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# *In vitro* propagation of *Boerhaavia diffusa* L. through direct and indirect organogenesis

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**ABSTRACT**

*The present study was aimed to develop an efficient reproducible protocol for the large scale propagation of medicinally important Boerhaavia diffusa L using the shoot-tip, nodal, inter-nodal and leaves as explants. Highest percentage (97.2 ± 0.68 for nodal segments and 95.6 ± 0.82 for Shoot tip) of multiple shoot proliferation (14.4 ± 0.64 for nodal segments and 7.83 ± 0.38) was observed on MS medium augmented with 1.5 mg/l of BAP. The maximum number of shootlets regeneration (11.6 ± 0.38) was observed from MS medium supplemented with 3.0 mg/l of BAP. Maximum percentage (73.2 ± 0.47) of shootlets was observed in MS medium supplemented with 3.0 mg/l of BAP. Highest percentage, maximum number of rootlets/shootlet and mean length of rootlets were observed in ½ MS medium supplemented with 1.0 mg/l of IBA.*

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## INTRODUCTION

*Boerhaavia diffusa* (Nyctaginaceae) is a medicinal herb, commonly known as punarnava. It is common in India, Bangladesh and other tropical regions of Asia and grows wild in different forests and village groves. The whole plant of *B. diffusa* is a very useful source of the drug punarnava, which is documented in India Pharmacopoeia as a diuretic [1]. The roots and leaves with flowers have been found to be highly potent [2]. Roots of this plant contain anti-fibrinolytic agent and it is effectively used as a laxative and diuretic in dropsy and anasarca, ascites, jaundice, asthma and in scanty urine, anaemia, gonorrhoea and blood purifier. The active constituent of the drug is the alkaloid, punarnavine, the total alkaloid content of the roots being about 0.04%. Plant powder is used against abdominal tumour and cancer. It is

also useful in curbing epilepsy, dysentery and inflammatory renal diseases [3]. In Ayurveda, *B. diffusa* was used as an adjuvant in the treatment of pulmonary tuberculosis in addition to chemotherapy [4]. Flowers and seeds are used as contraceptives [5]. It was used in renal ailments as diuretic [6] and to treat seminal weakness and blood pressure [7]. It is also used in the treatment of stomach ache, anemia, cough, cold and a potent antidote for snake and rat bites and nephritic syndrome [8], hepatitis, gall bladder abnormalities and urinary disorders [9]. Pharmacological studies have demonstrated that punarnava possesses punarnavoside, which exhibits a wide range of properties - diuretic anti-inflammatory, antifibrinolytic [10], antibacterial [11), anti-hepatotoxic, anthelmintic febrifuge, anti-leprosy, anti-asthmatic, anti-urethritis, anti-lymphoproliferative activity [12], anti-metastatic [13], immunosuppressive activity [14], Anti-diabetic activity, Anti-oxidant activity [15] and immune-modulation [16]. The plant was also found to be a valuable source of medicinally important secondary metabolites including Punarnavine, borhavine [17], Hentriacontane, β-sitosterol and ursolic, punarvoside [10] and boeravinone [18]. The roots of *B. diffusa* are a rich source of a basic protein or antiviral agent, which is used for inducing systemic resistance in many susceptible crops against commonly occurring viruses viz., papaya ring spot virus [19], cucumber green mottle mosaic virus [20], mung bean yellow mosaic virus, bean common mosaic virus [21] and water melon mosaic virus [19]. Usually the plant is propagated through seeds and creeping stem nodes. Mass scale collection of this plant by local herbalists and Ayurvedic and Unany companies from natural habitats is leading to a depletion of this plant species. *B. diffusa* is propagated by seeds, but the seed viability is poor and has very low germination percentage. Under such a situation it is important to develop techniques for rapid and mass propagation of this species to meet up the commercial need and also for protecting from the depletion from the nature [22]. *In vitro* micropropagation technique has been proved to be very efficient for mass propagation of rare and endangered plant species [23]. Accordingly such technique has been reliably used in propagation of many medicinal plant species [24-28]. A massive study on the phytochemistry and pharmacology of *Boerhaavia diffusa* were available on hand and few attempts were made on *in vitro* regeneration of *Boerhaavia diffusa* using leaves and stem (nodes and shoot tip) as explants [22, 29-34]. With this background the present study was aimed to develop an efficient reproducible protocol for the large scale propagation of medicinally important *Boerhaavia diffusa* using the shoot-tip, nodal, inter-nodal and leaves as explants.

