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Rapid *in vitro* Propagation of *Boerhaavia diffusa* (L.) through Nodal Segments

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ABSTRACT: A rapid and efficient protocol for the large scale propagation of a potential medicinal plant *Boerhaavia diffusa* L., through *in vitro* culture of nodal segment explants obtained from aseptic seedlings. *In vitro* multiple shoot (14-15) induction was observed from axillary bud explants cultured on MS medium fortified with BAP (2.0 mg/l) and Kn (3.5 mg/l). The multiple shoots were separated and subcultured for their elongation on same medium supplemented with gibberelic acid (0.5 mg/l). Rooting on *in vitro* produced elongated shoots was achieved on half MS medium having IBA (0.5 mg/l). Rooted plantlets were hardened in plastic pots containing sterilized soil and vermiculite (3:1). Well established plantlets were acclimatized to the field with 70% survival rate.

Key words: Medicinal plants, Nodal segments, Micropropagation, *in vitro* rooting

Introduction

Boerhaavia diffusa Linn., commonly known as Punarnava in Sanskrit and is a herbaceous plant of the family Nyctaginaceae. The word Punarnava literally means, one which renews the body, that is, which brings back the youth. The whole plant or its specific parts like leaves, stem and roots are known to have medicinal properties and have a long history of uses by indigenous and tribal people in India. Punarnava is pungent, bitter and laxative. It is a cooling, stomachic, diaphoretic, expectorant, antipyretic and cardiotonic (Chopra *et al.,* 1956). It stimulates the function of heart and kidney and has been found specific for diabetes, Jaundice, breast cancer resistance and general debility (Ahmed-Belkacem *et al.,* 2007; Pari and Satheesh, 2004 ; Nalamolu *et al*., 2004). It is also used in epilepsy, abdomen pain due to congestion of blood and effective for seminal weakness and blood pressure (Gaitonde *et al.,* 1974). Pharmaceutical studies have demonstrated that punarnava possesses diuretic and anti-inflammatory activities (Bhalla *et al.,* 1968).

Micropropagation is one of the innovative methods of asexual propagation that has proved to be effective for *in vitro* propagation of medicinal and aromatic plants and in commercial exploitation of valuable plant derived pharmaceuticals (Faisal *et al*., 2006). *In vitro* plant regeneration is also the most important step for successful implementation of various biotechnological techniques used for plant improvement programme.

As there are no previous reports with respect to the regeneration of this plant from nodal explants. The present study was undertaken to standardize a protocol for *in vitro* regeneration from nodal explants of *Boerhaavia diffusa* L. for conserving this important medicinal plant species.

Materials and Methods

Plant material and sterilization

Healthy nodal cuttings (0.7-1.0 cm) with dormant axillary buds were collected from mature plants grown in Rajasthan university campus. Nodal segments were washed first under running tap water followed by a liquid detergent Teepol (5% v/v), for 10 minutes. After repeated wash, the nodal segments were kept in Bavistin (1% w/v), a fungicide for 10 minutes, followed by surface disinfestations with 70% ethanol (10-15 sec.) then with 0.1% (w/v) mercuric chloride solution for 2-3 min. After repeated wash with sterile double distilled water (4-5 times), the nodal explants were cultured on to the sterile nutrient medium.

Medium and culture condition

MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 0.8% (w/v) agar were used in all the experiments. Plant growth hormones and their combination were added to the medium as given below. The pH of the medium was adjusted 5.8 before autoclaving. All the cultures were maintained in the culture chamber at 24 ±2°C under 16Hrs photoperiod. Cool-white fluorescent tubes provided a light intensity of 3000 lux with 60-65% relative humidity.

Shoot induction and multiplication

Nodal segments were placed on MS medium augmented with various cytokinins (BAP, Kin) at different concentration (0.5-8.0 mg/l), either individually or in combination. Different strengths of basal media, that is, ¼MS, ½MS, MS were also examined with optimal concentration of BAP (0.5-8.0 mg/l) and Kin (0.5-8.0 mg/l) to detect the basal media suitable for shoot regeneration. Cultures were subcultured onto the same fresh medium after 3 weeks. The frequency of explant-producing shoots, number of shoots per explant was scored after 6 weeks of inoculation.

