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COMPARATIVE STUDY OF DIFFERENT NUTRIENT SUPPLEMENTS ON *IN VITRO* REGENERATION AND BOERAVINONE B PRODUCTION IN *BOERHAAVIA DIFFUSA* L.

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ABSTRACT

Shoot cultures of medicinally acclaimed plant *Boerhaavia diffusa* were initiated *in vitro* and the influence of different strengths of Murashige and Skoog (MS) medium, plant growth regulators, carbon/nitrogen/phosphate (CNP ratio) and varying concentrations of carbon sources on biomass regeneration and boeravinone B production were analyzed. Experiments were performed to examine the effect of media manipulation strategies through HPTLC analysis on a useful secondary metabolite- boeravinone B in the plant species. MS full strength medium fortified with zeatin (Zn) (1 mg/L) and naphthalene acetic acid (NAA) (0.5 mg/L) indicated complete success with an average of 13.0 ± 0.33 shoots per explant and 10 -14 roots per explant were obtained on medium supplemented with NAA (0.5 mg/L). Well rooted plantlets were hardened and acclimatized with 95 % survival rate under greenhouse conditions. CNP ratio of 1:1:1 (control) emerged as the best combination for biomass production (4.58 DW) in this plant species. A CNP ratio of 2:1:1 exhibited higher boeravinone B content of 16.78 %, wherein the concentration of carbon source was increased two folds. Likewise, by increasing the table sugar concentration upto 5 %, highest boeravinone B content (19.15 %) was recorded, which is 3.9 times more than that in the field grown plants. Since, sucrose (5.58 DW) and table sugar (5.63 DW) at 3 % concentration showed similar results for biomass production so sucrose in the medium could be replaced by a cheaper carbon source (table sugar) to make process cost-effective. This protocol can be used for maximizing biomass and metabolite content for its commercial exploitation and applications in herbal drug-based industries.

Key-words: Carbon sources, CNP ratio, HPTLC profiling, Punarnava.

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INTRODUCTION

In recent years, advancement in utilization of secondary metabolites for medicinal purpose has significantly aroused and keen interest has been devoted to the enhancement of production of these metabolites especially by utilizing various media manipulation techniques under *in vitro* conditions (Ramachandra and Ravishankar 2002).

In context to this, the present study focuses upon the medicinally important herb *Boerhaavia diffusa* (Punarnava) that belongs to family Nyctaginaceae and is widely prevalent in the tropical and sub-tropical regions of the world. It is a perennial herb which is used in various traditional medicinal systems due to the presence of medicinally important phytochemicals like alkaloids (punarnavine), flavonoids (boeravinone A-F), saponins, carbohydrates, tannins and phenols in them (Chaudhary and Dantu 2011). It has been demonstrated that the plant extracts of *B. diffusa* possess anticonvulsant (Kaur and Kumar 2009), anti-inflammatory (Agrawal *et al*. 2011), diuretic (Desai *et al*. 2008), antifibrinolytic (Sengul *et al*. 2009) and antibacterial properties (Umamaheswari *et al*. 2010). Recently drug formulation of *B. diffusa* has been used as an adjuvant for suppressing secondary tumor formation in anticancer therapy (Milic 2008). Due to its slow vegetative propagation, less seed viability and low seed germination rate, this species is on the verge of extinction and has become vulnerable. Under such circumstances, plant tissue culture holds a great prominence for plant conservationists and pharmaceutical industries as a suitable method for obtaining large quantity of genetically homogenous and healthy plant material. Such *in vitro* propagation techniques have been used efficiently for the conservation of endangered plant species (Fay 1992).