## EXPERIMENTAL SECTION

Young shoots of *Boerhaavia diffusa* were collected from the herbal garden attached to the Department of Biotechnology, Muthayyammal College, Rasipuram, Namakkal, Tamil Nadu, India and. Young shoots washed with running tap water for 5 min and treated with 0.1% bavistin for 1 min and rinsed twice with distilled water. Then the explants were surface sterilized with 0.1 (w/v) $HgCl_2$ solutions for 3-8 min aseptically. After rinsing 3-4 times with sterile distilled water nodal segments, shoot tips, leaves and inter-nodal segments were separated and trimmed into 1cm in length and used as the explants. The explants were placed vertically (Shoot tip and nodal segments) on solid basal Murashige and Skoog [35] medium supplemented with 3% sucrose, 0.6% (w/v) agar (Hi-Media, Mumbai) and different concentration (1.0 mg/l to 5.0 mg/l) of BAP and Kin alone for direct regeneration. Inter-nodal and leaves segments were cut into 0.7 cm in length and cultured horizontally on Murashige and Skoog solid medium supplemented with 3% sucrose, gelled with 0.7% agar and different concentration (1.0 mg/l to 5.0 mg/l) of 2, 4 - D either alone or in combination. After few weeks, the *in vitro* proliferated callus cultures were sub-cultured onto MS medium

supplemented with BAP, Kin, and NAA either alone or in combinations for organogenesis. Each and every experiment was performed with ten replicates and repeated thrice. The callus cultures were maintained for a period of over 10 months by periodic sub-culturing with 2 to 4 weeks intervals on to fresh multiplication medium. For rooting, the *in vitro* raised shootlets were sub-cultured on ½ strength MS medium supplemented with various concentrations of auxins. The pH of the medium was adjusted to 5.8 before autoclaving at 121$^{o}$C for 15min. The cultures were incubated at 25 ± 2$^{o}$C under cool fluorescent light (2000 lux 12 h/day photoperiod). For acclimatization, the plantlets with well developed roots (5 cms) were removed from culture tubes, washed in running tap water to remove the remnants of agar and plantlets were planted separately one to 10 cm diameter polycup filled with potting mixture river sand, garden soil and farm yard manure (1:1:1). Plants were in mist chamber with relative humidity of 70% plants were irrigated with 10 x liquid MS media (5ml) at 8 hrs intervals for 2 weeks and establishment rate was recorded and tabulated.

## RESULTS

The rate of explants survival were varied with concentration of mercuric chloride and sterilization time, 0.1% $HgCl_2$ for 4 min showed highest percentage (92%) of survival rate of explants. 0.1% $HgCl_2$ for less than 3 min showed high percentage (90%) of microbial contamination, above 3 to 4 min exposure showed moderate percentage (75 – 80) of survival it was observed that at 5 min sterilization with 0.1% $HgCl_2$, hundred percentage microbes free explants but the mortality rate explants was high up to 95 to 100%.

Proliferation of multiple shoots was observed with high frequency from nodal segments and shoot tips within three days of inoculation. These explants were capable of directly developing multiple shoot on MS medium containing different concentrations and combinations of cytokinin, BAP and Kin. MS medium supplemented with various concentration of BAP and Kin stimulated the shootlets formation with varied percentage (Table -1). Highest percentage of shootlets formation (97.2 ± 0.68 for nodal segments and 95.6 ± 0.82 for Shoot tip) maximum number of shootlets per explants (14.4 ± 0.64 for nodal segments and 7.83 ± 0.38 for shoot tip) were observed on MS medium augmented with 1.5 mg/l of BAP (Fig. 1. a-e).

