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Global Journal of Biotechnology & Biochemistry 3 (2): 83-86, 2008
ISSN 1990-9241
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In vitro flowering, somatic embryogenesis and regeneration in *Boerhaavia diffusa Linn*. - A medicinal plant

[1]M.S. Sudarshana, [2]M.H. Niranjan and [3]S.T. Girisha

[1,2]Department of studies and Research in Botany, Manasa gangotri, Mysore-06, India
[3]Department of Biotechnology, Jnanabharati campus, Bangalore University, Bangalore-56, India

Abstract: *In vitro* callogenesis was achieved from leaf explants of *Boerhaavia diffusa* Linn. on basal medium supplemented with different concentrations of BAP, NAA, Kn and IAA. Regeneration of shoot buds were observed on MS medium supplemented with BAP and NAA at different concentrations. In some of the replicates the shoot buds were elongated and terminated in flower buds. *In vitro* flowering occurred within two weeks of subculture. The highest percentage of flowering was obtained on medium supplemented with BAP (2 mg l^{-1}). Excised shoots after transferring to rooting liquid medium (0.5-2.0 mg l^{-1} IAA or NAA) roots were initiated. The regenerated plantlets have been successfully established in vermiculite soil. Subsequently, they were shifted to the field conditions. Regenerated plantlets resemble the parent plants morphologically and cytologically. Histological study of the cultures showed the presence of meristemoids, somatic embryos and embryoids.

Key words: *Boerhaavia diffusa* · Organogenesis · Medicinal plant · *In vitro* flowering

INTRODUCTION

Boerhaavia diffusa (Nyctaginaceae) commonly known as punarnava is a medicinal herb. The root of the plant is considered laxative and diuretic [1]. It is used to cure jaundice, ascites, anasarca, asthma and in scanty urine. The active constituent of the drug is the alkaloid, punarnavine, the total alkaloid content of the roots being about 0.04%.

In recent years much interest has been evinced in the propagation of medicinal plants by tissue culture [2, 3]. There are only a few reports in the members of Nyctaginaceae [4] and less in those members which are medicinal [5]. As the regenerative potentiality is maximum in the leaf explants [6], this was used as the explant material to determine the efficacy of the various media to induce callus and organogenesis. Plant tissue cultures have rapidly made progress to obtain somaclonal variants and for producing a novel secondary product of genetic variability [7].

Initiation of flowering may become a valuable research tool to conduct *in vitro* hybridization and to cut short the long period for flowering and seed formation. Flowering *in vitro* has been promoted by cytokinins at optimum concentrations.

As there are no reports with respect to the regeneration of this plant the present study was undertaken to standardize a protocol for *in vitro* regeneration, embryogenesis and flowering from leaf explants besides carrying out histology of the callus cultures.

MATERIAL AND METHODS

Plant Material: Healthy leaves of *B. diffusa* were collected from 5-6- week old plants growing in Manasagangotri campus, University of Mysore, Mysore. Leaves were made into segments of 1.0 sq.cm and thoroughly washed under running tap water for 30 mins. Explants were disinfected with 1% solution (v/v) teepol for 10min followed by surface sterilization using 0.1% HgCl2 for three minutes and rinsed thrice in sterile distilled water to remove traces of HgCl2.

Culture Medium: Surface sterilized explants were placed on solid media like WM [8], MS [9] and B-5 [10] containing various concentrations and combinations of auxins and cytokinins. Sucrose (3%) is used as the carbon source. The pH was adjusted to 5.8 before autoclaving. The cultures were maintained at 22±1°C under a 16 h

Corresponding Author: S.T. Girisha, Department of Biotechnology, Janabharati campus, Bangalore University, Bangalore-56

photoperiod. Cool-white fluorescent tubes provided a light intensity of 80µ mol $m^{-2}s^{-1}$. Callus induction was observed within 10 days and the callus was subcultured once in 2 weeks. Shoots were differentiated in 5 weeks and some of the elongated shoots terminated in flower buds. The shoots were excised and transferred to liquid medium for rooting. The rooted plantlets were removed from cultures and hardened in plastic cups containing vermiculite soil and kept in the green house for further acclimatization and finally transferred to the field.

For histological studies embryogenic callus was fixed in FAA (Formalin acetic alcohol; 5:5:90). Sections were taken at 9-11µ thickness using a rotary microtome. The Ref. slides were processed and stained in Heidenhain's haematoxylin following customary methods and mounted in canada balsam [11].