Reports on phytochemical and pharmacological aspects of *B. diffusa* are available; however, reports on successful *in vitro* regeneration are few and insufficient (Bhansali *et al*. 1978; Shrivastava and Padhya 1995; Nagaranjan *et al*. 2005). An attempt has been made to optimize the protocol for *in vitro* propagation of this valuable herb by investigating the effect of different plant growth regulators, carbon sources (table sugar, sucrose, fructose, maltose, dextrose) and varied ratio of carbon, nitrogen and phosphate on biomass regeneration and production of one of the active phytoconstituents of the herb - boeravinone B. This metabolite is naturally present

in different parts of the plant species and has been reported to be used for its anti-inflammatory, anti-apoptotic and anti-ageing properties (Bairwa *et al*. 2013; Bairwa and Jachak 2015; Biradar *et al*. 2018). The present study also aims at enhancing boeravinone B production under *in vitro* conditions through media manipulation mechanisms. Further, interest was in comparing varied content of boeravinone B in the different culture treatments. The qualitative and quantitative analysis of boeravinone B was done through HPTLC (High performance thin layer chromatography), since it is simple, accurate, rapid and a cost effective method in comparison to HPLC (High performance liquid chromatography). From literature review, it has been observed that only limited studies have been performed and reported on identification and quantification of boeravinone B in whole plant parts (Gomes *et al*. 2013; Bairwa *et al*. 2014; Gomes *et al*. 2014; Vaidya *et al*. 2014). No reports on analysis and quantification including enhancement of boeravinone B production using *in vitro* regenerated tissues have been attempted.

MATERIALS AND METHODS

Plant material identification and authentication: Plant material of *Boerhaavia diffusa* was collected from vegetatively propagated 3-month-old plants habituated at Trikuta Hills Herbal Garden, Shri Mata Vaishno Devi University (SMVDU), Katra, Jammu and Kashmir (Latitude 28°66′ North, Longitude 77°21' East and Altitude 754 m). After examining its habit, vegetative structure, flower morphology followed by microscopic examination, the plant was identified as *Boerhaavia diffusa* (L.) by Dr. Bikarma Singh, Scientist, Regional Research Laboratory Herbarium, Indian Institute of Integrative Medicine (CSIR-IIIM), Jammu and the identified and authenticated plant material under accession number - RRLH23492 has been deposited at Janaki Ammal Herbarium of CSIR- IIIM, Jammu for future reference.

Explant surface sterilization: Actively growing healthy young shoot tips (1.5 - 2 cm) of *Boerhavia diffusa* chosen as explants, were thoroughly washed under tap water to remove superficial contaminants, sterilized with 1 % (v/v) Tween 20 (HiCare Solutions, Delhi) for 2 min. Further, the explants were treated with 1 % (v/v) sodium hypochlorite (Himedia Chemicals, Mumbai) for 2 min. Secondary treatment was given with 0.2 % (w/v) mercuric chloride (Himedia Chemicals, Mumbai) for 3 min under laminar air flow cabinet (Khera Instruments Pvt. Ltd., Delhi) and finally washed with sterile distilled water 3-4 times for removing traces of sterilants.

Growth medium and aseptic culture conditions: The most commonly utilized basal Murashige and Skoog [(MS) Himedia Chemicals, Mumbai] medium (Murashige and Skoog 1962) was used. Throughout the experiment, the medium pH was maintained at 5.8 with 0.1 N sodium hydroxide (NaOH)/1 N hydrochloric acid (HCl) (Himedia Chemicals, Mumbai) and 0.7% agar [(w/v) (Himedia Chemicals, Mumbai)] was used as the gelling agent. The medium was dispensed in culture tubes and autoclaved at 121 °C for 15 min at 15 psi. The cultures were incubated in a growth room maintained at 25 ± 2 °C temperature with 16/8 hr day/night photoperiod provided by cool white fluorescent light (3000 - 3500 lux) (Phillips, India).

Influence of medium strength and different plant growth regulators (PGR's): MS medium of varying strengths (quarter, half, full, double) were utilized to test their impact on shoot proliferation and boeravinone B production. Reproducible and effective protocol of *in vitro* regeneration depends upon the type of plant growth regulators (PGR's) used. Therefore, shoot tip explants were inoculated vertically on MS medium fortified with different concentrations and combinations of PGR's; auxin [1-Napthaleneacetic acid (NAA)] and cytokinins [Thiadiazuron (TDZ); Kinetin (Kn); 6 - Benzyl aminopurine (BAP); Zeatin (Zn) (Himedia Chemicals, Mumbai)] (Table 1) along with 3 % sucrose [(w/v), (Himedia Chemicals, Mumbai)]. Explants inoculated on MS medium without the supplementation of PGR's was considered as the control. After one month, cultures were sub-cultured onto medium of original composition and all data were recorded on weekly basis.

