BIOLOGIA PLANTARUM 45 (2): 299-302, 2002

BRIEF COMMUNICATION

Plant regeneration through somatic embryogenesis in leaf derived callus of *Plumbago rosea*

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Abstract

Regeneration of *Plumbago rosea* L., a rare medicinal plant, via somatic embryogenesis in callus cultures derived from leaf explants was described. Optimum callus formation was achieved on semi-solid Murashige and Skoog (MS) medium supplemented with 0.25 mg dm^{-3} kinetin and 2.0 mg dm^{-3} 1-naphthaleneacetic acid (NAA). Somatic embryogenesis was achieved upon transferring the 4-week-old callus to a medium containing 1.0 mg dm^{-3} kinetin (Kn), 0.5 mg dm^{-3} gibberellic acid (GA_3) and 0.1 mg dm^{-3} NAA. Embryo maturation and germination was achieved on the half-strength MS basal salts supplemented with 0.01 - 0.25 mg dm^{-3} Kn and 2 % (m/v) saccharose. An average of 50 - 60 plantlets were obtained from 150 mg of embryogenic callus within 4 week of subculture. Out of the 50 plantlets about 28 survived in the greenhouse.

Additional key words: gibberellic acid, naphthaleneacetic acid, *in vitro*, medicinal plant.

Plumbago rosea Linn. (family *Plumbaginaceae*) is a rare medicinal plant, distributed in the tropics. It is a rich source of an alkaloid, plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone), used as anticancer drug. Roots of older plants yielded the maximum amount of alkaloids. Propagation through seed is difficult due to poor seed setting, germination and death of young seedlings under natural condition. Vegetative propagation through stem cuttings is possible but is seriously damaged by red ants and a soil fungus. *In vitro* propagation of *Plumbago* species were achieved through axillary bud culture and shoot bud regeneration via the callus phase (Satheesh Kumar and Bhavanandan 1988, Rout *et al.* 1999a,b, Chetia and Handique 2000, Saxena *et al.* 2000). Cultivation and improvement programmes of the *Plumbago* species are limited. To date, there are no reports on plant regeneration via somatic embryogenesis of *Plumbago* species. The present investigation is the first report on induction of somatic embryogenesis from leaf callus of *Plumbago rosea* Linn.

Leaves were collected from 4-week-old *in vitro* grown plants of *Plumbago rosea* and used as explant source. They were placed on semi-solid basal Murashige and Skoog (1962; MS) medium supplemented with different concentrations and combinations of BA (0 - 3.0 mg dm^{-3}), Kn (0.0 - 3.0 mg dm^{-3}), NAA (0.0 - 3.0 mg dm^{-3}) and IAA (0.0 - 3.0 mg dm^{-3}) for callusing. The pH of the media was adjusted to 5.8 using 0.1 M HCl or 0.1 M NaOH before autoclaving. Routinely, 25 cm^3 of the liquid medium containing 0.8 % (m/v) agar (*Qualigen*, Bombay, India) was dispensed into culture tubes (25 × 150 mm) (*Borosil*, Bombay, India) and plugged with non-absorbent cotton wrapped in one layer of cheese-cloth. To maintain the callus culture, the proliferated calluses (150 ± 10 mg) were regularly sub-cultured at 4-week intervals on fresh medium with the same composition. The cultures were incubated under a 16-h photoperiod (irradiance of 55 μmol m^{-2} s^{-1}) provided by cool, white fluorescent

Received 27 February 2001, *accepted* 8 August 2001.

Abbreviations: BA - 6-benzylaminopurine; GA_3 - gibberellic acid; Kn - kinetin; IAA - indole-3-acetic acid,; NAA - 1-naphthaleneacetic acid; MS medium - Murashige and Skoog (1962) medium.

Acknowledgement: The authors wish to acknowledge the help of the Department of Forests and Environment, Government of Orissa for providing necessary facilities.

