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In vitro Micropropagation of *Plumbago indica* L. Through Induction of Direct and Indirect Organogenesis

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Abstract

Leaf and nodal segments of two months old field grown seedlings of *Plumbago indica* L. were cultured on agar solidified MS supplemented with different concentrations and combinations of NAA, IAA, 2,4-D and picloram, and BAP and Kn. The nodal segments produced either multiple shoot buds (MSBs) or callus of different nature depending on the combinations of plant growth regulators (PGRs). The callus of light green and nodular shape, on further subculture on wide range of PGRs supplemented media, differentiated into MSBs. These MSBs underwent rapid elongation on different PGRs supplemented media. The elongated shoot buds were rooted on transferring in rooting medium and were acclimated in field with 90% survivability. The leaf segments, however, produced only white and friable calluses failed to undergo any differentiation in some of the media combinations.

Introduction

Medicinal plants are important source of traditional and synthetic medicines containing different types of organic compounds having therapeutic properties. Approximately 80% of people in developing countries still rely on traditional medicines for their primary health care. This usually involves the use of plant extracts (Vieira and Skorupa 1993). Many medicinal plant species are disappearing at an alarming rate, as a result of rapid agricultural and urban development, deforestation and indiscriminate collection. The tissue culture technique has been proved very efficient in rapid mass propagation and conservation of these rare and endangered medicinal plants (Fay 1992, Sagare et al. 2000, Lakshmi and Mythili 2003).

Plumbago indica L., commonly known as 'Raktochita' is a rare medicinal herb of Bangladesh. Its root, root bark and milky juice of whole plant are used for medicinal purposes by the village people particularly of tribal areas as it contains two important alkaloids namely, napthoquinone and plumbagin (Ghani 1998). Roots are used for procuring abortion and the milky juice is used for treatment of

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scabies, leucoderma etc. This plant species has become rare in Bangladesh and needs to be propagated rapidly to meet up the medicinal demand and also for conservation purposes. Many important medicinal herbs throughout the world have been successfully propagated *in vitr*o, either by organogenesis (Erdei et al. 1981, Shoyama et al. 1983, Hatano et al. 1986, Matsumoto et al. 1986, Hiraoka and Oyanagi 1988, Nishioka 1988, Tsay et al. 1989, Huang et al. 2000, Chen et al. 2001, Chueh et al. 2001) or by somatic embryogenesis (Hiraoka et al. 1986, Kitamura et al. 1989, Tsay and Huang 1998, Sagare et al. 2000). The present work was therefore undertaken with a view to establishing an efficient protocol for *in vitro* rapid propagation of this medicinal plant species.

Materials and Methods

Two months old seedlings of *Plumbago indica* L. were collected from the nursery of Bangladesh Council for Scientific and Industrial Research (BCSIR), Chittagong and were established in garden pots of Botany Department, Chittagong University. Leaf and nodal segments of approximate 0.5 - 1 cm were used as explants. Surface sterilization of the explants was done by submerging them in 0.2% (w/v) $HgCl_2$ solution for ten min with occasional agitation followed by a dip in 70% ethanol for 30 sec. The segments were then washed thoroughly with sterile distilled water and cultured in MS supplemented with different concentrations and combinations of NAA, IAA, 2,4-D and picloram, and BAP and Kn. All the media were solidified with 0.8% (w/v) agar and pH was adjusted to 5.8 prior to autoclaving for 20 min at 121ºC under 1.2 kg/cm^2 pressure. Culture vessels with inoculated explants were maintained under a regular cycle of 14 hr light and 10 hr dark at 25 ± 2ºC.

Multiple shoot buds (MSBs) that developed from either nodal explant or from callus elongated on MS supplemented with different PGRs and were rooted on transferring in rooting media *viz.* (i) MS + 3% (w/v) sucrose + 0.5 mg/l IAA, (ii) half strength of MS + 1.5% (w/v) sucrose + 0.5 mg/l IAA, and (iii) one fourth strength of MS + 0.75% (w/v) sucrose + 0.5 mg/l IAA. The well rooted plantlets were finally transferred to pots with sandy-loam soil and humus at 2 : 1.

