



IMPACT OF PHYTOHORMONES IN MICROPROPAGATION OF MEDICINALLY POTENT PLANT: *BOERHAVIA DIFFUSA L*

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ABSTRACT

A protocol for plant regeneration in *Boerhavia diffusa* L. has been standardized using *in vivo* nodal stem segments. Experiments were carried out to examine the effect of various auxins, cytokinins, inorganic and organic salts on subsequent axillary bud proliferation, multiple shoot induction, elongation of shoots, root formation and complete plantlet regeneration. Multiple shoot proliferation (14.5 ± 0.645) was achieved on Murashige and Skoog (MS) medium supplemented with benzylaminopurine (2.0 mg l^{-1}) and kinetin (3.5 mg l^{-1}). Elongation of *in vitro* raised multiple shoots were accomplished on MS medium fortified with gibberellic acid (0.5 mg l^{-1}) in addition to optimized enzyaminopurine and kinetin. The elongated shoots ($7.250 \pm 0.313 \text{ cm.}$) were rooted on half strength MS salts medium augmented with IBA (0.5 mg l^{-1}) with 60-80% response. Plantlets were hardened and acclimatized in the field with 72% survival rate.

Keywords: *In vitro* propagation, Phytohormones, nodal segments, *Boerhavia diffusa* L.

INTRODUCTION

Traditional methods of plant propagation have been found to be partially successful, whereas modern plant biotechnology techniques are proving to be a powerful complement to conventional methods¹.

Our relationship with plants is a multifaceted as our relationship with the man. Herbs have been used to promote and safe guard health, relieve pain and discomfort, heart diseases, wounds and refresh mind by providing us oxygen.

Boerhavia diffusa L. is an important medicinal plant belonging to family Nyctaginaceae. Every part of this plant is credited with some medicinal properties and is employed in traditional Ayurvedic system of natural therapy. It consists of several metabolites of immense value. The roots and leaves with the flowers have been found to be more potent than the stem alone.

Punarnava is pungent, bitter, hot and laxative. It is a cooling stomachic, antipyretic and cardiotoxic. It stimulates the function of heart and kidney and has been found specific for diabetes, jaundice and general debility². Pharmaceutical studies have demonstrated that punarnava possesses diuretic and anti-inflammatory activities³. It is also used in urinary disorders and in oedema⁴. Micropropagation represents the optimum efficiency in terms of vegetative plant propagation and allows large scale production in a lesser time and small space. The rate of micropropagation may vary from species to species.

The commercial bulk of *B. diffusa* represents a heterogeneous population. Consequently, it quite often results in poor quality roots and biomass.

A large number of publications on the chemistry, pharmacology and several other aspects have been made but here are only few reports on *in vitro* regeneration of *Boerhavia diffusa* L⁵⁻⁷. The present study was undertaken to standardize a protocol for *in vitro* regeneration from nodal explants.

MATERIAL AND METHODS

Plant Material

Single, juvenile nodal segments of *B. diffusa* were procured from adult trees (1-2 years old) growing in a natural environment in the Botanical Garden, Department of Botany, University of Rajasthan, Jaipur (Rajasthan, India). Nodal segments 1–2 cm. long containing axillary buds were taken from elongating shoots of the plant. They were selected at the mid season of monsoon time (August in the north-western hemisphere, Rajasthan, India). Explants were surface-sterilized by immersion in ethanol (70%; v/v) for 1 min. and in commercial bleaching solution (0.6% active chlorine) for 30 min. with one drop of Tween-20 as surfactant. Finally, all explants were washed three times with sterilized distilled water to get rid of traces of surfactants.