Root formation

The isolated shoots were harvested and transferred to ½ MS medium supplemented with indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) at different concentrations (0.5-8.0 mg/l). Data were recorded on percentage of rooting and root length after 6 week after transferring on to rooting media.

Acclimatization

Plantlets with well-developed shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to plastic pots containing sterile garden soil and vermiculite (3:1) under diffused light. Potted plantlets were covered with an inverted beaker to ensure high humidity and watered every 3 days. Inverted beakers were opened after 2 weeks in order to acclimatize plants to field conditions. After 4 weeks, acclimatized plants were transferred to pots containing normal soil and maintained in a greenhouse under normal conditions.

Data analysis

All experiments were set up in completely randomized design. Data presented in the table are treatments of 10 replicates (culture tubes) and all experiments were repeated thrice. Data were analyzed using analysis of variance (ANOVA) and means were compared using Tukey's test at the 0.05 level of significance. Results of subculture experiment were expressed in terms of mean values ±SE and were recorded after 4 weeks.

Results and Discussion

Axillary bud provides a rapid multiplication system in which the number of potential plants is increased exponentially by repetitive reculturing. Nodal segments with axillary buds from mature plants were used as primary explants. MS medium lacking growth regulators failed to induce shoots formation from nodal explants.

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Both cytokinins (BAP, Kinetin) facilitated axillary bud initiation. Number of newly formed buds depends on the concentration and type of cytokinin used (Table I&II).

To analyse the impact of BAP on shoot proliferation, it was supplemented to the MS medium at 0.5-8.0 mg/l. However, at concentration of BAP (2.0 mg/l), 7-8 shoots were produced by the nodal segments. Further increase in the BAP concentrations was not found to elicit favorable responses (Table-I). However, the number gradually increased up to 14-15 with the formation of adventitious shoots on MS medium supplemented with BAP (2.0 mg/l), Kn (3.5 mg/l) (Table-II; Fig.1 A & B). Increased concentration of BAP and Kn in combination minimized the number of shoots. These *in vitro* obtained multiple shoots were healthy and dwarf in nature. These shoots were elongated on same medium supplemented with gibberelic acid (0.5 mg/l) (Fig.1C). These *in vitro* raised elongated shoots measuring 5.0-6.0 cm failed to root on a hormone free MS medium. Therefore various auxins were attempted for rooting trials (Table III). Amongst all the auxins (NAA, IAA, IBA, 2,4-D) (0.5-8.0 mg/l) treatments, optimal rooting (60-70%) was obtained on IBA (0.5 mg/l) incorporated into ½ MS medium (Fig.1D). Generally 7-8 roots developed from the cultured shoots. IAA showed less rooting without root hairs. Miniature plantlets with well-developed roots were taken out from culture vessels. After thorough washing of the roots in distilled water to remove adhered agar and salts to avoid chances of infection under pot condition, they were transferred to pots containing vermin-compost and sterilized soil (1:3). Initially high humidity was maintained by covering the plantlets with inverted glass beakers (Fig.1E). After 15 days, humidity was simultaneously reduced and they were gradually exposed to sunlight for acclimatization in *in vivo* conditions. After 2-3 weeks, plants were finally transferred to pots containing garden soil. After two month of transplantation, they were survived well in nature (Fig.1F).

The caulogenic effect of BAP in combination with Kn observed in the present study is in consonance with other workers (Das and Pal, 2005; Faisal *et al*., 2006; Sujatha and Ranjitha, 2007; Sanatombi and Sharma, 2007; Rai et al., 2009; Lal and Singh, 2010)

Further *in vitro* raised multiple shoots after separation from the bunch of shoots were elongated on MS medium incorporated with BAP and Kn along with gibberelic acid, elongation of shoots on gibberelic acid favoured with the earlier reports of (Nyode *et al.,* 2003; Koubouris and Vasilakakis, 2006).

The optimal role of IBA for rooting in the regenerated plantlets has also been reported by several workers (Vadawala *et al*., 2006; Zhu *et al*., 2006; Barik *et al*., 2007; Faisal *et al*., 2007; Zale *et al*., 2008; Zhang.*et.al*.,2008).