Table 1: Percentage of leaf explants showing callus initiation in *B.diffusa*

	2, 4-D	IAA	BAP	Kn
MS	78.0	15.2	69.0	45.2
B-5	70.6	12.0	55.3	38.4
WM	30.3	5.3	18.7	20.0

Table 2: Response of leaf segments of *B. diffusa* to various growth hormones on MS medium

	Culture with No. of shoots (mg l^{-1})	
Growth regulators (%)	Shoots	Culture±S.D.
BAP (1.0)	15	1.4±2.80
BAP (2.0)	31	0.9±2.90
BAP (0.5) +NAA (0.5)	35	1.7±2.70
BAP (1.0) +NAA (0.5)	65	2.1±1.99
BAP (3.0) +NAA (1.0)	85	2.6±1.44
Kn (2.0) +NAA (0.5)	35	1.0±2.4
Kn (3.0) + NAA (1.0)	30	0.8±1.8

RESULTS

MS medium was found to be the most suitable for both callus induction and regeneration in *B.diffusa.* Of the growth regulators tried to initiate callus, 2, 4-D elicited better response (Table 1). Nearly in all the growth regulators the explants proliferated callus within 15 days (Fig. a). Series of subcultures were carried out and the effects of growth regulators on the callus that derived on MS medium are mentioned in Table 2. Shoot buds (Fig. b) and roots were differentiated after 5 weeks of callus initiation. Maximum percentage of shoot proliferation was occurred on a medium containing BAP (3.0 mg l^{-1}) and NAA (1.0 mg l^{-1}).

The callus after 3-4 subcultures loses its potentiality to produce shoot buds but root formation was evident even after 5-6 subcultures. The regenerated shoots were excised and transferred to the MS liquid medium supplemented with different concentrations of IAA, IBA and NAA. At low concentrations (0.5 mg l^{-1}) of IAA maximum proliferation of roots was observed.

***In vitro* Flowering:** *In vitro* flowering was observed within 15 days after subculture on BAP (2 mg l^{-1}) supplemented medium (Fig. c). High frequency of flowering per explant was more on MS medium supplemented with 2 mg l^{-1}BAP compared to other concentrations of BAP or NAA tested. *In vitro* flowers are small when compared to *in vivo* flowers.

Histology: Serial sections of 3-day-old incubated leaf segment showed the division of spongy and vein cells to form a compact mass of cells (Fig. d). The leaf section of the 5th day revealed the initiation of root primordia from the vascular traces.

The callus that subcultured on the medium supplemented with BAP (2 mg l^{-1}) and NAA (1 mg l^{-1}) was found to contain embryogenic cells. The subcultured callus proliferated on MS medium having BAP (3 mg l^{-1}) and NAA (1 mg l^{-1}) showed different types of embryoids interspersed with a shoot bud (Fig. e). A six-week-old callus showed shoot buds each with a pair of primordial leaves (Fig. f) enclosing the shoot apex.

Morphology of *in vitro* Plants: A preliminary screening of the regenerated plants did not show any variation with the donor plants. The cytological analysis of the root tips of the regenerated plants were diploid.

DISCUSSION

The present investigation has clearly demonstrated that, MS medium is the most suitable medium for callus induction as well as for plant regeneration in *B. diffusa*. The most effective auxin for callus induction was 2, 4-D, followed by BAP, Kn and IAA. There are several reports [12, 13] which suggest that the leaves are the best sources of explants for the induction of multiple shoots. The data presented in Table 2 shows that, high frequency and maximum number (85%) of multiple shoots were elicited on MS medium containing 3.0 mg l^{-1}BAP + 1.0 mg l^{-1}NAA followed by 1.0 mg l^{-1}BAP + 0.5 mg l^{-1}NAA. A significant observation was that after fourth subculture it loses its potency to regenerate. It is due to the loss of endogenous factors at critical stages of growth during serial subcultures [14].

