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Clonal Propagation Through Nodal Explant Culture of *Boerhaavia diffusa* L. - A Rare Medicinal Plant

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Abstract

Multiple shoots were induced on MS fortified with 2.0 mg/l BAP and 0.2 mg/l NAA within 30 days of culture. Maximum (93%) explants produced multiple shoots with an average 12 shoots per culture after two successive subcultures at 14 days interval in the same medium. Cent per cent rooting was achieved in half strength of MS supplemented with 1.0 mg/l IBA. Following 21 days *in vitro* rooting period and seven days of *ex vitro* acclimatization, plantlets were successfully established in natural condition. The survival rate of regenerated plants was 90 per cent.

Introduction

Boerhaavia diffusa L., commonly known as '*Punarnava*' in Bengali, is an important medicinal herb of the family Nyctaginaceae. The whole plant of *B. diffusa* is used as medicinal purpose because it contains different alkaloids and organic acids of medicinal importance. Roots of this plant contain antifibrinolytic agent and it is effectively used as a diuretic in dropsy and anasarca, jaundice, anaemia, gonorrhoea and blood purifier. Plant powder is used against abdominal tumour and cancer. It is also useful in curbing epilepsy, dysentery and inflammatory renal diseases (Ghani 2003). Flowers and seeds are used as contraceptives in Ayurveda (Joshi 2000). *B. diffusa* was used as an adjuvant in the treatment of pulmonary tuberculosis in addition to chemotherapy (Kant et al. 2001). Tribal people of the Chittagong Hill Tracts use this herb for urinary troubles and as stimulant. Usually the plant is propagated through seeds and creeping stem nodes. But due to the indiscriminate collection of huge amount of this plant by local herbalists and Ayurvedic and Unany companies, this plant species is on the verge of extinction. Under such a situation it is important to develop techniques for rapid mass propagation of this species to meet up the commercial need and also for protecting the genetic erosion. *In vitro* micropropagation technique has been proved to be very efficient for mass propagation of rare and endangered

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plant species (Fay 1992, Mikulík 1999). Accordingly such technique has been reliably used in propagation of many medicinal plant species (Sivakumar and Krishnamurthy 2000, Selvakumar et al. 2001, Wawrosch et al. 2001, Das and Handique 2002). A few work have been reported on *in vitro* propagation of *B. diffusa* but with limited success (Nagarajan et al. 2005, Roy 2008). With all these considerations the present research study was undertaken to develop an efficient and repeatable *in vitro* propagation protocol for *B. diffusa* through nodal explant culture.

Materials and Methods

Rootstocks of *Boerhaavia diffusa* L. were collected from Khagrachhari hilly areas and planted in earthen pots in the experimental field of the Institute of Biological Sciences, Rajshahi University. Leaves, internodal and nodal segments (0.5 - 1.0 cm) of young healthy sprouts were used for *in vitro* culture. Explants were first thoroughly washed under running tap water for 20 min and then treated with liquid detergent (Tween 80) for ten min, followed by dipping in savlon solution (5% v/v) for ten min. The explants were then washed with distilled water. After a 45 second treatment with 70% ethanol the explants were surface sterilized with an aqueous solution of 0.1% $HgCl_2$ for four min and rinsed with sterile distilled water for four - six times. The surface sterilized explants were implanted in 0.6% (w/v) agar solidified MS decant in culture tubes (2.5 × 15 cm) devoid of plant growth regulators (control) or fortified with NAA, IAA and IBA : 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l and BAP and Kn: 1.0, 2.0, 3.0 and 4.0 mg/l either singly or in combination with various concentrations. pH of the medium was adjusted to 5.7 ± 0.1 before adding agar and the medium was autoclaved at 121ºC and 1.1 kg/cm^2 pressure for 20 min. Initially the explants were cultured individually for 30 days and then subcultured at a regular interval of 14 days. When shoots attained a height of four - six cm these were then subcultured individually for rooting in phytagel solidified half strength of MS with IBA, NAA and IAA (0.1 - 1.0 mg/l + 3% sucrose) and compared to the control (MS without growth regulators). Microcuttings were elongated in the same medium. Culture vessels (shooting and rooting) containing inoculated explants were maintained in the culture room under a regular cycle of 16 hr photoperiod at 3000 lux light intensity of cool white fluorescent light at 25 ± 2ºC.

Well-rooted plantlets were removed from culture tubes and washed thoroughly in running tap water to remove all trace of medium attached to the roots. Those were then transplanted in plastic pots containing 1 : 1 autoclaved garden soil and compost. In order to maintain a high humidity the pots were then covered with transparent polythene bags and acclimatized in the laboratory temperature for two weeks. The pots were irrigated weekly intervals with

distilled water. Polythene covers were removed gradually, and the plants were then transferred to earthen pots containing garden soil. After two weeks the plantlets were successfully established in field conditions. Each experiment was repeated thrice using 15 replicates (total 45 cultures per treatment). The data were analyzed statistically. Means were compared using DMRT.