Results and Discussion

Within 25 - 30 days of culture both leaf and nodal segments gave differential response to different combinations of PGRs. Type of response was dependent on both explant and PGRs supplements of the medium (Table 1). The nodal segments underwent direct organogenesis producing of MSBs on (i) 2.0 mg/l BAP + 0.1 mg/l IAA, (ii) 2.0 mg/l BAP + 1.0 mg/l IAA, (iii) 2.5 mg/l BAP + 1.0 mg/l NAA, (iv) 2.0 mg/l BAP + 2.0 mg/l NAA, and (v) 1.0 mg/l BAP +0.1 mg/l NAA supplemented media. Maximum number of MSBs were recorded on 2.0 mg/l

BAP +1.0 mg/l IAA supplemented medium (7 ± 0.18 per explant). Induction of direct organogenesis has been reported in a number of medicinal plants *in vitro* and it variably depends on appropriate level of both endogenous and exogenous hormones (Erdei et al. 1981, Shoyama et al. 1983, Hatano et al. 1986, Matsumoto et al. 1986, Hiraoka and Oyanagi 1988, Nishioka 1988, Tsay et al. 1989,

Table 1. Development of MSBs/callus from nodal and leaf segments of *P. indica* cultured on PGRs supplemented MS.

PGRs conc. and combinations (mg/l)	Explant*	Time (days)	Types of response
3.0 BAP + 1.5 Kn + 1.0 NAA	NS	25 - 30	Light green nodular callus
	LS	-	-
1.5 BAP + 0.5 IAA + 1.5 2, 4-D	NS	25 - 30	White friable callus
	LS	25 - 28	"
2.0 BAP	NS	25 - 30	"
	LS	25 - 30	"
2.0 BAP +2.0 IAA	NS	25 - 30	"
	LS	-	-
2.5 BAP + 1.0 NAA	NS	25 - 30	MSBs
	LS	25 - 30	White friable callus
2.0 BAP + 1.0 IAA	NS	25 - 30	MSBs
	LS	-	-
2.0 BAP + 0.1 IAA	NS	25 - 30	MSBs
	LS	-	-
2.0 BAP + 0.1 IAA	NS	25 – 30	MSBs
	LS	-	-
2.0 BAP + 0.5 picloram	NS	25 - 30	Light green nodular callus
	LS	-	-
3.0 BAP + 2.0 Kn	NS	25 - 30	Light green nodular callus
	LS	-	-
2.0 BAP + 2.0 NAA	NS	25 - 30	MSBs
	LS	-	-
1.0 BAP + 0.1 NAA	NS	25 - 30	MSBs
	LS	-	-

*NS = Nodal segment. LS = Leaf segments. - = No response.

Huang et al. 2000, Chen et al. 2001, Chueh et al. 2001). On the other hand, nodal segments produced callus of different nature in some of the media combinations. Light green nodular callus (Fig. 1A) was produced on (i) 3.0 mg/l BAP + 1.5 mg/l Kn + 1.0 mg/l NAA, (ii) 2.0 mg/l BAP + 0.5 mg/l picloram while white and friable callus was produced on (i) 2.0 mg/l BAP, (ii) 2.0 mg/l BAP +2.0 mg/l IAA, and (iii) 1.5 mg/l BAP + 0.5 mg/l IAA + 1.5 mg/l 2, 4-D supplemented media. Leaf explants produced only white and friable callus (Fig. 1B) on three PGR

combinations *viz*. (i) 2.0 mg/l BAP, (ii) 2.5 mg/l BAP + 1.0 mg/l NAA, and (iii) 1.5 mg/l BAP + 0.5 mg/l IAA + 1.5 mg/l 2, 4-D supplemented media.

Table 2. Response of leaf and nodal segments induced callus in MS supplemented with different PGRs.

