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In vitro propagation of *Boerhaavia diffusa* L.: An important medicinal plant of family Nyctagimaceae

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Abstract

***Boerhaavia diffusa* L., also known as santhi, or punarnava is an important medicinal plant, belonging to the family Nyctaginaceae. This species is said to be distributed throughout Malwa plateau in central India, as per ayurvedic literature, but due to extensive commercial exploitation, the species has become vulnerable. For callus induction, leaf explants were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 2,4-dichlorophenoxy acetic acid (2, 4-D 2.26 µM-9.04 µM) and N^6-benzyladenine (BA 1.11 µM-4.44 µM), either alone or in combinations. Calli formed within 10-12 days of culture, followed by shoots regeneration within 20-25 days. Direct organogenesis was achieved from nodal explants in MS media fortified with 2,4-D (2.26 µM-9.04 µM) along with BA (1.11 µM-4.44 µM) within 20 days. Multiple shooting was observed during subculture of *in vitro* regenerated shoots when 2,4-D was replaced with α-naphthalene acetic acid (NAA). Rooting was achieved in MS medium fortified with 2.85 µM IAA, within 7-10 days and also on half strength MS medium containing 2.85 µM Indole-3-acetic acid (IAA). For hardening, regenerated plants, with roots (3-4cm) were initially maintained on half-strength MS liquid medium ($MS_{1/2}$) without growth regulators followed by quarter strength MS ($MS_{1/4}$) liquid medium for 10 days. For acclimatization sterile mixture of soil, sand and manure (2:1:1) was used. Survival rate of regenerated plants was nearly 100%.**

Key words: *Boerhaavia diffusa*, vulnerable, organogenesis, plant regeneration, *in vitro* flowering

Introduction

Boerhaavia diffusa L., generally known as Punarnava or santhi is a perennial creeping, diffusely branched herb belonging to the Nyctaginaceae family. It is widely distributed throughout India. It is used in the traditional medicine system of Ayurveda and Unani. *B. diffusa* is known for its pharmacological properties like anti-lymphoproliferative, antiurethritis, antiasthmatic, antibacterial, anti-inflammatory, antileprosy, antidiabetic, immune-modulation and anti-metastatic (Mehrotra et al. 2002). Root extract was utilized as an adjuvant in the treatment of pulmonary tuberculosis, chemotherapy and is a very potent source of the alkaloid drug punarnavaine, which is documented as a diuretic in Indian Pharmacopoeia (Kant et al. 2001). Two rotenoids, Boeravinones G and H are isolated from the roots of this plant and also reported to contain a ribosome-inactivating protein BDP-30 (Srivastava et al. 2015). Several plant viruses like cucumber green mottle mosaic virus and papaya ring spot virus are disinfected via use of purified glycoprotein. Its dried root extract was found active against mung bean yellow mosaic virus, water melon mosaic virus and bean common mosaic virus (Awasthi and Menzel, 1986). In 6-7 weeks old male Swiss albino mice, this plant has been reported to have cancer chemopreventive property against skin papillomagenesis (Bharali et al. 2003).

Roots are used in the preparation of various ayurvedic formulations. Indiscriminate exploitation of roots of this plant and due to low seed viability, this plant species has been categorized as vulnerable in central India. Under these circumstances, there is a need to develop easy and efficient method for mass propagation of this species. Tissue culture methods have been used for conservation and mass multiplication of rare and endangered plant species such as *Ilex khasiana* (Dang et al. 2011), *Ajuga bracteosa* (Kaul et al. 2013), *Heliotropium kotschyi* (Sadeq et al. 2016) *Salacia oblonga* (Deepak et al.