Figure 1: Micropropagation of *Boerhaavia diffusa* L. (A)Shoot bud proliferation (B) Multiplication of shoots, (C) Elongation of shoots, (D) *In vitro* rooting of isolated shoots, (E) Potted plantlets were covered with an inverted beaker, (F) Potted plants, as seen after two month of transplantation

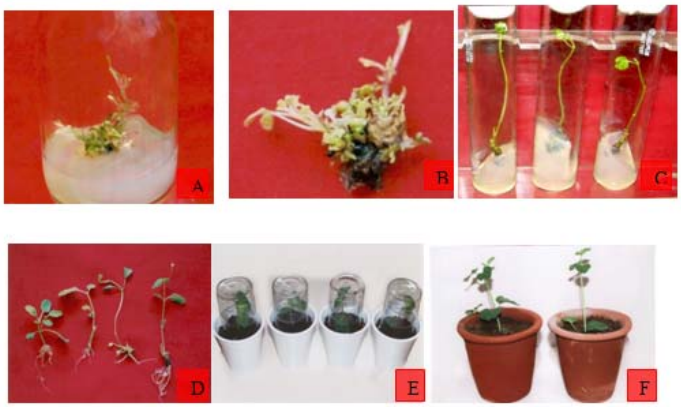

Table-I: Effect of cytokinins on axillary bud proliferation

Medium	:	MS + Sucrose (3.0%) + BAP / Kn (0.5-8.0 mg/l)
Inoculum	:	Nodal segments
Incubation	:	At 26±2°C under 16 hours photoperiod for three-four weeks

Cytokinin levels (mg/l)	**No. of shoot buds per explant *Mean ± $t_{0.05}$ S.E. ($\overline{X}$)**
Control (0)	Nil
BAP	
0.5	3.75±0.478
1.0	4.5±2.88
1.5	5.75±0.25
2.0	7.25±0.478
2.5	5.0±0.707
3.0	3.5 ±0.645
3.5	3.25± 4.18
4.0	2.0±0.408
5.0-8.0	Nil
Kn	
0.5	Nil
1.0	1.25±0.25
2.0	2.0±0.408
3.0	3.25±4.18
4.0	4.5±2.88
5.0-8.0	Nil

*Values are 95% confidence limits for mean

Table-II: Effect of bap in combination with kn on axillary bud proliferation

Medium	:	MS + Sucrose (3.0%) + BAP (2.0 mg/l) + Kn (0.5-8.0 mg/l)
Inoculum	:	Nodal segments
Incubation	:	At 26±2°C under 16 hours photoperiod for three-four weeks

Kn levels (mg/l)	**No. of shoot buds per explant** ***Mean ± $t_{0.05}$ S.E. ($\overline{X}$)**
0.5	3.0± 0.408
1.0	4.0 ±0.408
1.5	5.0 ±0.408
2.0	6.0± 0.408
2.5	8.5± 0.5
3.0	9.75± 0.25
3.5	14.5± 0.645
4.0	8.0± 0.408
4.5	4.0± 0.408
5.0	3.25± 0.478
5.5	2.75± 0.478
6.0	2.5± 0.288
7.0-8.0	Nil

*Values are 95% confidence limits for mean

Table-III: Effect of various auxins on root induction

Medium	:	Half MS + Sucrose (3.0% mg/l) + IAA / IBA / NAA / 2,4-D	(0.5-8.0 mg/l).
Inoculum	:	Excised *in vitro* regenerated shoots	
Incubation	:	At 26±2°C in 16 hour photoperiod for three-four weeks	

Auxin levels (mg/l)	**Rooting response**	**Remarks**
IBA		
0.5	60-80%	
1.0	30-50%	Optimal rooting, roots were lengthy branched, and white in colour.
2.0	30-40%	
3.0	20-30%	
4.0	10-20%	
5.0	10-20%	
IAA		
0.5	20-30%	
1.0	Nil	Meager root induction
2.0-8.0	Nil	
NAA		
0.5-8.0	Nil	No response
2,4-D		
0.5-8.0	Nil	No response

In conclusion, the present results of micropropagation of *Boerhaavia diffusa* L. through tissue culture techniques were encouraging as these eliminates labour intensive, shorten time period required for the development of plantlets and overcome the problems arising due to indiscriminate harvest from wild population and rapid loss of germinability of the seeds. The finding described here will provide

help in efforts on large scale production of identical plants of *Boerhaavia diffusa* L.

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