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Development of Protocol for Efficient Clonal Propagation of *Boerhaavia diffusa* L. Through *in vitro* Culture of Different Explants

Biotechnology

KEYWORDS : Regeneration, Multiple shoots, Boerhaavia diffusa, Explants, Nodal segments

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ABSTRACT

Protocol for efficient clonal propagation of Boerhaavia diffusa L. through in vitro culture of different explants has been developed. Nodal segments and shoot tips were cultured on MS medium supplemented with, BAP, kinetin and NAA either alone or in combinations. Nodal segments when cultured for five weak on MS + 1.75mg/l BAP alone and along with 0.5mg/l KN gave 74% response for shoot induction. However, when MS medium was supplemented with 1.75mg/l BAP+ 0.5mg/l NAA, nodal segments produced similar result. It was observed that on the same medium the number of shoots was increased due to de novo development. MS medium supplemented with 1.75mg/l BAP+ 0.5mg/l IAA gave no better result. The highest average numbers of roots (18-20) were obtained on ½ MS +1.0mg/l IBA+1.0mg/l IAA. More than ninety percent of the cultures responded in rooting medium within 26 to 27 days of culture. The rooted plantlets were acclimatized in the laboratory conditions. Finally they were shifted to the field among which 75% survived.

Abbreviation: BAP – 6-benzylaminopurine; IAA – indole - 3-acetic acid; IBA – indole-3-butyric acid' Kn – kinetin; MS – Murashige & Skoog; NAA – naphthaleacetic acid.

Introduction:

Boerhaavia diffusa L. belongs to family Nyctaginaceae. It is a herbaceous species of immense medicinal value. It has been mentioned in Atharvaveda with the name "*punarnava*". According to Atharvaveda the plant is digestive, diuretic, and anti-inflammatory and is effective in jaundice and stomach ailments. Due to presence of different alkaloids and organic acids of medicinal importance, this plant is used in gonorrhea, anaemia, dysentery and inflammatory renal diseases. Tribals use this plant in urinary troubles and as stimulant (Ghani 2003).

Plants usually grow in the campus, institutional garden, on the railways tracks and on the walls of abandoned building. Normal propagation is through seeds and nodes of the creeping stem. However, due to indiscriminate collection by the local people, workers of the vaidaya and the suppliers, the species is on the verge of extinction. So it needs conservation.

The potential use of micropropagation in *ex vitro* conservation of threatened plants has already been demonstrated for a number of species (Fay 1992; Mikulik 1999). Micropropagation allows the establishment of a large stock of plants within a short period of time from a minimum of original plant material, thus imposing a minimum impact on the endangered native populations (Fay 1992). This technique is also being used for the micropropagation of medicinal plants viz: Yadav *et al* 1990; Ray *et al* 1995; Ahmad *et al* 2001; Chen *et al* 2001; Das and Handique 2002; Roy 2008. However, we get few reports of *in vitro* regeneration regarding B. *diffusa* L. (Roy, 2008; Biswas et. al., 2009). Considering all the above the present work was carried to develop efficient methods for clonal propagation of *B. diffusa* L. through *in vitro* culture of nodal and shoot bud explants.

Materials and Methods:

Young and healthy branches from field grown plants of *B. diffusa* L. were collected from Botanical Garden, Department of Botany, B.R.A. Bihar University, Bihar, India. From these plants nodal segment and shoot tips were excised. These explants were properly surface sterilized by thoroughly washing under running tap water in a conical flask whose mouth was covered with cheese cloth for one hour. Nodal segments and shoot tips were treated with 70% alcohol for 20-30 seconds followed by 0.1% $HgCl_2$ for six to seven minutes. Rinsing of the explants was done 5 times with sterile glass distilled water. The materials kept in sterile water in conical flasks were shaken manually for 3-4 minutes to remove the traces of $HgCl_2$ completely. MS basal salt supplemented (Murashige and Skoog, 1962) with different concentration of BAP (0.25-2.5mg/l); KN (0.25-2.5mg/l) and NAA (0.25-0.5mg/l) were used either alone or in combination for the initiation of multiple shoots from the explants. The above culture media were containing 3% sucrose, 0.8% agar. The pH of the media was adjusted to 5.8±0.2 before autoclaving at 15 psi for 15 minutes. For rooting, well grown plantlets were excised and cultured on half strength MS medium supplemented with different concentration and combination of IBA, NAA, and IAA. The culture was maintained at *in-vitro* under conditions of 25 ± 2°C room temperature, 60 ± 5% relative humidity (RH) and 60 ± 5 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF). The PPF was provided by fluorescent lamps (TDL 36 W/84 Cool White 3350Im, Philips, Thailand) under a 16 h d^{-1} photoperiod. *In vitro* rooted plantlets were taken out from the culture test tube carefully, and and gently washed in double sterile distilled water to eliminate attached medium from their roots. Such plants were transferred in poly bag containing soil + sand + compost 1:1:1 ratio. These poly bags were covered with transparent polyethylene cover and were placed on a sponge, floating on water in a tub to maintain moisture. After one and half months the plants were planted in an earthen pot in green house condition. Finally they were transferred to the field, where plants survived and grew in good manner.