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2016) and *Genipa americana* (deSouza et al. 2019). Tissue culture protocols have been used not only for propagation of endangered plant species but also to accelerate the selection process and enhancing efficiency of desirable traits at cellular level in crops (Heidary et al. 2018; Huynh et al. 2017). Various techniques of callus induction have been applied, considering different plant parts such as, leaf tissues, shoot tips, seeds and embryo etc in order to improve different traits. Clonal propagation of *B. diffusa* from shoot tip and nodal explants has been achieved using various growth regulators (Bhansali et al. 1978; Biswas et al. 2009). Synthetic seed development using shoot tip and nodal segments along with assessment of genetic fidelity through genetic fingerprinting of regenerated plants was also reported by Ray and Bhattacharya (2008). Sudarshana et al. (2008) observed embryonic cells in leaf calli cultured on MS medium supplemented with BA (2 mg/l) and NAA (1 mg/l). Jenifer et al. (2012) reported development of adventitious roots, directly from leaf explants of *B. diffusa,* cultured on MS media supplemented with various concentrations of NAA and IBA (0.25, 0.5, 1.0, 2.0 or 4.0 mg/l). Hairy root cultures in *B. diffusa* have been worked out using shoot tips and leaves from *in vitro* regenerated plants from nodal segments (Shrivastava and Padhya 1995). The present study describes an efficient and reproducible protocol for propagation of *B. diffusa* through nodal and leaf explants.

Materials and methods

Source of explants, sterilization and culture conditions

Leaf and nodal explants were used for cultures. The plant material was collected from medicinal plants garden located at College of Agriculture, Rajmata Vijayaraje Scindia Agriculture University, Campus Indore, Madhya Pradesh. Plant materials were first washed with running tap water for 15 min, followed by treating with Tween 20 solution (0.5% v/v) for 15 min and then washing with autoclaved distilled water. Further sterilization was carried out under laminar air flow. Surface-disinfection was done, using freshly prepared 0.1% (w/v) mercuric chloride solution for 15 min. After this, the explants were quickly rinsed for 10 s using 70% ethanol (v/v) followed by 3-4 washings in sterile distilled water. 5-7 mm explants were aseptically cultured on 25 mm x 150 mm glass tubes containing 25 mL of Murashige and Skoog medium. Non-absorbent cotton plugs wrapped with one layer of cheesecloth were used to cover the culture tubes. After adjusting the pH to 5.8 with 0.1 N HCl or 0.1 N NaOH, 0.8% (w/v) agar (Hi-Media, India) was added to the liquid medium. The tubes were autoclaved at 121°C and 1.06 kg/cm^2 pressure for 15 min. The cultures were incubated at relative humidity of 60%, temperature 25 ± 2°C and 16/8-h light/dark photoperiod (cool-white fluorescent light, irradiance 50 mmol m^{-2} s^{-1}).

Callus induction and shoot proliferation from leaf explants

For callus induction, leaf explants were cultured on MS medium supplemented with different concentrations of 2,4-dichloro phenoxy acetic acid (2,4-D) (2.26-4.52 µM) and N^6-benzyladenine (BA 1.1- 2.2 µM). Three replicates were maintained for each treatment and every treatment was repeated thrice. After 2 weeks of culture initiation, explants forming callus were scored and percentage response was calculated. For shoot induction from leaf explants, the calli were transferred to the medium containing various concentrations of α-naphthalene acetic acid (NAA) and BA.

Shoot proliferation from nodal explants

For micropropagation, nodal explants were cultured on MS medium containing different concentrations of 2,4-D (2.26 µM-9.04 µM) or NAA (2.64-3.98 µM), either alone or in combinations with BA (1.11 µM-4.44 µM). Shoot multiplication was done by excising single shoots from multiple shoots produced from single explants. These single shoots were maintained in different culture tubes on the same medium before roots induction.

Roots formation from in vitro grown shoots and acclimatization

Rooting of *in vitro* regenerated shoots was achieved in MS medium enriched with IAA (2.85-5.70 µM) and also in MS basal medium. Regenerated plants with roots (3-4cm) were initially maintained on $MS_{1/2}$ liquid medium (basal medium) for 15 days. Then the plantlets were transferred to $MS_{1/4}$ liquid medium and maintained for 10-15 days. After 20-25 days, the regenerated plants were shifted to pots containing mixture of autoclaved soil: manure: sand (1:2:1). During acclimatization, the pots were kept in shade and humid conditions.