Results and Discussion

In terms of multiple shoot proliferation nodal explants responded better than other explants *viz.* internodes and leaves. For this reason further experiments were carried out using only nodal segments. Similar findings of axillary bud proliferation have also been reported in other medicinal plants (Chandramu et al. 2003, Lara et al. 2003, Anand and Jeyachandran 2004, Hassan and Roy 2004, Sultana and Handique 2004). The nodal explants underwent direct organogenesis when cultured on MS using various concentrations of BAP (1.0 - 4.0 mg/l) and Kn (1.0 - 4.0 mg/l) separately or in combinations with low concentrations of NAA (0.1 - 0.4 mg/l), IAA (0.2 - 1.5 mg/l) and IBA (0.2 -1.5 mg/l). When used singly BAP showed the strongest effect than Kn in terms of shoot induction. Of the different treatments 3.0 mg/l BAP was most effective. In this concentration 53% explants were responded and the numbers of shoot as well as length of shoot per explants were recorded 2.20 ± 0.12 and 1.45 ± 0.24, respectively (Table 1). BAP is considered to be one of the most useful cytokinins

Table 1. Response of nodal segments of *B. diffusa* on MS with different concentrations of BAP and Kn after 60 days of culture. Values are the mean of three replicates with 15 explants.

Growth regulators (mg/l)		Explants inducing shoots (%)	Number of shoot/culture	Length (cm) of shoot/culture
BAP	Kn	Mean	Mean ± SE	Mean ± SE
1.0		31c	1.64 ± 0.24b	1.0 ± 0.23bc
2.0		51ab	2.17 ± 0.11a	1.15 ± 0.12ab
3.0		53a	2.20 ± 0.12a	1.45 ± 0.24a
4.0		29c	1.02 ± 0.09c	0.93 ± 0.08bc
	1.0	33c	1.04 ± 0.11c	0.63 ± 0.07c
	2.0	40bc	1.08 ± 0.06c	0.84 ± 0.16bc
	3.0	47b	1.11 ± 0.07c	1.07 ± 0.22abc
	4.0	31c	1.02 ± 0.09c	0.67 ± 0.16c

Means with the same letters within columns are not significantly different at 5% level.

for achieving the micropropagation and showed highest effect in respect of multiplication of axillary buds (Martin 2002, Joshi and Dhar 2003). On the other hand combination of BAP or Kn with NAA and IAA was also assayed. However, combinations of IBA with cytokinins did not give any positive response. The best

response was achieved using 2.0 mg/l BAP in combination with 0.2 mg/l NAA after 60 days of culture (Figs. 1, 2). In this combination 93% explants showed proliferation and the highest mean number of shoot and shoot length (cm) per culture were 12.51 ± 0.45 and 5.10 ± 0.13, respectively (Table 2). Synergistic effect of BAP in combination with an auxin has been reported and most of the cases BAP and NAA were used for the induction of multiple shoots of various medicinal plants (Sudha et al. 1998, Huang et al. 2000, Chen et al. 2001). The number of shoots per explant increased when the media were replaced after

Table 2. Response of nodal segments of *B. diffusa* on MS with BAP, NAA and IAA after 60 days of culture. Values are the mean of three replicates with 15 explants.

Growth regulators (mg/l)			Per cent response	Number of shoot/culture	Length (cm) of shoot/culture
BAP	NAA	IAA	Mean	Mean ± SE	Mean ± SE
2.0	0.1		71b	7.86 ± 0.43b	3.20 ± 0.17b
2.0	0.2		93a	12.51 ± 0.45a	5.10 ± 0.13a
2.0	0.3		65b	6.35 ± 0.34c	3.16 ± 0.26c
2.0	0.4		51c	5.04 ± 0.22d	2.42 ± 0.14b
2.0		0.2	40cd	2.02 ±0.14ef	1.07 ± 0.10e
2.0		0.5	49c	2.46 ± 0.16ef	1.23 ± 0.14e
2.0		1.0	62b	3.24 ± 0.22e	1.54 ± 0.16d
2.0		1.5	31d	1.62 ± 0.17f	0.70 ± 0.09f