**Table 1 Effect of different concentrations of cytokinin for multiple shoot induction from shoot tip and nodal explants of *Boerhaavia diffiusa***

| MS+Cytokinin Conc. in mg/l | | Mean % of Shoot formation ± S.E | | *Mean Number of Shoots / explants* | | *Mean Length of Shootlets* | |
|---|---|---|---|---|---|---|---|
| **BAP** | **Kin** | **Node** | **Shoot tip** | **Node** | **Shoot tip** | **Node** | **Shoot tip** |
| **0.5** | **0.0** | 63.7 ± 0.36 | 54.6 ± 0.62 | 2.8 ± 0.24 | 2.3 ± 0.23 | 4.4 ± 0.41 | 3.2 ± 0.31 |
| **1.0** | **0.0** | 79.3 ± 0.64 | 68.5 ± 0.73 | 6.6 ± 0.43 | 4.5 ± 0.26 | 5.3 ± 0.36 | 4.3 ± 0.27 |
| **1.5** | **0.0** | 97.2 ± 0.68 | 95.6 ± 0.82 | 14.4 ± 0.64 | 7.8 ± 0.38 | 5.6 ± 0.26 | 5.6 ± 0.26 |
| **3.0** | **0.0** | 73.6 ± 0.56 | 71.4 ± 0.64 | 7.4 ± 0.53 | 5.2 ± 0.34 | 6.2 ± 0.34 | 5.2 ± 0.19 |
| **4.0** | **0.0** | 64.8 ± 0.49 | 68.4 ± 0.36 | 7.2 ± 0.38 | 4.8 ± 0.41 | 5.2 ± 0.28 | 5.4 ± 0.21 |
| **5.0** | **0.0** | 52.4 ± 0.64 | 59.3 ± 0.61 | 5.8 ± 0.41 | 2.7 ± 0.36 | 4.4 ± 0.36 | 4.6 ± 0.34 |
| **0.0** | **0.5** | **46.3** ± 0.38 | 48.2 ± 0.53 | 2.1 ± 0.22 | 1.1 ± 0.31 | 3.8 ± 0.47 | 2.4 ± 0.36 |
| **0.0** | **1.0** | 71.3 ± 0.64 | 73.4 ± 0.67 | 2.4 ± 0.38 | 1.8 ± 0.26 | 4.4 ± 0.26 | 4.1 ± 0.35 |
| **0.0** | **1.5** | 92.6 ± 0.83 | 88.2 ± 0.69 | 3.6 ± 0.37 | 2.2 ± 0.41 | 4.8 ± 0.34 | 4.9 ± 0.22 |
| **0.0** | **3.0** | 72.6 ± 0.47 | 71.8 ± 0.52 | 3.4 ± 0.32 | 2.4 ± 0.52 | 5.3 ± 0.42 | 5.2 ± 0.37 |
| **0.0** | **4.0** | 61.5 ± 0.28 | 63.4 ± 0.53 | 2.6 ± 0.42 | 2.3 ± 0.41 | 4.9 ± 0.26 | 4.4 ± 0.32 |
| **0.0** | **5.0** | 45.7 ± 0.53 | 49.7 ±0.38 | 2.2 ± 0.36 | 1.8 ± 0.23 | 4.2 ± 0.37 | 3.5 ± 0.41 |

Highest percentage of callus (83.6 ± 0.59 in inter-nodal segments and 91.7 ± 0.46 in leaves) induction was observed on MS medium supplemented with 0.5 mg/l of 2, 4-D (Fig. 1. f). Calli obtained from the inter-nodal and leaf explants were tested for shoot regeneration (Table 1). The maximum number of shootlets regeneration (11.6 ± 0.38) was observed from MS medium supplemented with 3.0 mg/l of BAP (Fig. 1. g, h). Maximum percentage (83.2 ± 0.47) of shootlets was observed in MS medium supplemented with 3.0 mg/l of BAP.