Data analysis

Morphological responses of explants such as percent

response for callus formation, shoot formation, mean value of shoots per explant and percentage of roots formation were recorded. The response of explants forming shoots was observed after 3rd week of culture and the mean value of shoots per explant was calculated after 5 weeks. All experiments were repeated thrice using completely randomized design (CRD) consisting minimum fifteen replicates per treatment Results were statistically analyzed, standard error and mean values were calculated for each experiment.

Results and discussion

Effect of plant growth regulators on callusing and shoot proliferation from leaf segments

MS medium fortified with 2,4-D alone or in various combinations with BA was found best for white and compact callus formation from leaf pieces, within two weeks. Explants cultured on MS medium containing NAA (2.64-3.98 µM), along with BA (1.11-2.22 µM) formed calli after 2-3 weeks of culture. Explants cultured on MS medium fortified with 2,4-D (2.26 µM) and BA (1.11 µM) resulted in giving maximum response (83 ± 0.57 %) for callus formation (Table 1). Least callus induction (47 ± 1.15 %) was observed for MS medium supplemented with NAA (2.64-3.98 µM) along with BA (1.11-2.2 µM). Increasing concentration of 2,4-D (more than 2.26 µM) in combination with BA (1.11 or 2.22 µM) or alone did not result in increase in callus mass. The responding calli were transferred to shoot induction medium after 15-18 days. Shoots formation occurred from calli on transfer to MS media containing NAA (2.64-3.98 µM), either alone or in combination with BA (1.11-4.44 µM) (Fig. 1 a, b). Shoot primordia could be observed upon surface of calli, within 7-8 days of transfer to the shoots induction medium. Maximum percentage of shoots formation (86.6 ± 1.52 %) and maximum mean no. of shoots per explants (4.57 ± 0.64) was noted from the calli developed on MS medium containing 2,4-D (2.26 µM) and BA (1.11 µM), upon transfer to MS medium enriched with 2.64 µM NAA and 1.11 µM BA (Table 1). Multiple shoots proliferation observed in continuous cultures in the same medium. Maximum of 5 shoots were observed from the calli derived from leaf pieces. For shoot multiplication, individual shoots were excised and maintained in different culture tubes on the same medium for 10-15 days. 4-5 cm long proliferated shoots were transferred to rooting medium.

Effect of plant growth regulators on callusing and shoot proliferation from nodal explants

Direct shoots regeneration (without any intermediate callusing) was observed from nodal explants cultured

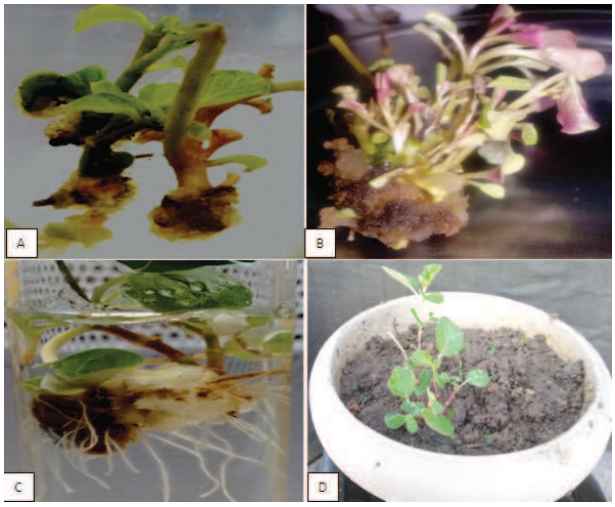

Fig. 1. Callus induction and shoots regeneration from leaf explants. (A-B) Multiple shoots formation on MS medium supplemented with NAA(2.64 µM)+BAP(1.11 µM), (C) Rooting from regenerated plantlet in half strength MS liquid medium and (D) *In vitro* regenerated plantlets transferred to sterile mixture of soil, sand & manure (2:1:1)