***In vitro* rooting:** To find out the effective strength of auxin for *in vitro* rooting of this plant, *in vitro* shoots were isolated and inoculated in MS medium strengthened with various concentrations of auxins (Indole Butyric acid (IBA), Indole Acetic acid and 1-Napthylacetic acid [(NAA): (0.5; 1.0; 1.5, 2 mg/L), (Himedia Chemicals, Mumbai) (Table 2).

Hardening and acclimatization: For acclimatization, the well developed *in vitro* plantlets having at least 2 to 4 cm roots were carefully removed from culture tubes and washed with distilled water in order to remove the remnants of agar adhered to the roots of the plantlets. They were then transplanted into perforated plastic cups containing autoclaved river sand and garden soil (1:1). In order to prevent desiccation and to avoid rapid changes in environment, cups were wrapped in plastic bags and incubated in a plant growth chamber (Spire Automation and Innovation, India) under 25 ± 2 °C temperature, 70 – 80% humidity, 16/8-hr photoperiod and irrigated twice a week. The bags were gradually perforated after 1 week and finally removed. Hardened plantlets were then transferred to earthen pots and successfully acclimatized to field conditions. Survival rate and growth parameters (shoot length, mean number of leaves, etc.) were recorded and statistically evaluated after three weeks of

incubation in growth chamber and also after transfer to the field conditions.

Effect of carbon, nitrogen and phosphate (CNP) ratio and different carbon sources on biomass regeneration and boeravinone B Production: *In vitro* regenerated shoot tips regenerated in best combination and concentration of PGR's of appropriate size (1.5-2 cm) were used as source material for further experiments. Carbon, nitrogen and phosphate (CNP) are the essential constituents of MS medium. In this study, the ratio of these three elements was changed to find out their effect on proliferation and production of secondary metabolite in *B. diffusa* cultures. 3% (w/v) sucrose (carbon source), nitrogen (NH_4NO_3 + KNO_3 equivalent to 840 mg/L nitrogen) and phosphate (KH_2PO_4 equivalent to 170 mg/L phosphate) at a concentration of 1:1:1 was used as control. In various treatments, the concentration of each component was first decreased to one half and then increased to two folds with respect to standard concentration (1:1:1) present in MS medium by keeping constant the concentration of other components to investigate their effect on culture development (Supplementary Table S2). Further, for evaluating the effect of carbon sources on biomass regeneration and boeravinone B production, different types (sucrose, fructose, table sugar, maltose, dextrose) at varied concentrations (1%, 3%, 5%) (Supplementary Table S3) along with the best combination and concentration of auxin and cytokinin were utilized. Cultures were maintained under controlled conditions and MS medium with 3% sucrose was regarded as control.

Qualitative and quantitative analysis of boeravinone B content through HPTLC: Field grown donor plant and all the *in vitro* regenerated plant samples were shade dried, powdered and extracted (200mg/20ml) with methanol for 5 hours using soxhlet extraction method. Finally, the extracts were filtered using a 0.45 µm PVDF syringe filter, air dried and further dissolved in HPLC grade methanol (Sigma Aldrich Co., USA) prior to HPTLC study. Quantification of boeravinone B in different extracts was done by using CAMAG HPTLC analysis. Activated and pre-washed TLC aluminium plates pre-coated with silica gel 60F - 254 (E. Merck) of 20 x 10 cm size were used as stationary phase and toluene: ethyl acetate: methanol (7:1:2) (v/v) was used as mobile phase for the analysis. Deuterium and tungsten lamp was used as source of radiation. Development of CAMAG twin trough chamber was done by its pre-saturation with mobile phase for 20 mins. Slit dimension was kept as 5.00 X 0.45 mm with 100 µm/step data resolution. Detection was carried out at 254 nm wavelength at a speed of 20 mm/s by using denistometric scanner (CAMAG TLC scanner operated by Win CATS software). Samples were automatically injected in the injector and 15 µl of each sample was spotted on the plate by automated sample applicator or µl syringe (Hamilton, Switzerland) in the form of bands of width 6 mm. Temperature was maintained between 23-27 °C and 9.4 mm distance was kept between each track.