The *in vitro* raised shootlets were sub-cultured on ½ strength MS medium augmented with various concentrations of IAA and IBA. At 9$^{th}$ day, the *in vitro* raised shootlets were produced *in vitro* rootlets without any callus proliferation (Fig. 1. i). Highest percentage, maximum number of rootlets/shootlet and mean length of rootlets were observed in ½ MS medium supplemented with 1.0 mg/l of IBA (Table 2). After 15 days, *in vitro* raised plantlets were hardened in polycups containing a mixture of sterile garden soil: sand (3:1) covered with polypropylene bags and irrigated with 10 x diluted MS liquid medium.

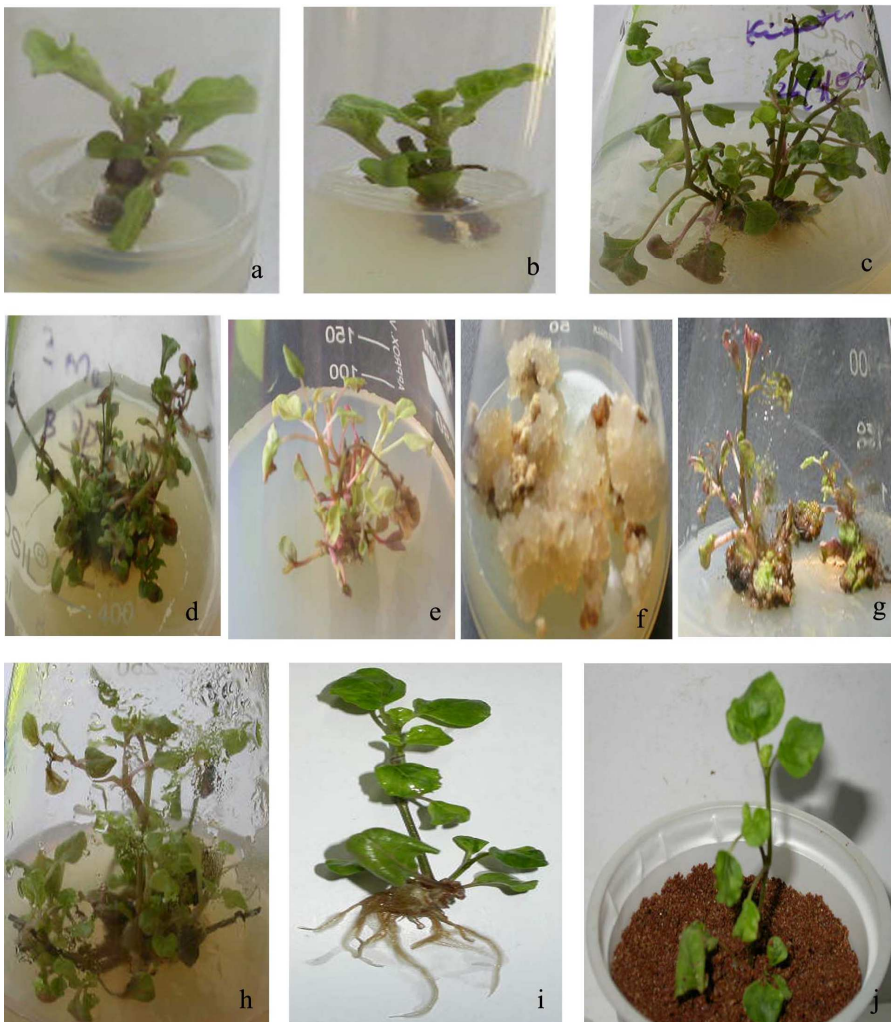


**Fig. 1. *In vitro* Propagation of Boerhavia diffusa L**

**a - Multiple shootlets initial stage – Shoot tip; b – Multiple shootlets initial stage – Nodal segments; c – Multiple shoot lets after 10 days - Shoot tip; d and e - Multiple shoot lets after 10 days – Nodal segments; f – Callus induction from Leaves and Inter-nodal segments; g and h – Multiple Shootlets regeneration from the semi-friable callus of Leaves and Inter-nodal segments; i - *In vitro* derived plantlet with shootlet and rootlets and j - Hardened plants in Poly Cups.**

The plants were kept in a culture room for 15 days. Sixty five percentages of plants were successfully established in polycups (Fig. 1. j). After 15 days the polycups hardened plants, were transferred to pots and kept in green house. Sixty percentages of plants were well established in the green house condition.

