IBA in root induction has been reported in many species (Epstein et al. 1993). The slow movement and slow degradation of IBA facilitates its localization near the site of application and thus functions better in inducing roots (Nickell 1982). Maximum frequency (97 %), number of roots/shoot (around 15) and mean root length (13.41 cm) was achieved within ten days when shoots were cultured on MS with IBA.

The ultimate success of *in vitro* propagation lies in successful establishment of plants in the soil. Normally, in absence of greenhouse facilities *in vitro* plantlets loose tremendous amount of water through leaf surfaces with poorly deposited cuticular wax and poorly developed or non-active stomatal system (Wardley et al. 1983). This problem was taken care of by regular sprinkling of water and irrigating the regenerated plantlets twice a day. The rooted shoots demonstrated 100 % survival rate in the net house (Fig. 1F). However, a 96 % transplantation success of *in vitro* hardened plantlets in the field (Table 1) was

observed in comparison to the 65 - 90 % survival of plantlets recorded in the experiments of previous workers (Rout 2002, Selvakumar et al. 2001). The high survival rate of *in vitro* plants of *P. zeylanica* in present studies indicates that this procedure could be easily adopted for large-scale multiplication and cultivation. The *in vitro* propagated plantlets resembled the general growth and morphological characteristics of the donor plants.

The *in vitro* raised- and seed grown plants were uprooted from the field (Fig. 1G) for root harvesting. A significantly higher number of roots (19.0 ± 0.6) per plant were observed compared to the seed generated stock with roots (5.1 ± 1.4). There were a threefold increase in root biomass on fresh weight basis of *in vitro* roots (153.1 ± 2.4 gm) in comparison with seed generated plants (47.3 ± 0.2 g). Similar observations have been reported by Roja and Heble (1996) for *in vitro* generated plants of *Rauwolfia serpentina* with thick root stumps and fresh weight (60.56) compared to long slender root and fresh weight (11.92) per plant in conventionally grown counterparts. Present results are in agreement with earlier report by Satheesh Kumar and Bhavanandan (1988) who obtained a higher number of roots (18.0 ± 0.5) and fresh weight (137.4 ± 3.4 gm) in *in vitro* raised *Plumbago rosea* as compared to what were observed in rooted cuttings (14.0 ± 1.7; 47.9 ± 1.6 g), respectively. Further qualitative analysis through thin layer chromatography, emulated the presence of plumbagin (Yellow spots) on the silica gel plate with R_f 0.76 on mobile phase - petroleum ether: ethylacetate (7 : 3) in both *in vitro* generated and seed-grown plants.

Thus, a reproducible protocol for *P. zeylanica* was established through nodal and leaf explants. This protocol can be exploited for conservation and commercial propagation of this medicinal plant in the Indian subcontinent.

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