Standard stock solution of boeravinone B and calibration curve preparation: Standard stock solution of boeravinone B (Natural Remedies, Bangalore) was prepared by dissolving 10 mg of standard drug powder in 10 ml (1 mg/ml) of HPLC grade methanol and working solution was prepared by diluting 1 ml of stock solution in 10 ml of methanol to get a final 100 µg ml/L concentration. Eight point calibration curves were utilized ranging from 100 - 800 ng and curves were established by plotting areas of peaks against various concentration of standard solution. Quantification of boeravinone B content in different samples was done by utilizing linear regression equation of calibration curves and each sample was quantified in triplicates in order to maintain consistency.

Experiment design and statistics: Cultures were regularly sub-cultured after four weeks and all the experiments were repeated thrice and six replicates per treatment were used. Data was scored after every seven days of inoculation for leaf number, shoot length and shoot number in each experiment. Growth expressed as growth index (GI) was calculated for the all treatments on the basis of fresh weight (FW) and dry weight (DW) (GI= final weight-initial weight/initial weight) in order to assess the increase in biomass regeneration. ANOVA (SPSS version 17.0) (SPSS Inc., Chicago, USA) was used to analyze the differences in means of data recorded and Duncan's multiple range test (DMRT) was used to compared means of different treatments at p value ≤ 0.05.

RESULTS AND DISCUSSION

Medium composition is a determining factor for growth and successful plant regeneration depends on the right choice of nutrient medium. As nutrient requirement of plants vary from species to species, therefore standardization of a particular medium on the basis of concentration of different mineral nutrients is very difficult. Also, concentration of nutrient salts in growth medium significantly influences the shoot growth and relevant studies so far show that half and full strength of MS medium is more suitable for *in vitro* regeneration of many plant species (Mustafa *et al*. 2013). Shoot tip explants cultured on different concentrations of MS medium have been frequently used for micropropagation in large number of plants for *in vitro* multiplication and callus induction of *Drosera* genus (Perica and Berljak 1996), micropropagation of *Coptis teeta* and *Terminalia belerica* (Rathore *et al*. 2008).

In the present study also it was observed that half and full strength (control) MS medium showed

similar results for biomass production, however full strength medium had the best results when compared to the other treatments.Whereas a two fold salt strength was found to be detrimental leading to weak shoot growth with fewer shoot and leaf number (Supplementary Table S1).