Experimental Design and Statistical Analysis

The experimental design was completely randomized, corresponding to factorial MS basal medium × Growth regulator (Cytokinin and Auxin), in triplicate with 18 plant of each. For statistical analysis, means ± SE were calculated. Data were analyzed using a two-way analysis of variance (ANOVA). The values were compared using Tukey´s test ($p < 0.05$), using SPSS version 16.0' software.

Result and discussion:

Nodal and shoot tip explants were cultured on various concentration of two cytokinins and one auxin either alone or in combinations. The data were obtained after five weeks of inoculation and have been presented in the table 1. In the nodal explants highest percentage (74) of response for shoot induction was observed in MS + 1.75mg/l BAP + 0.5mg/l NAA (table 1). At this concentration an average of 10.4±0.22 shoots regenerated from the nodal explants whereas 7.2±0.3 shoots regenerated from shoot tips. Both the explants revealed poor response on the medium supplemented with either BAP or Kn alone. However, comparatively better response was observed on BAP supplemented medium in comparison to Kn at the same concentration. Similarly addition at BAP + Kn or higher concentration of BAP had no promoting impact. Medium containing BAP and NAA was found more suitable than BAP alone. Here nodal explants were more suitable than that of the shoot tips. Similar findings of axillary bud proliferation have also been reported in *Boerhaavia diffusa* L. as well as other medicinal plants (Chandramu *et al* 2003; Lara et al 2003; Anand and Jayachandran 2004; Hassan and Roy 2004; Sultan and Handique 2004; and Roy 2008). BAP is considered to be one of the most useful cytokinins for obtaining the induction of axillary buds. It showed highest effect in respect of multiplication of axillary buds (Martin 2002; Josi and Dhar 2003.). A rapid rate of propagation depends on the sub-culturing of proliferating shoot culture (Rout et al 2000).

In order to induce roots in the above tissue culture raised plantlets individuals plant was excised and cultured on half strength MS medium supplemented with various concentration (0.25 – 2.5mg/l) of IBA, IAA,NAA either alone or in combinations. Roots were induced in all the cultures supplemented with the above auxins, but highest number of roots was induced in the medium supplemented with 1.0mg/l IBA+1.0mg/l IAA. Maximum number (18-20) and longest (8.5±0.20) roots were obtained in this condition after 26th days of inoculation. Even similar concentration of IBA+NAA+IAA (1.0mg/l) gave no better response with respect to initiation of roots. Similarly higher concentration (2.5mg/l) of BAP or IAA gave zero response. Better rooting in IBA supplemented medium has also been reported in other medicinal plant as well as in *Boerhaavia diffusa* L. (Lee 1994; Rout et al 1999; Lui and Li 2001; and Roy 2008). Rooted plants were taken out from the medium and washed properly. They were acclimatized in earthen pot containing soil, sand and compost under artificial moist chamber. In the beginning the growth was slow but finally they become healthy.

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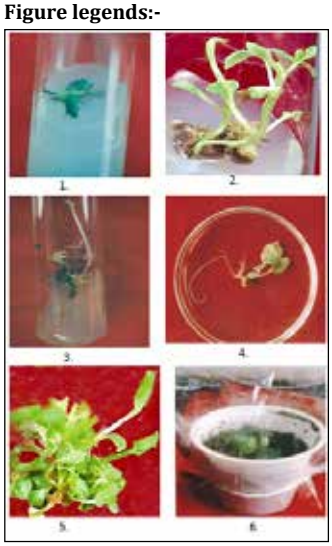
Figure legends:-



Figures 1-6: In vitro propagation of B. diffusa L. from nodal explants

1. Initial culture of nodal explant
2. Multiple shoots from the explant
3. Explant showing rooting
4. Rooted plantlet in Petridish
5. Multiple shoots six week old
6. Full grown plant in pot covered with polybag

Table no. 1. Effect of various concentrations of growth regulators, supplemented in MS basal medium either alone or in combination, on nodal and shoot tip explants of *Boerhaavia diffusa* L. after five weeks of inoculation.