Inoculation of explants, Incubation, complete plantlet formation and hardening of plantlets

Prior to inoculation, surface sterilized explants were dipped in 0.1% aqueous solution of mercuric chloride (HgCl_2 ; w/v) for 2 min. followed by rinsing with sterilized distilled water at least thrice. Explants were cultured aseptically on MS basal media⁸ treated with BA, Kn and GA_3 ($0.5\text{-}5.0 \text{ mg l}^{-1}$) and IAA, IBA and NAA ($0.5\text{-}5.0 \text{ mg l}^{-1}$). Sucrose (3.0%; w/v) was used as carbon source and agar (0.8%; w/v) as a gelling agent. The pH of the media was adjusted to 5.8 ± 0.2 before autoclaving at 15 psi for 20



min. 30 ml of media were dispensed in 100 ml Borosil flask, and explants were placed vertically on the surface of the culture medium. Cultures were incubated at $25 \pm 2^\circ\text{C}$ in 16 hrs. photoperiod with $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity provided by white, florescent tubes Philips™ TLD 36 W/54 and $55 \pm 5\%$ relative humidity. *In vitro* regenerated shoots of 7-8 cm. in length were excised from the shoot cluster and transferred to root induction medium consisting MS basal medium fortified with varying concentrations of IBA, 30 gm. /l sucrose, solidified with 0.8% agar.

Regenerated plantlets with well developed roots were taken out from the culture vials and gently washed in distilled water to eliminate attached medium from their roots and then transferred to polycups containing sterilized soil and vermicompost (3:1). They were provided with two drops of $\frac{1}{2}$ MS salts at alternate day, each polycup containing plantlets covered with inverted glass beaker to maintain high relative humidity. After 15 days, humidity is reduced by opening the glass beakers for few min. and after another 2 weeks, plantlets were gradually shifted to shade. Finally they were transplanted to field after four weeks.

Experimental Design and Statistical Analysis

A completely randomized design was used with three factors: explant age (juvenile, adult), basal culture medium (MS), and cytokinins used (BAP, Kn). Each

treatment was applied to 32 replicates. For statistical analysis, means \pm SE were calculated. Data were analyzed by analysis of variance (ANOVA) to detect significant differences at $P < 0.05$ using Tukey's multiple range tests, using SPSS version 16.0' software.

RESULTS AND DISCUSSION

The success of *in vitro* morphogenesis depends on the physiological status of the plant (Juvenile status of the explants), media regime, choice of explants, various growth regulators, appropriate additives at optimum concentration at various physical factors i.e. light, temperature and humidity. The rate of shoot multiplication by axillary bud culture depends upon hormonal level as well as on the initial supply of reserve food material⁹.

The multiple shoot formation using nodal segments has proved to be an effective method for mass multiplication of plants *in vitro*. During the present study, axillary buds of *Boerhavia diffusa* proliferated on MS medium supplemented with BAP (2.0 mg l^{-1}) in combination with Kn (3.5 mg l^{-1}) after 10 days of inoculation. Further on two subculturing, proliferated shoot buds produced multiple shoots (14.5 ± 0.645) with varying length on the same medium (Table 1, Figure 1 A, B, C). Similar results have also been reported in case of *Mentha piperata* L.¹⁰; *Ocimum basilicum* L.¹¹; *Mucuna pruriens* L.¹²; *Artemisia vulgaris* L.¹³; *Capsicum annum* L.¹⁴.

Table 1: Effect of different concentrations of cytokinin (BAP & Kn) for shoot multiplication from nodal segments of *B. diffusa*

BAP (mg l^{-1})	Kn (mg l^{-1})	No. of shoots per explants	
		Mean $\pm t_{0.05}$ S.E.	Length of shoots (cm.)
0.5		3.75 \pm 0.478	2.125 \pm 0.295
1.0		4.5 \pm 2.88	2.375 \pm 0.324
1.5		5.75 \pm 0.25	2.75 \pm 0.478
2.0		7.25 \pm 0.478	3.125 \pm 0.227
2.5		5.0 \pm 0.707	3.0 \pm 0.408
3.0		3.5 \pm 4.18	2.750 \pm 0.313
3.5		3.25 \pm 4.18	2.70 \pm 0.313
4.0		2.0 \pm 0.408	1.75 \pm 0.366
	0.5	1.778 \pm 0.278	1.375 \pm 0.263
	1.0	2.00 \pm 0.267	1.875 \pm 0.295
	1.5	2.125 \pm 0.295	1.250 \pm 0.164
	2.0	2.375 \pm 0.183	1.275 \pm 0.183
	3.0	2.625 \pm 0.183	1.875 \pm 0.295
	3.5	5.00 \pm 0.327	2.250 \pm 0.313
	4.0	3.250 \pm 0.250	1.75 \pm 0.164
2.0	0.5	2.375 \pm 0.183	1.375 \pm 0.263
2.0	1.0	3.25 \pm 0.75	1.875 \pm 0.295
2.0	1.5	5.5 \pm 0.645	2.0 \pm 0.408
2.0	2.0	8.0 \pm 0.408	3.5 \pm 0.645
2.0	3.0	9.75 \pm 0.25	4.5 \pm 2.88
2.0	3.5	14.5 \pm 0.645	7.250 \pm 0.313
2.0	4.0	8.5 \pm 0.5	3.25 \pm 0.478

Values represent mean \pm SE of 28 replicates per treatment in three repeated experiments.

Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level.



Figure 1



Figure 1 A-I: Micropropagation of *Boerhavia diffusa* through nodal stem segment culture

- A: Axillary shoot bud proliferation on MS medium supplemented with BAP (2.0 mg/l) + Kn (3.5 mg/l) after 10 days of inoculation
 B: Multiplication of shoots on the same medium and hormone after second subculturing of the proliferated shoot buds
 C: Exposed view of the same
 D: Shoot elongation on BAP (2.0 mg/l)+ Kn (3.5 mg/l) with gibberellic acid (0.5 mg/l)
 E: *In vitro* rooting on half strength MS medium along with IBA (0.5 mg/l)
 F: Exposed view of *in vitro* regenerated plantlets
 G: Hardening of plantlets by providing high relative humidity through covering plantlets with inverted glass beakers
 H: Acclimatization of plantlets
 I: Transplanted hardened plantlets in the large earthen pots

In contrast to this, Ray and Bhattacharya (2008)¹⁵ reported multiple shoots on the BAP in *Eclipta alba* L. Similarly, Sharma and Mohan (2006)¹⁶ also obtained multiple shoots on the BAP (2.0 mg l⁻¹) in *Chlorophytum borivillanum* (Sant.f.). In addition to this, Sen *et al.* (2009)¹⁷ noticed multiple shoot formation on MS medium enriched with BAP (0.5 mg l⁻¹) in *Phyllanthus amarus* Schum. and Thonn. However, effectiveness of BAP in axillary shoot regeneration has also been reported in a number of other plant species¹⁸.

Multiple shoots after separation from the shoot cluster, transferred to the shoot elongation medium comprising with a combination of BAP (2.0 mg l⁻¹) + Kn (3.5 mg l⁻¹) with gibberellic acid (0.5 mg l⁻¹), proved to be optimum for shoot elongation (Figure 1 D). Elongation of shoots on gibberellic acid has also been reported earlier¹⁹. In contrast to this, Seetharam *et al.* (2007)²⁰ reported shoot elongation on MS medium with BAP (2.0 mg l⁻¹) and NAA (1.0 mg l⁻¹). Moreover, Sivanesan and Murugesan (2005)²¹ have also reported that shoot elongation was obtained in *Withania somnifera* L. (Dunal) on MS medium with BAP (2.0 mg l⁻¹).

The ability of plant tissues to form roots depends on interactions of different endogenous and exogenous factors. The role of auxins in root development is well established and reviewed²²⁻²³.

During the present studies, rooting was achieved when well developed elongated shoots (7.250 ± 0.313 cm.) of *Boerhavia diffusa* L. transferred to half strength MS medium fortified with IBA (0.5 mg l⁻¹) (Figure 1 E, F). Similarly, root induction in the *in vitro* regenerated shoots on half strength MS medium supplemented with IBA has also been reported earlier²⁴⁻²⁶.

In contradiction to this, Roy (2008)⁶ observed maximum of 90 % micro shoots rooted in MS medium supplemented with IBA and IAA (1.0 mg l⁻¹ each).

The quality of the regenerated plants with sufficient root length, are important factors, which control the success rate during the transition of plants from *in vitro* to *in vivo* conditions²⁷. During the present study, *in vitro* regenerated plantlets with well developed root system were transferred to pots having soil and vermicompost (3:1) and hardened (Figure 1 G, H). Initially they were gradually exposed to natural environment for their acclimatization. After 2-3 weeks, the plants were finally transplanted to large earthen pots containing normal garden soil (Figure 1 I). Similar hardening procedure has been used earlier²⁴. About 72% of these plantlets showed survival rate.

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