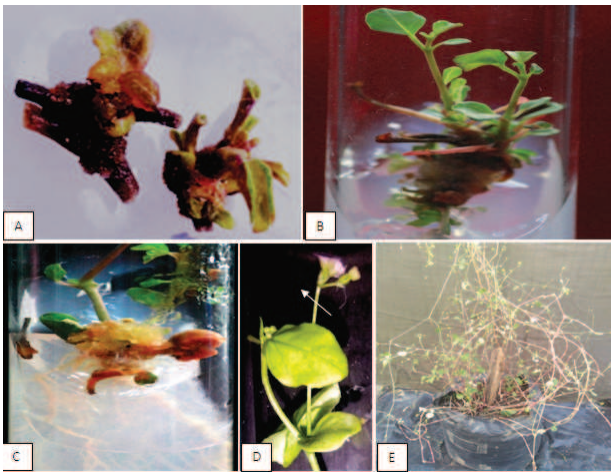

Fig. 2. Multiple shoots induction from nodal explants and root induction in *in vitro* regenerated shoots of *B. diffusa.* (A-B) *In vitro* shoot regeneration on MS medium containing 4.44 µM BA, (C) Root differentiation in *in vitro* regenerated shoots, (D) *In vitro* flowering on MS medium containing NAA (2.64 µM) + BAP (1.11 µM) and (E) *In vitro* regenerated plantlets transferred to sterile mixture of soil, sand & manure (2:1:1)

Table 1. Effect of plant growth regulators on shoots proliferation from the calli derived from leaf explants (after 35 days of culture)

Callus induction (μM)	% callus formation± SE	Conc. of PGR used for shoot induction in the calli raised on callus induction medium(1) (μM)	% Shoot formation ± SE	Mean no. of shoots/explants ± SE	Mean length of shoots ± SE
2,4-D (2.26)	73±0.57	BAP(4.44)	60.00±0.57	1.94±0.12	1.79±0.09
		NAA(2.64)+BAP(1.11)	62.20±0.33	1.81±0.07	1.65±0.03
2,4-D (4.52)	76±0.66	BA (4.44)	51.10±0.33	1.21±0.03	2.23±0.37
		NAA(2.64)+BA (1.11)	57.70±0.33	1.64±0.08	1.52±0.26
2,4-D (2.26)+BA (1.11)	83±0.57	BA (2.22)	84.40±0.66	3.43±0.11	3.15±0.33
		BA (4.44)	80.00±0.57	3.38±0.22	3.14±0.43
		NAA(2.64)+BA (1.11)	86.60±1.52	4.57±0.71	3.02±0.12
		NAA(3.98)+BA (2.22)	82.20±0.88	3.65±0.05	3.12±0.15
2,4-D (4.52)+ BA (2.22)	69±1.76	BA (2.22)	64.40±0.88	4.12±0.12	2.57±0.15
		BA (4.44)	68.80±0.33	2.38±0.09	2.72±0.23
		NAA(2.64)+BA (1.11)	73.30±0.57	2.10±0.17	2.87±0.50
		NAA(3.98)+BA (2.22)	66.60±0.57	2.53±0.01	2.60±0.21
NAA(2.64)+BA (1.11)	47±1.15	NAA(2.64)+BA (1.11)	55.50±0.88	3.11±0.22	3.13±0.56
		NAA(3.98)+BA (2.22)	53.30±0.57	2.21±0.34	3.07±0.39
NAA(3.98)+BA (2.22)	47±1.0	NAA(2.64)+BA (1.11)	51.10±0.66	1.95±0.25	2.99±0.17
		NAA(3.98)+BA (2.22)	51.10±0.88	2.43±0.21	3.26±0.40

Values are mean ± S.E. of three independent experiments, each with a minimum of 15 replicates