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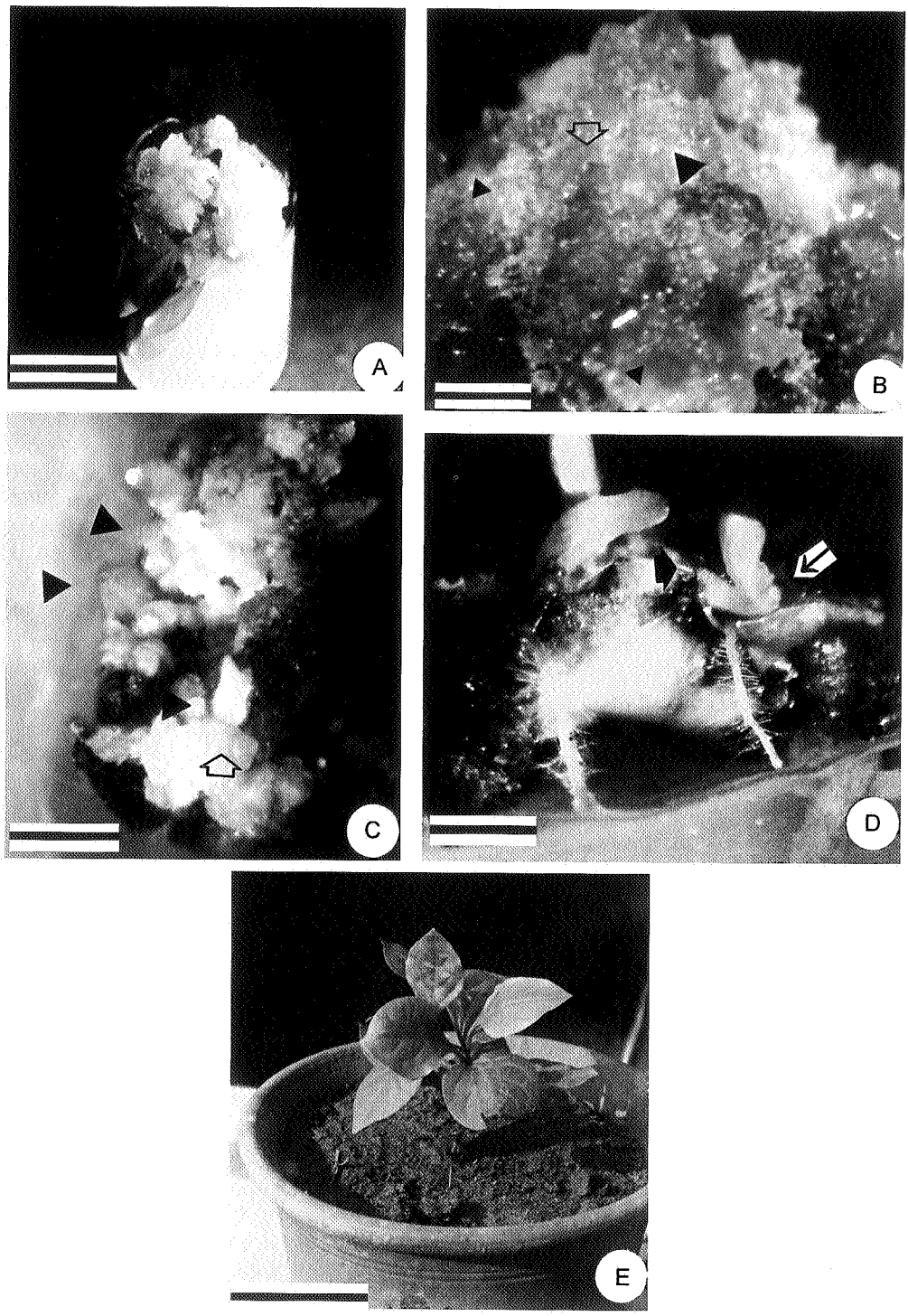

Fig. 1*A*. Induction of callus from leaf explants of *Plumbago rosea* on MS medium supplemented with 0.5 mg dm^{-3} Kn and 2.0 mg dm^{-3} NAA after 4 weeks of culture (*bar* = 10 mm).
Fig. 1*B* and *C*. Development of globular embryogenic callus (*arrows*) from leaf-derived callus of *P. rosea* cultured on MS medium supplemented with 1.5 mg dm^{-3} Kn, 0.5 mg dm^{-3} GA_3 and 0.1 mg dm^{-3} NAA after 4 weeks (*A*) and six weeks (*B*) of culture (*bar* = 5 and 10 mm for *B* and *C*, respectively).
Fig. 1*D*. Clump of somatic embryos were germinated (*arrows*) on half strength MS medium supplemented with 0.1 mg dm^{-3} Kn and 2 % saccharose after 10 d of culture (*bar* = 15 mm).
Fig. 1*E*. Somatic embryo derived plantlet grown in the pot (*bar* = 50 mm)

lamps (*Philips*, Bombay, India) and maintained at a constant temperature of 25 ± 2 °C. To induce somatic embryogenesis, the proliferated friable callus (150 ± 10 mg) were transferred to various media containing BA (0.0 - 3.0 mg dm^{-3}), Kn (0.0 - 3.0 mg dm^{-3}), GA_3 (0.1 - 1.0 mg dm^{-3}), NAA (0.0 - 0.5 mg dm^{-3}) or IAA (0.0 - 0.5 mg dm^{-3}) at various concentrations and combinations with 3 % (m/v) saccharose. All cultures were incubated at the above mentioned conditions.