Growth regulator (mg/l)		% explants showing response		Average no. of shoot/ explant	
Cytokinin	Auxin	Nodal	Shoot tips	Nodal	Shoot tips
		Mean		Mean ±t0.05 S.E.	
BAP	NAA				
0.25	--	42	36	2.4±0.17	--
0.50	--	44	38	2.5±0.18	2.0±0.2
1.0	--	48	40	3.0±0.22	2.5±0.24
1.50	--	52	44	3.5±0.23	3.0±0.16
1.75	--	58	46	4.6±0.22	3.2±0.18
2.00	--	50	42	3.2±0.24	2.5±0.02
Kn					
0.25	--	6.0	--	--	--
0.50	--	8.0	05	--	--
1.00	--	18	12	2.6±0.17	--
1.50	--	26	16	2.6±0.22	--
1.75	--	34	22	3.5±0.24	2.0±0.18
2.00	--	28	17	2.0±0.3	--
BAP+ Kn					
0.25+0.50	--	38	32	3.4±0.24	2.2±0.22
0.50+0.50	--	40	36	3.6±0.16	2.5±0.23
1.00+0.50	--	48	43	4.0±0.18	3.4±0.22
1.50+0.50	--	53	46	4.5±0.22	3.6±0.16
1.75+0.50	--	60	48	5.4±0.23	3.6±0.18
2.00+0.50	--	52	41	3.2±0.24	2.5±0.15
BAP +	NAA				
0.25 +	0.25	42	36	3.5±0.18	2.5±0.22
0.50 +	0.25	44	38	4.4±0.23	3.0±0.20
1.00 +	0.25	52	43	4.8±0.16	3.2±0.16
1.50 +	0.25	56	46	5.2±0.23	3.5±0.24
1.75 +	0.25	66	50	6.4±0.26	4.2±0.23
2.00 +	0.25	54	40	5.2±0.18	3.6±0.16
0.25 +	0.50	46	40	3.8±0.24	2.0±0.18
0.50 +	0.50	52	45	4.0±0.23	3.0±0.22
1.00 +	0.50	60	52	4.5±0.16	3.5±0.23
1.50 +	0.50	68	56	7.2±0.30	4.2 ±0.16
1.75 +	0.50	74	60	10.4±0.22	5.4±0.20
2.00 +	0.50	62	52	6.2±0.18	4.2±0.22
KN +	NAA				
0.25 +	0.50	10	05	--	--
0.50 +	0.50	14	08	3.2±0.17	2.0±0.23
1.00 +	0.50	26	16	3.4±0.18	2.2±0.22
1.50 +	0.50	32	20	3.8±0.20	2.0 ±0.16
1.75 +	0.50	40	26	4.6±0.18	3.0±0.17
2.00 +	0.50	32	20	3.2±0.22	1.8±0.18

Table no.2. Effect of different concentration of auxin supplemented in MS basal medium (half strength) either alone or in combination on root initiation of tissue culture raised plants of *Boerhaavia diffusa* L.

Growth regulator (mg/l)	% response for rooting	Number of roots/culture	Average root length (cm) Mean ±t0.05 S.E.
0.25 IBA	30	06-08	3.0±0.23
0.50 IBA	42	10-12	4.5±0.22
1.00 IBA	66	14-16	6.0±0.18
1.50 IBA	52	09-11	2.0±0.16
2.00 IBA	48	05-06	--
2.50 IBA	--	--	--
0.25 IAA	--	--	--
0.50 IAA	--	--	3.0±0.22
1.00 IAA	26	05-06	3.5±0.23
1.50 IAA	12	03-04	--
2.00 IAA	--	--	--
2.50 IAA	--	--	
IBA + IAA			5.0±0.22
0.25 0.5	44	11-12	6.0±0.24
0.50 0.5	68	12-14	8.0±0.18
1.00 0.5	72	16-18	6.0±0.23
1.50 0.5	58	12-13	4.0±0.16
2.00 0.5	52	10-11	--
2.50 0.5	--	--	6.0±0.30
0.25 1.0	52	12-14	7.0±0.22
0.50 1.0	60	15-16	8.5±0.20
1.00 1.0	86	18-20	5.0±0.24
1.50 1.0	70	14-16	4.0±0.23
2.00 1.0	56	10-12	--
2.50 1.0	--	--	
IBA + NAA + IAA			6.0±0.30
1.0 0.5 0.5	44	10-12	7.0±0.23
1.0 0.5 1.0	68	16-18	5.0±0.18
1.0 1.0 0.5	52	14-16	4.0±0.16
1.0 1.0 1.0	58	12-14	

Data were taken after 26 days of inoculation. Values are mean of three replicates with 18 plants.

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