Table 2. Effect of plant growth regulators on callusing and shoot proliferation from nodal explants (after 35 days of culture)

Inoculation medium (μM)	% callus formation± SE	Conc. of PGR used for shoot induction on inoculated explants (μM)	% Shoot formation ± SE	Mean no. of shoots/explants ± SE	Mean length of shoots ± SE
BA(2.22)	-	NAA(2.64)+BA (1.11)	86.60±0.57	2.63±0.24	3.79±0.19
		NAA(3.98)+BA (2.22)	80.00±1.00	2.37±0.26	3.87±0.08
BA(4.44)	-	NAA(2.64)+BA (1.11)	82.20±0.88	2.48±0.36	3.82±0.25
		NAA(3.98)+BA (2.22)	75.50±0.33	2.27±0.22	4.12±0.42
2,4-D (2.26)+BA(1.11)	47±1.15	BA (2.2)	77.70±0.33	3.31±0.03	3.79±0.06
		BA (4.44)	80.00±1.00	3.27±0.34	4.21±0.25
		NAA(2.64)+BA (1.11)	95.50±0.66	3.40±0.10	4.35±0.11
		NAA(3.98)+BA (2.22)	86.60±0.57	2.66±0.33	4.22±0.47
2,4-D (4.52)+ BAP (2.22)	50±0.57	BA (2.2)	77.70±0.88	2.95±0.07	3.30±0.11
		BA (4.44)	80.00±0.57	3.08±0.12	3.25±0.23
		NAA(2.64)+BA (1.11)	82.20±0.66	3.19±0.05	3.14±0.16
		NAA(3.98)+BA (2.22)	80.00±0.57	3.26±0.17	3.04±0.15
NAA(2.64)+BAP(1.11)	30±1.0	NAA(2.64)+BA (1.11)	73.30±1.00	2.75±0.15	4.15±0.20
		NAA(3.98)+BA (2.22)	71.10±0.66	2.59±0.26	4.06±0.09
NAA(3.98)+BAP(2.2)	32±1.20	NAA(2.64)+BA (1.11)	73.30±0.57	2.79±0.10	3.80±0.03
		NAA(3.98)+BA (2.22)	71.10±0.88	2.77±0.18	3.99±0.20

Values are mean ± S.E. of three independent experiments, each with a minimum of 15 replicates

on media containing BA alone (2.22-4.44 µM). Presence of auxins NAA (2.64-3.98 µM) or 2,4-D (2.26-4.52 µM), induced slight callusing at the basal portion of cultured explants which was in contact with media, after 10 days of culture (Table 2) (Fig. 2a). Bud break could be observed within 10-15 days of culture. Shoots and leaf formation was observed in most of the plant growth regulator (PGR) combinations used for shoot induction. Shoots formation as observed on various combinations has been presented in table 2 (Fig. 2 a,b). Addition of NAA along with BA resulted in early response for shoots formation as compared to the medium containing BA alone. Maximum mean no. of shoots per explant was 3.40±0.10 for MS media enriched with NAA 2.64 µM and BA 1.11 µM. The number of shoots per nodal explant increased from 3^{rd}-5^{th} week upon subculture. Maximum 12 shoots were observed from single nodal explants. For multiple shooting and rooting of *in vitro* regenerated shoots, the same methodology was followed as in the case of shoots obtained from leaf calli. Combinations of BA with low concentrations of NAA have been used to induce shoot formation in several plant species (Vij and Kaur, 1998; Rani et al. 2003; Sivanesan and Jeong, 2007; Ragi and Shibu 2014). In the present study, we observed that BA (4.44 µM) alone could induce bud break. Faisal et al. (2006), and Lahiri et al. (2012) observed in *Mucuna pruriens* that BA alone was most effective for inducing maximum shoots from cotyledonary nodal explants and from callus tissues. In *Balanites aegyptiaca,* for induction of bud break and multiple shoot proliferation, MS medium containing BA (12.5 µM) was found most effective (Siddique and Anis, 2009).