For germination, the group of somatic embryos were transferred to MS basal salts supplemented with different concentrations and combinations of Kn, NAA (0.0, 0.01, 0.05, 0.1, 0.2 and 0.25 mg dm^{-3}) and 2 % (m/v) saccharose. Rooted plantlets (3 - 4 cm) were transferred to 5 cm earthern pots containing a mixture of sterilized garden soil, sand and cow-dung manure (2:1:1, v/v) and kept in a climate-controlled greenhouse for acclimatization. Twenty five cultures were used per treatment and each experiment was repeated at least three times. The data pertaining to mean percentage of cultures showing callus proliferation, embryogenic callus growth, mean number of somatic embryos per culture, percentage of somatic embryos germinated and percentage of plantlets surviving in soil were statistically analysed by the Duncan multiple range test (Harter 1960).

Table 1. Effect of Kn, BA, NAA and IAA [mg dm^{-3}] on callus induction from leaf explants of *Plumbago rosea* after 4 weeks of culture. Means ± SE having same letter in a column were not significantly different by at $P < 0.05$ (Post-Hoc Multiple Comparison test). 25 cultures per treatment, repeated thrice.

Kn	BA	NAA	IAA	Explants showing callus [%]
0	0	0	0	0
0.25	0	0.5	0	0
0.25	0	1.0	0	60.7 ± 1.0e
0.25	0	1.5	0	72.8 ± 0.8i
0.25	0	2.0	0	82.4 ± 1.1k
0.5	0	1.0	0	68.2 ± 0.6h
0.5	0	2.0	0	76.8 ± 1.2j
0.5	0	3.0	0	64.4 ± 1.0g
0	0.25	1.0	0	50.6 ± 0.8b
0	0.5	2.0	0	53.8 ± 0.4c
0	0.5	3.0	0	60.4 ± 0.8e
0.5	0	0	1.0	44.6 ± 1.0a
0.5	0	0	2.0	50.2 ± 0.7b
0.5	0	0	2.5	62.4 ± 1.0f
0.5	0	0	3.0	56.2 ± 0.8d
0	0.25	0	1.0	45.6 ± 0.7a
0	0.5	0	1.0	50.2 ± 1.1b
0	0.5	0	1.5	56.4 ± 1.3d
0	0.5	0	2.0	61.8 ± 1.0f
0	0.5	0	3.0	60.4 ± 1.2e

Callus initiation was obtained on the cut regions as well as abaxial surfaces of the explants within 12 - 15 d. There was no callus formation when explants were cultured in media without auxin or cytokinin. Friable white callus was developed on the abaxial surfaces of the explants on MS basal medium supplemented with 0.25 - 0.5 mg dm^{-3} Kn and 1.0 - 3.0 mg dm^{-3} NAA (Table 1). The highest frequency (82.4 %) of callus formation, however, was recorded on leaf segments when cultured on MS media containing 0.25 mg dm^{-3} Kn and 2.0 mg dm^{-3} NAA (Fig. 1*A*) which subsequently turned brown within 8 weeks of culture. The browning of the callus was overcome by adding 0.25 mg dm^{-3} ascorbic acid in the culture medium. The medium having Kn + IAA, BA + NAA or BA + IAA also promoted callus growth from leaf explant but the rate of callus growth was very slow. Higher concentration of either IAA or NAA in the culture medium did not show any positive effect of callus growth. Most of the media that induced rapid callus growth included NAA in combination with Kn.