**Table 2: Effect of 2, 4-D on Callus production from the Leaves and Inter-nodal segments of *Boerhavia diffusa***

| MS + 2, 4 – D in mg/l | Mean percentage of callus induction ± S.E. | | Type of Callus | |
|---|---|---|---|---|
| | Inter-nodal | Leaves | Leaves | Inter-nodal |
| 0.0 | 00.0 ± 0.0 | 00.0 ± 0.0 | NIL | NIL |
| 0.5 | 83.6 ± 0.59 | 91.7 ± 0.46 | Friable | Semi-friable |
| 1.0 | 78.9 ± 0.87 | 77.4 ± 0. 67 | Friable | Semi-friable |
| 1.5 | 73.8 ± 0.73 | 74.3 ± 0.68 | Friable | Semi-friable |
| 2.0 | 68.9 ± 0.81 | 68.9 ± 0.62 | Friable | Semi-friable |
| 2.5 | 67.3 ± 0.54 | 64.4 ± 0.64 | Semi-friable | Semi-friable |
| 3.0 | 64.4 ± 0.68 | 58.7 ± 0.56 | Semi-friable | Semi-friable |

**Table 3: influence of plant growth regulators on Organogenesis of *Boerhavia diffusa* L**

| MS + Cytokinin Conc. in mg/l | | Mean % of Shoot formation ± S.E | *Mean Length of Shootlets* | *Mean Number of Shootlets* |
|---|---|---|---|---|
| BAP | Kin | | | |
| 1.0 | 0.0 | 43.8 ± 0.62 | 4.2 ± 0.38 | 6.2 ± 0.46 |
| 2.0 | 0.0 | 67.4 ± 0.59 | 6.4 ± 0.71 | 7.1 ± 0.53 |
| 3.0 | 0.0 | 83.2 ± 0.47 | 7.3 ± 0.53 | 11.6 ± 0.38 |
| 4.0 | 0.0 | 68.4 ± 0.53 | 5.4 ± 0.42 | 6.4 ± 0.23 |
| 0.0 | 1.0 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| 0.0 | 2.0 | 28.7 ± 0.74 | 2.3 ± 0.24 | 3.5 ± 0.47 |
| 0.0 | 3.0 | 32.1 ± 0.63 | 2.7 ± 0.56 | 3.9 ± 0.61 |

Table 4: **Effect of Auxins on rooting on *in vitro* derived shootlets of *Boerhavia diffusa* L**

| MS + Auxin Conc. in mg/l | | Mean % of Rootlet formation ± S.E | *Mean Length of Rootles* | *Mean Number of Rootlets* |
|---|---|---|---|---|
| IBA | IAA | | | |
| 0.5 | 0.0 | 84.8 ± 0.71 | 3.6 ± 0.42 | 8.3 ± 0.53 |
| 1.0 | 0.0 | 94.8 ± 0.48 | 6.7 ± 0.27 | 12.1 ± 0.72 |
| 1.5 | 0.0 | 86.7 ± 0.62 | 5.4 ± 0.42 | 9.4 ± 0.83 |
| 0.0 | 0.5 | 68.4 ± 0.54 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| 0.0 | 1.0 | 74.8 ± 0.61 | 3.1 ± 0.37 | 5.2 ± 0.71 |
| 0.0 | 1.5 | 62.7 ± 0.58 | 2.4 ± 0.58 | 4.7 ± 0.48 |