In vitro flowering

Flowers developed (Fig. 2d) from *in vitro* regenerated shoots derived from both the explants on MS medium supplemented with NAA (2.64 µM) and BA (1.11 µM). *In vitro* flowers were similar in morphology as observed *in vivo* grown plants. Nadaguda et al. (1990) reported BA along 5 % coconut milk to be most effective for flowering and multiple shoots formation in bamboo. Age of explants is an important factor which regulates flowering in *in vitro* cultures. Mature explants have higher probability of flowering as compared to juvenile explants (Cheruvathur et al. 2015). Termination of flowering during root induction may be due to removal of cytokinin from the medium (Scorza and Janick, 1980; Sudarshana et al. 2008). Viable seeds were also obtained which germinated on media as well as in soil.

Rooting and hardening

Roots formation was observed when 4-5 cm long *in vitro* regenerated shoots were transferred to MS medium supplemented with IAA (2.85 µM) or MS basal medium. Maximum roots induction from *in vitro* regenerated shoots derived from nodal explants was 86.66±0.33% and from calli derived from leaf pieces was 80.00±0.57%, on transfer to MS medium supplemented with IAA 2.85 µM (Figs. 1 and 2c). Roots formation occurred in the cultures on medium containing IAA within 10-15 days of transfer whereas it took about 30 days for rooting when MS basal medium was used for the induction of roots. Tap roots originated from the basal portion of nodal explants whereas 8-10 lateral (adventitious) roots emerged from calli derived from leaf pieces. Effect of media and PGRs on root formation from *in vitro* regenerated shoots developed from nodal segments and leaf derived calli, have been listed in Table 3.

Table 3. Effect of IAA on rooting of *in vitro* regenerated shoots from nodal and leaf explants

Conc. of IAA (µM) used for roots induction	% root formation from nodal explants±S.E.	% root formation from leaf explants±S.E.
0.00	66.66±0.33	63.33±0.33
2.85	86.66±0.33	80.00±0.57
5.70	76.66±0.88	69.25±0.88

Values are mean ± S.E. of three independent experiments, each with a minimum of 15 replicates

For hardening, regenerated plants with roots (3-4cm in length) were maintained on $MS_{1/2}$ liquid medium without any growth regulators for 10 days, followed by transfer to $MS_{1/4}$ liquid medium for next 10 days. An autoclaved soil, sand and manure (2:1:1) mixture was used for acclimatization of plantlets. Pots were kept in shade and humid conditions for 20 days before being transferred to ambient climatic conditions. Survival rate of completely regenerated plantlets on transfer to field conditions was nearly 100 % (Figs. 1d and 2e). Biswas et al. (2009) reported that combination of IBA (4.90 µM) and IAA (5.70 µM) was more effective for root induction in *B. diffusa*. In the present study, IAA (2.85 µM) alone or MS medium without any plant growth regulator induced root induction. Shrivastava and Padhya (1994), reported decrease in root induction when the concentration of IBA increased from 0.5 µM onwards.

A rapid plant regeneration protocol involving indirect and direct organogenesis from leaf and nodal explants of *B. diffusa* was established. *In vitro* regenerated plants produced multiple shoots and flowers. We observed roots induction from regenerated shoots originated from nodal explants (86%) and calli derived from leaf pieces (80%), on MS medium enriched with IAA (2.85 µM). Survival rate of *in vitro* grown plants in soil was nearly 100%. Commercial production of micropropagated plantlets may be carried out using this protocol keeping in view its immense medicinal value.

Authors' contribution

Conceptualization of research (SC, AP); Designing of the experiments (AP, OV); Contribution of experimental materials (SC); Execution of field/lab experiments and data collection (AP, OV); Analysis of data and interpretation (SC, AP, OV); Preparation of manuscript (SC, AP, OV).

Declaration

The authors declare no conflict of interest.

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