Numerous white globular clumps of somatic embryos were developed over the entire surface of the callus on MS basal medium supplemented with 1.0 - 1.5 mg dm^{-3} Kn, 0.25 - 0.5 mg dm^{-3} GA_3 and 0.1 mg dm^{-3} NAA after 4 weeks (Fig. 1*B*) and 6 weeks (Fig. 1*C*) of culture. The embryogenic callus production was maximum on medium containing 1.5 mg dm^{-3} Kn, 0.5 mg dm^{-3} GA_3 and 0.1 mg dm^{-3} NAA. The frequency of embryogenic callus production varied from 32.6 to 86.8 % in leaf-derived calluses depending on the cencentration of auxins and cytokinins tested (Table 2). The ability of the cultures

Table 2. Effect of growth regulators [mg dm^{-3}] on somatic embryogenesis in callus culture of *P. rosea* after 4 weeks of subculture. Means having same letter in a column were not significantly different at $P < 0.05$. Means of three repeated experiments; each experiment consisted of 25 replicates.

BA	Kn	GA_3	NAA	IAA	Cultures forming embryo [%]	Number of somatic embryos [$culture^{-1}$]
0	0	0	0	0	0	0
1.0	0	0	0.1	0	0	0
1.0	0	0.25	0.1	0	0	0
0	1.0	0.25	0.1	0	32.6 ± 1.0a	26.2 ± 0.8a
0	1.0	0.25	0	0.1	0	0
0	1.5	0.5	0.25	0	0	0
0	1.0	0.5	0.1	0	62.2 ± 0.7c	44.8 ± 1.1c
0	1.5	0.5	0	0.1	0	0
0	1.5	0.5	0.1	0	86.8 ± 0.8e	66.4 ± 0.6e
0	1.0	1.0	0.1	0	55.4 ± 0.6b	34.8 ± 0.5b
0	1.5	1.0	0.1	0	66.2 ± 0.5d	48.4 ± 0.8d
1.5	0	1.0	0.1	0	0	0
2.0	0	1.0	0.1	0	0	0
1.5	0	1.0	0	0.1	0	0

to become embryogenic declined with an increase in the concentration of either Kn or NAA. Similar development of embryogenic calluses subjected to high contents of cytokinins and low contents of auxin in combination with GA_3 was reported in *Rumex acetosella* (Culafic *et al.* 1987), *Spinacia oleracea* (Xiao and Branchard 1983) and *Eryngium foetidum* (Ignacimuthu *et al.* 1999). The number of somatic embryos/culture varied from 26.2 to 66.4 depending on the growth regulators used (Table 2). The production of somatic embryos per culture was maintained for prolonged periods (data not shown). The inclusion of GA_3 in the induction medium helps the production of higher percentage of embryogenic calli for longer period was reported in *Trifolium pratense* (Davey and Power 1988), hybrid rose (Rout *et al.* 1991) and in *Simarouba glauca* (Rout and Das 1994).

Table 3. Effect of different concentrations of kinetin on somatic embryo germination of *Plumbago rosea* within 2 weeks of culture. Means ± SE of three repeated experiments; each experiment consisted of 25 replicates.

Kn [mg dm^{-3}]	Germination [%]
0	0
0.01	36.2 ± 0.8
0.05	48.4 ± 0.6
0.10	62.0 ± 1.1
0.20	40.8 ± 1.3
0.25	22.4 ± 0.7

About 36 - 62 % of the somatic embryos showed germination on medium having 0.01 - 0.25 mg dm^{-3} Kn within 2 weeks of transfer (Table 3). The somatic embryos were germinated into plumule and root (62 %) on MS medium supplemented with 0.1 mg dm^{-3} Kn within 10 d of culture (Fig. 1*D*). Addition of NAA in the germination medium showed inhibition of embryo germination. On an average 50 - 55 somatic embryos germinated into plantlets per 150 mg of embryogenic callus. It was reported that the inclusion of low concentration of cytokinin in the germination medium stimulates the germination of somatic embryos (Chang and Hsing 1980, Rout and Das 1994). The efficiency of somatic embryo germination was strongly dependent on the composition of auxin, cytokinin and gibberellic acid concentrations during the initial induction of embryogenic phase; for subsequent growth and development of the embryos into plantlets auxin and GA_3 were not required. Our findings agree well with the results of *Asparagus officinalis* (Kunitake and Mii 1990) and hybrid rose cultivar (Rout *et al.* 1991). Of the 50 plantlets that were subjected to acclimatization, 72 % survived under greenhouse condition. The plants grew well in the natural environment (Fig. 1*E*). There was no differences among plantlets in morphology.

In conclusion, an efficient protocol was developed for plant regeneration via somatic embryogenesis in callus culture of an important medicinal plant *Plumbago rosea*. Further investigation are necessary for enhancing the alkaloids plumbagin through genetic transformation study.

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