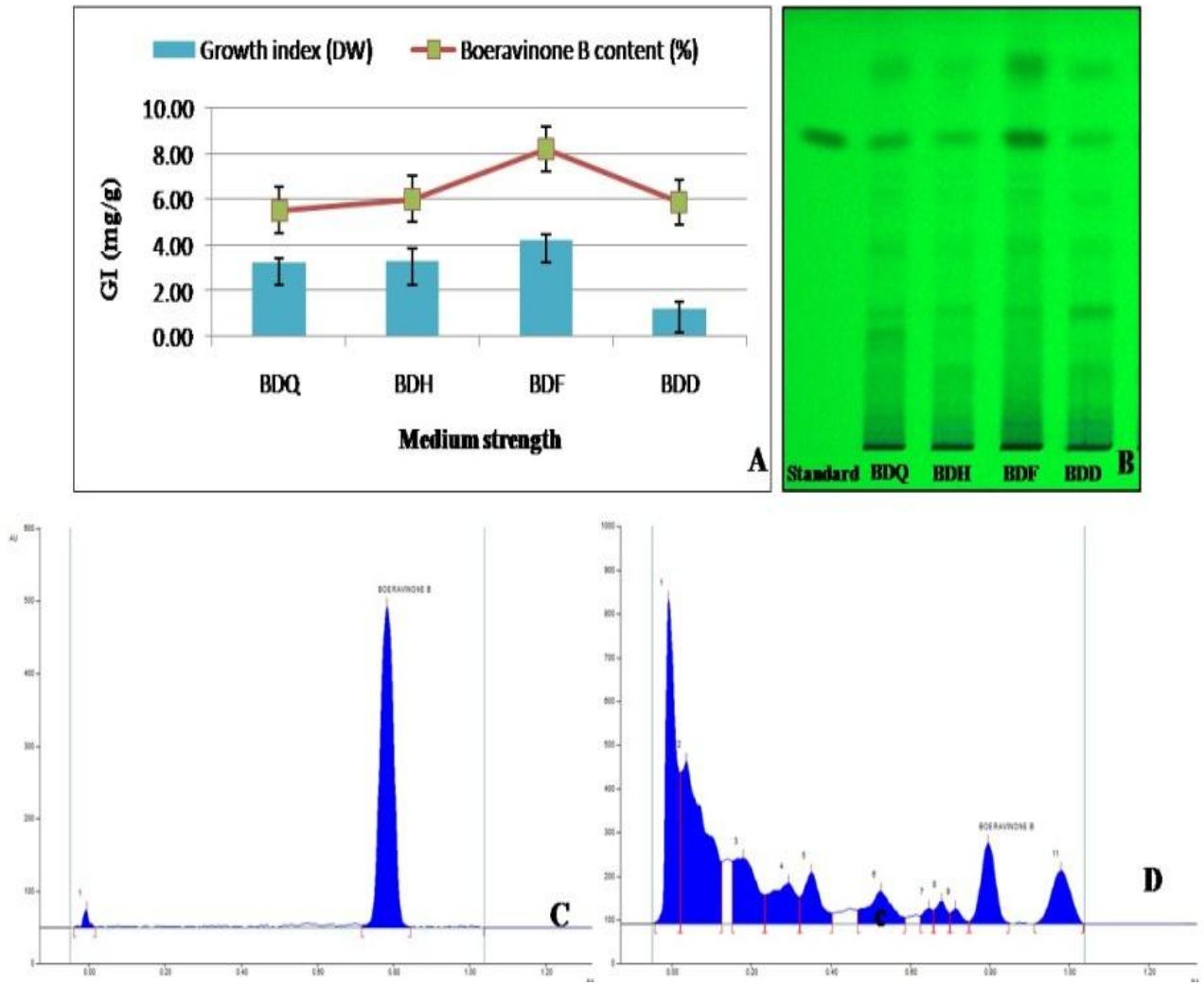

Figure 1: Effect of different strength of MS salt: BDQ (quarter strength MS); BDH (half strength MS); BDF (full strength MS); BDD (double strength MS) (A) Graphical representation of GI (DW) and boeravinone B production in MS medium of various salt strength; (B) HPTLC plate at 254 nm under fluorescent light showing the presence of boeravinone B in all treatment as compared to standard marker; (C) HPTLC chromatogram of boeravinone B (start position 0.7 Rf and end position 0.86 Rf); (D) HPTLC chromatogram of extract of MS full strength medium.

Reduction in biomass production expressed as growth index (GI) was in the order of full > half > quarter > double (Figure 1 A). Moreover, when different strengths treatment were analysed for the presence of boeravinone B, it was observed in all the treatments as compared to standard boeravinone B (Figure 1 B, C). In case of quantitative analysis, maximum yield of this specific secondary metabolite was obtained from shoots cultured on full strength MS medium (8.2 %) (Figure 1 A, D).

Shoot multiplication: Multiple shoots (with an average of 13.0 ± 0.33) from shoot tip explants were produced within 3-4 weeks in MS medium fortified with 1 mg/L Zn and 0.5 mg/L NAA and 3% sucrose. Among the different treatments used, this treatment was found to be optimal for growth and production of boeravinone B and significantly resulted in a nearly two fold increase (14.13%) in boeravinone B content as compared to control (MS medium devoid of PGR's) (7.53%) and threefold increase as compared to 3 month old field grown donor plant (4.83%) (Table 1, Figure 2).