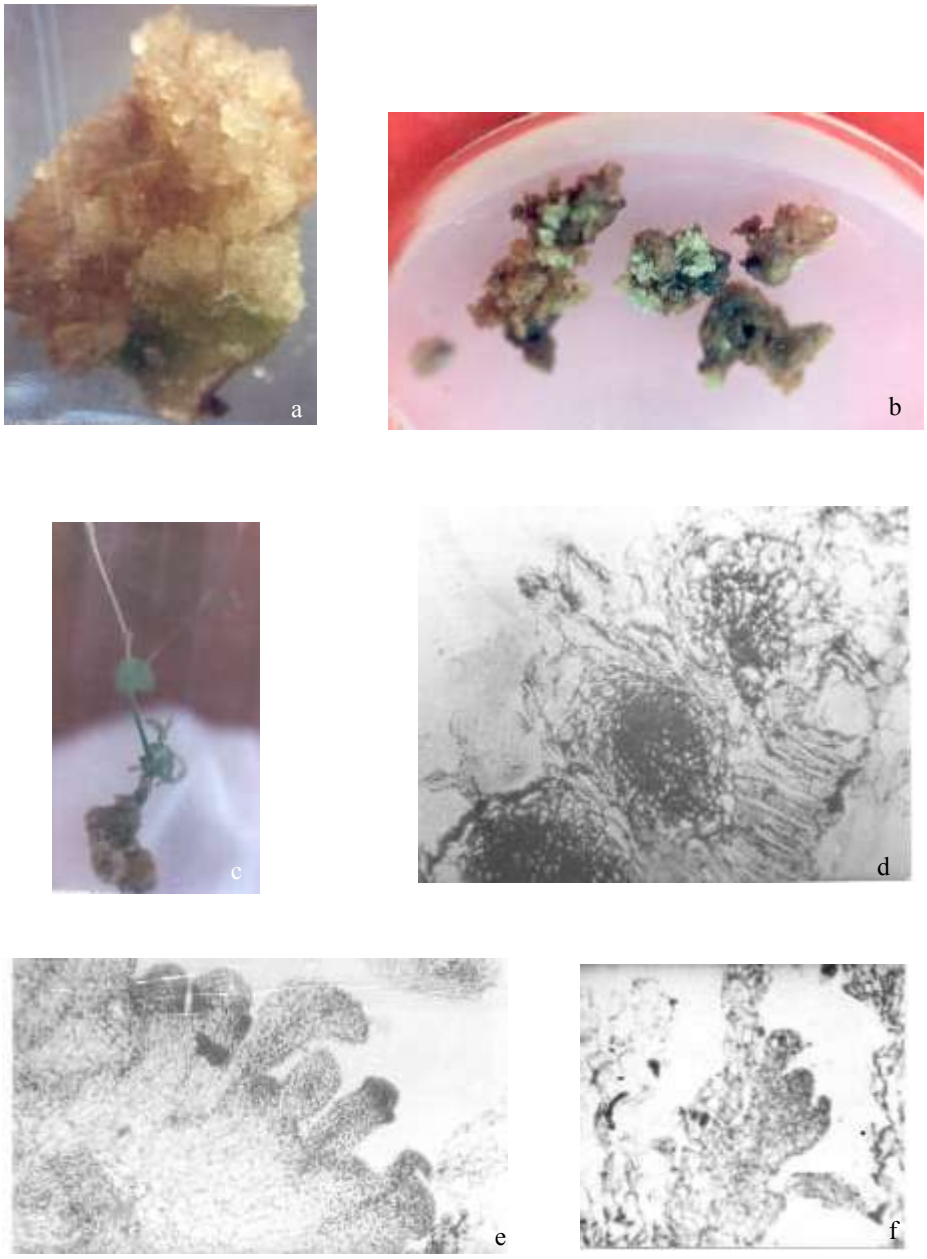

Fig. a: Leaf callus proliferation
Fig. b: Differentiation of shoot buds
Fig. c: *In vitro* flowering
Fig. d: Callus proliferation from the spongy and vein cells
Fig. e: Embryoids with a shoot bud
Fig. f: A shoot bud with a pair of leaf primordia

In some subcultures containing 2 mg l^{-1} BAP the shoot buds after elongation terminated in flowers indicating BAP as cytokinin supported flowering similar to the work of Ralph and Jules [15] in *Passiflora suberosa* where cytokinin is an absolute requirement for flowering. The medium supplemented with IAA (0.5-1.0 mg l^{-1}) induced maximum rooting contrary to the work of Rout *et al*. [16] where in the excised shoots of *Plumbago rosea* induced poor rooting with an intervening callus.

Histologically, a dome of meristematic tissue developing at the periphery of cell aggregates was noticed from which different types of embryoids developed. Similar reports has been observed in carrot [17] where in the embryoids arise by the activity of groups of cells. Present work is also supported by Tisserat *et al*. [18] who pointed out that a single cell origin had not been demonstrated in many cases where apparently normal bipolar embryoids were formed from aggregate of cells. This is in contrary to the report of Konar *et al*. [19] wherein the somatic embryoids in *Rananculus sceleratus* were developed from individual epidermal cells.

The ability to regenerate plants from leaf explants of *B.diffusa* has interesting and medicinal applications. The results show that *B. diffusa* can be propagated by somatic embryogenesis besides organogenesis. It is also possible that regenerated plants represent an enriched source of totipotent cells.

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ABBREVIATIONS

MS = Murashige and skoog's,
WM = White's medium,
B-5 = Gamborg's,
BAP = Benzyl Amino purine,
Kn = Kinetin,
IAA = Indole Acetic Acid,
NAA = Naphthalene Acetic Acid.