PGRs conc. and combinations (mg/l)	Colour and nature of callus	Nature of response after 45 days of culture
3.0 BAP + 1.5 Kn + 1.0 NAA	Light green nodular	Underwent differentiation producing MSBs
	White friable	Multiplied only
1.5 BAP + 0.5 IAA + 1.5 2, 4-D	Light green nodular	Underwent differentiation producing MSBs
	White friable	Multiplied only
3.0 BAP + 2.0 Kn + 1.0 NAA	Light green nodular	Underwent differentiation producing MSBs
	White friable	Multiplied without any differentiation
2.5 BAP + 2.0 NAA	Light green nodular	Underwent differentiation producing MSBs
	White friable	Multiplied only
2.5 BAP + 1.5 Kn + 1.5 NAA	Light green nodular	Underwent differentiation producing MSBs
	White friable	Multiplied only

After two subsequent subcultures in a broad spectrum of auxin and cytokinin supplemented media, the light green nodular callus underwent differentiation producing huge number of MSBs (Fig. 1C). Medium fortified with 3.0 mg/l BAP + 1.5 mg/l Kn + 1.0 mg/l NAA proved to be best for induction of MSBs in this species. Thus indirect organogenesis took place and a combination of higher concentration of cytokinin and lower concentration of auxin proved to be effective for such kind of differentiation of the callus tissue. This finding confirms the results reported by Skoog and Miller (1957) that the relative ratio of cytokinin to auxin was an effective determining factor of organogenesis in tobacco pith callus culture. White and friable callus that developed from either leaf or nodal explants proliferated but failed to undergo any kind of differentiation in any of the PGR combinations (Table 2).

In order to induce rapid elongation, the MSBs originated either through direct- or indirect organogenesis were grown on different PGR supplemented media (Table 3). Medium fortified with 0.5 mg/l BAP + 1.0 mg/l IAA proved to be efficient for rapid elongation (Fig. 1D). Strong and stout root system developed on the elongated shoot buds when these were grown individually on rooting media. One fourth strength of MS supplemented with 0.75% (w/v) sucrose + 0.5

mg/l IAA proved to be efficient for induction of strong and stout root system (Fig. 1E, Table 4). This finding revealed that nutritional stress along with IAA was propitious for sprouting of roots. Similar finding was also reported by Hossain and Bhadra (2002). Well rooted plantlets were then transferred to