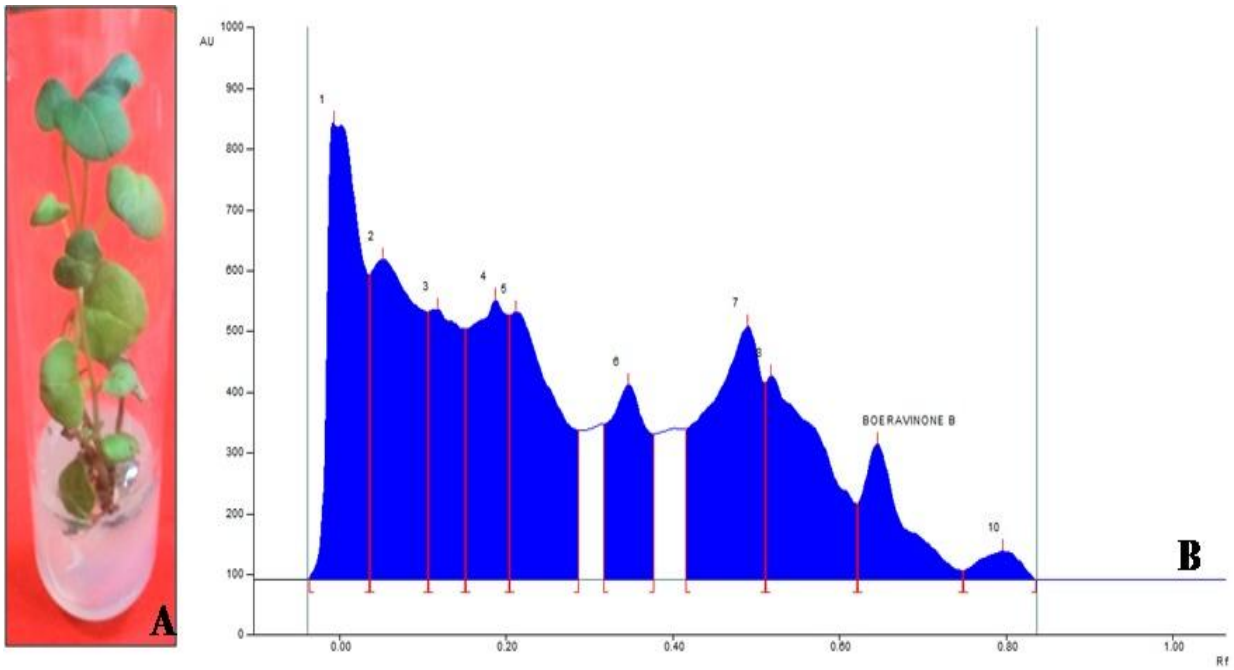

Figure 2: (A) *In vitro* regenerated plantlet of *Boerhaavia diffusa* in MS medium containing 1 mg/L Zn, 0.5 mg/L NAA and 3 % sucrose after 4 weeks of culture; (B) HPTLC chromatogram of shoot extract cultured on MS + Zn (1 mg/L) and NAA (0.5 mg/L)

Table 1:Effect of plant growth regulators on multiple shoot proliferation of *Boerhaavia diffusa* after 30 days of culture under *in vitro* conditions