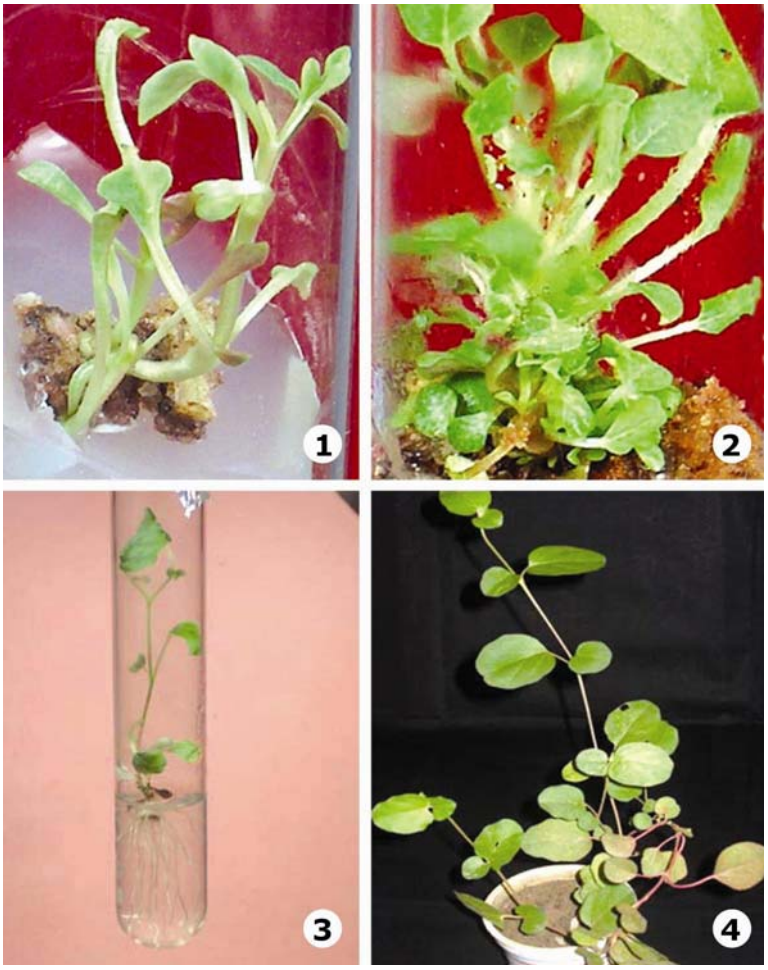
Means with the same letters within columns are not significantly different at 5% level.

Table 3. Effect of different concentrations of IBA, NAA and IAA on *in vitro* rooting of *B. diffusa* in half strength MS after 30 days of culture. Values are the mean of three replicates with 15 explants.

Auxins	Mg/l	Half strength MS		
		Rooting (%)	No. of roots/shoot	Length of root (cm)
Control	-	-	-	-
IBA	0.1	60d	5.37 ± 0.55bcd	2.43 ± 0.20cd
	0.5	93b	8.64 ± 0.26b	4.35 ± 0.32b
	1.0	100a	10.48 ± 0.06a	5.33 ± 0.11a
NAA	0.1	50de	4.02 ± 0.29de	2.23 ± 0.10d
	0.5	71c	5.82 ± 0.14c	2.83 ± 0.14c
	1.0	60d	4.46 ± 0.46d	2.21 ± 0.19d
IAA	0.1	40f	2.02 ± 0.06f	1.60 ± 0.10e
	0.5	47ef	2.46 ± 0.21ef	1.83 ± 0.17de
	1.0	53de	2.77 ± 0.15de	2.11 ± 0.13de

Means with the same letters are not significantly different at 5% level.

every 14 days of inoculation. A rapid rate of propagation depends on the subculturing of proliferating shoot culture (Rout et al. 2000). In the control no plants produced any shoots. Roy (2008) however, reported 90% shoot induction in MS +1.5 mg/l BAP and 0.5 mg/l NAA and in this combination the highest number, namely, 12 shoots regenerated from nodal explants. He also found that addition of coconut milk in the medium enhanced the number of shoots per culture.



Figs. 1-4: *In vitro* propagation of *B. diffusa* from nodal explants. 1. Multiple shoot induction. 2. Improved shoot proliferation. 3. Rooting of *in vitro* grown shoots. 4. *In vitro* grown plants transplanted in pot.

In order to induce roots the individual *in vitro* grown shoot buds were cultured on half-strength MS fortified with different concentrations of IBA (0,0 0.1 - 1.0 mg/l), NAA (0.1 - 1.0 mg/l) and IAA (0.1 - 1.0 mg/l). In all the concentrations of the plant growth regulators used in the experiments induced

rooting (Table 3). Without growth regulators no induction of roots was achieved. A 100% microcuttings rooted in the medium supplemented with 1.0 mg/l IBA. Maximum number (10.48 ± 0.06) and the longest (5.33 ± 0.11) roots were obtained in this concentration within three weeks of inoculation (Fig. 3). It indicates that stress condition favours the root induction in *B. diffusa*. Induction of roots in IBA supplemented medium has also been reported in some other medicinal plants (Lee 1994, Rout et al. 1999, Liu and Li 2001). Roy (2008) observed maximum of 90 per cent microshoots rooted in MS fortified with 1.0 mg/l each of IBA and IAA. The plantlets in pots were kept covered by transparent polybags for hardening outside the growth chamber. After seven days the polybags were completely removed and 90 per cent of the transplanted plants survived (Fig. 4). Initially the growth performance was slow after transplantation; thereafter it improved gradually and after two weeks new leaves began to emerge from the plants.

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