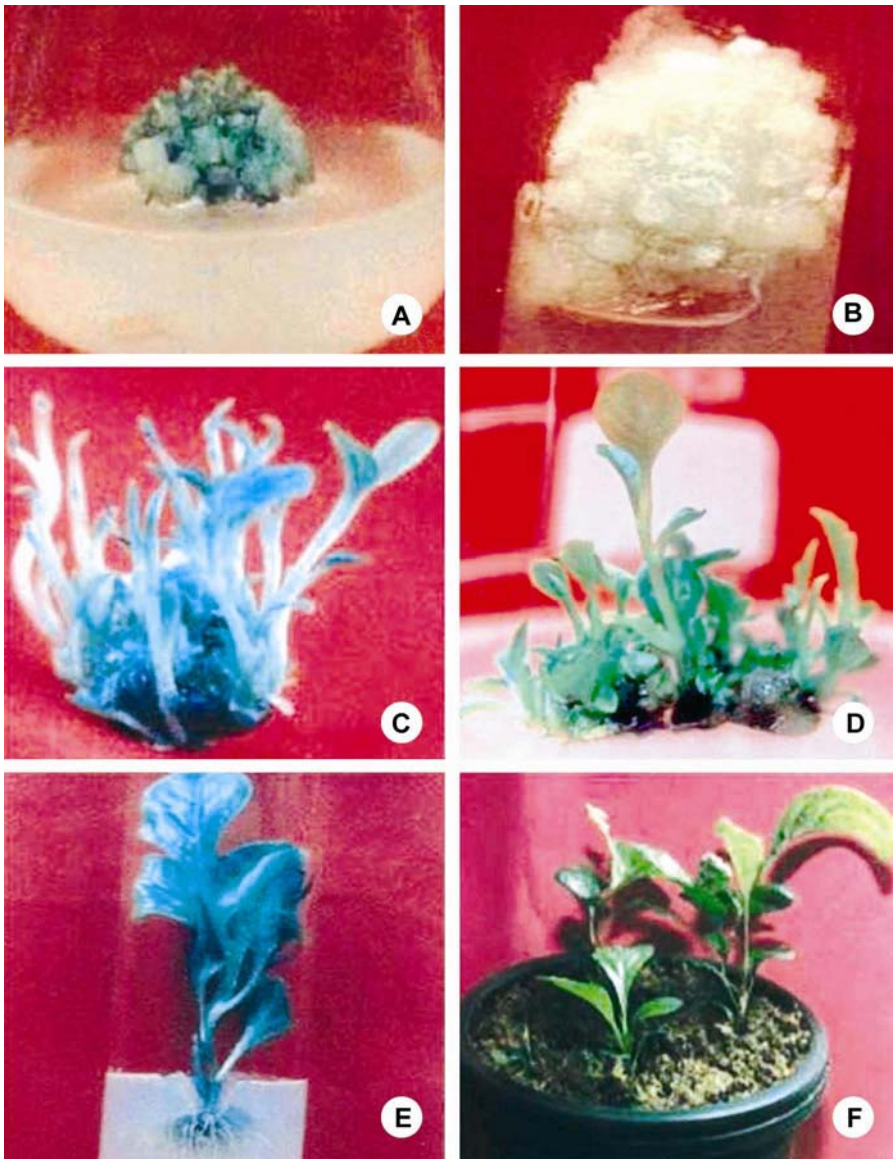

Fig. 1. A. Induction of light green nodular callus from nodal explant. B. Induction of white friable callus from leaf explant. C. Development of MSBs from green nodular callus. D. Elongation of shoot buds. E. Induction of strong and stout root system in individual plantlet. F. *In vitro* grown seedlings of *P. indica* are growing in pot in outside environment.

Table 3. Elongation of shoot buds developed from nodal segments and callus in MS supplemented with different PGRs.

PGRs conc. and combinations (mg/l)	Length (cm) of shoot buds* (Mean ± S.E.)
0.5 BAP + 1.0 IAA	3.23 ± 0.13
1.0 BAP + 0.5 IAA	2.34 ± 0.14
1.0 BAP + 1.0 IAA	1.29 ± 0.17
0.5 BAP + 0.5 NAA	1.34 ± 0.18
0.5 BAP + 1.0 NAA	1.29 ± 0.10
0.5 BAP + 2.0 NAA	1.39 ± 0.12
0.5 BAP + 3.0 NAA	1.25 ± 0.18
1.0 BAP + 0.5 NAA	1.31 ± 0.12
2.0 BAP + 0.5 NAA	1.17 ± 0.10
3.0 BAP + 0.5 IAA	1.20 ± 0.12

*Based on observations recorded on 15 cultured shoot buds in each medium.

Table 4. *In vitro* rooting.

Rooting medium containing sucrose and IAA	No. of roots/ shoot bud* (Mean ± S.E.)	Av. length (cm)* of roots (Mean ± S.E.)
MS + 3% (w/v) sucrose + 0.5 mg/l IAA	2. 73 ± 0.22	2.45 ± 0.32
½ MS + 1.5% (w/v) sucrose + 0.5 mg/l IAA	9.00 ± 0.72	4.50 ± 0.26
¼ MS + 0.75% (w/v) sucrose + 0.5 mg/l IAA	23.50 ± 2.54	2.50 ± 0.25

*Based on observations recorded on 20 cultured shoot buds in each medium with IAA.

outside natural environment through sequential phases of acclimatization. Over 90% of the plants survived after transplantation to the garden (Fig. 1F). The protocol developed for production of seedlings of *P. indica* can be used reliably for propagation in a commercial scale and *ex situ* conservation of this important medicinal plant species.

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