## DISCUSSION

High period of exposure with mercuric chloride leads the browning of explants and death. Our results were in agreement with Johnson observations [36]. They reported that surface sterilization exceeding 5 minutes was lethal to explants. In the present study also we observed above 5 min exposure with 0.1% mercuric chloride was lethal to nodal, inter-nodal and shoot tips of *Boerhaavia diffusa*. For young leaves 3 min sterilization with 0.1% mercuric chloride was optimal and showed highest percentage of explants survival. Biswas et al., [22] observed MS medium augmented with 3.0 mg/l of BAP was optimal concentration for the multiple shoot proliferation with 53%. They observed only 2.20 ± 0.12 shoot per nodal

segments. Roy [33] observed the highest percentage (90) of shoot induction and average of 5 ± 0.2 shoots / shoot tip and 12 ± 0.3 shoots / nodal of *B. diffusa* cultured on MS + 1.5 mg/l BAP + 0.5 mg/l NAA. In addition, he failed observe the multiple shootlets formation in the medium containing Kn alone. But in the present study we observed the highest percentage (97.2 ± 0.68 for nodal segments and 95.6 ± 0.82 for Shoot tip) of multiple shoot proliferation (14.4 ± 0.64 for nodal segments and 7.83 ± 0.38 for shoot tip) on MS medium augmented with 1.5 mg/l of BAP (Fig. 1. a-e). Our results were in agreement with the previous observation on *Canavalia virosa* [37], *Wadelia canlendulacea* [38], *Justicia jendarussa and Adenia hondala* [39], *Baliospermum montanum* [40], *Solanum anguivi*, *Phyllanthus amaraus* and *Jasminum calophyllum* [36]. The present study results are superior to the previous observation of Roy [33] and Biswas et al., [22]. Maximum length of shootlets 6.2 ± 0.34 and 5.6 ± 0. 26 were observed in MS medium augmented with 3.0 mg/l of BAP in nodal and shoot tip respectively (Table-1). Of the two cytokinin tested, BAP is considered one of the best cytokinin for achieving the multiplication and micropropagation of *B. diffusa*.

MS medium fortified with 2, 4-D elicited better callus response compared to other hormones tested. Our results were directly coincided and supplemented to Sudarshana et al., [34] observation in *B. diffusa.* In addition, they observed the callus induction with varied percentage in Kin, IAA and BAP supplemented medium. The present study is better than Sudarshana et al., [34] observation, they observed 78 percentage of callus induction from leaves segments cultured on MS medium augmented with 2,4-D. The callus obtained from leaf was semi-friable in nature and light pale yellowish brown, where as the callus obtained from the inter-nodal segment was dark yellowish brown and semi-friable. Similar kinds of observations were observed in *Passiflora mollissima*, *Withania somnifera, Rhinacanthus nasutus*, *Passiflora edulis Rauvolfia tetraphylla* and *Physalis minima* [41- 45]. The Kin supplemented medium also induced the callus with varied percentage; the calli derived leaves showed green in colour and semi-friable and delayed the shoot proliferation. The effect of Kin on callus induction was reported by Rout *et al*., [46] in *Plumbago zeylanica.* In the present study we observed callus induction on MS medium supplemented with Kin. Our result was directly consonance with the Rout *et al*., [47] observation on *Plumbago zeylanica*. Sudarshana et al., [34] observed highest percentage (85) of shootlets proliferation and 2.66 ± 1.44 mean numbers of shootlets per culture from the leaves derived calli of *B. diffusa* in MS medium augmented with 3.0 mg/l of BAP in combination with 1.0 mg/l of NAA. But in the present study we observed that when the callus was cultured with BAP combination with NAA showed the shootlets with basal callus. We observed the maximum number (7.3 ± 0.53) of shootlets per culture in MS medium augmented with 3.0 mg/l of BAP. Our results were in agreement with the previous observation on *Justicia jendarussa* and *Adenia hondala* [39], *Baliospermum montanum* [40], *Solanum anguivi*, *Phyllanthus amaraus* and *Jasminum calophyllum* [36].

We have developed an *in vitro* reproducible protocol for large scale multiplication of medicinally important plant *Boerhaavia diffusa* using nodal, shoot tip, leaves and inter-nodal segments. This protocol can be used reliably for propagation in a commercial scale and *ex situ* conservation of this important medicinal plant.

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