*MS medium+ PGRs (mg/L)	Mean leaf Number±SE	Mean Shoot Number±SE	Mean shoot Length±SE	Boeravinone-B content ± SE (%)
Field plant	-	-	-	4.83± 0.09
Control	5.4 ± 0.27^{f}	1.0 ± 0^{e}	4.3 ± 0.20^{b}	7.53 ± 0.13
BAP				
0.5	8.5 ± 0.17^{c}	2.1 ± 0.11^{c,d}	2.6 ± 0.10^{d}	
1.0	9.0 ± 0.19^{c}	2.0 ± 0.19^{c,d}	2.7 ± 0.13^{d}	6.13 ± 0.12
1.5	8.0 ± 0.18^{c}	2.0 ± 0.14^{c,d}	2.7 ± 0.16^{d}	
Kn				
0.5	8.1 ± 0.25^{a}	1.6 ± 0.17^{d,e}	3.6 ± 0.11^{b}	
1.0	8.0 ± 0.19^{d}	1.5 ± 0.18^{d,e}	3.8 ± 0.12^{b}	8.20 ± 0.21
1.5	8.1 ± 0.20^{a}	1.0 ± 0.17^{d,e}	3.7 ± 0.16^{b}	
TDZ				
0.5	4.3 ± 0.10^{g}	1.3 ± 0.17^{e}	1.3 ± 0.11^{f}	
1.0	4.0 ± 0.12^{g}	1.3 ± 0.19^{e}	1.2 ± 0.1^{f}	4.33 ± 0.19
1.5	4.9 ± 0.22^{g}	1.4 ± 0.20^{e}	1.1 ± 0.1^{f}	
Zn				
0.5	10.4 ± 0.12^{b}	3.6 ± 0.21^{b}	3.0 ± 0.21^{c}	
1.0	10.5 ± 0.17^{b}	3.8 ± 0.22^{b}	3.1 ± 0.25^{c}	11.54 ± 0.03
1.5	10.0 ± 0.11^{b}	3.7 ± 0.20^{b}	3.1 ± 0.20^{c}	
BAP + NAA				
1.0 + 0.5	7.8 ± 0.18^{d}	2.3 ± 0.25^{c}	2.0 ± 0.14^{e}	
1.0 + 1.0	7.6 ± 0.13^{d}	2.2 ± 0.19^{c}	2.1 ± 0.13^{e}	7.15 ± 0.14
1.5 + 1.5	7.6 ± 0.11^{d}	2.2 ± 0.19^{c}	2.1 ± 0.14^{e}	
Kn + NAA				
1.0 + 0.5	7.0 ± 0.18^{e}	1.3 ± 0.11^{e}	1.2 ± 0.07^{f}	
1.0 + 1.0	7.1 ± 0.14^{e}	1.2 ± 0.12^{e}	1.1 ± 0.1^{f}	9.60 ± 0.23

1.5 + 1.5	7.0 ± 0.15^{e}	1.2 ± 0.22^{e}	1.0 ± 0.11^{f}	
TDZ + NAA				
1.0 + 0.5	3.0 ± 0.19^{h}	1.1 ± 0.09^{e}	1.2 ± 0.1^{f}	
1.0 + 1.0	3.1 ± 0.14^{h}	0.9 ± 0.12^{e}	1.3 ± 0.1^{f}	3.88 ± 0.36
1.5 + 1.5	3.0 ± 0.16^{h}	1.0 ± 0.22^{e}	1.1 ± 0.1^{f}	
Zn + NAA				
1.0 + 0.5	13.0 ± 0.33^{a}	5.1 ± 0.25^{a}	5.3 ± 0.18^{a}	
1.0 + 1.0	12.4 ± 0.30^{a}	4.9 ± 0.21^{a}	5.0 ± 0.16^{a}	14.13 ± 0.11
1.5 + 1.5	12.0 ± 0.36^{a}	4.5 ± 0.22^{a}	4.7 ± 0.11^{a}	

Data represent Mean ± SE of 18 replicates of each treatment; Means followed by a different superscript in each column are significantly ($p \leq 0.05$) different from each other using DMRT; *Murashige and Skoog medium + BAP (1mg/L) and NAA (0.5 mg/L)

In vitro production of boeravinone B from shoot cultures of *B. diffusa* suggested that the pathway for its biosynthesis is not affected by lab conditions. Shoot multiplication was further enhanced by subsequent subcultures. There are few other reports (Roy 2008; Kumar *et al*. 2013) which also suggest that shoot tips were the best source of explants for the induction of multiple shoots in *B. diffusa*. In medium combination containing both cytokinin and auxin, explants produced a greater number of shoots as comparison to medium fortified with only cytokinin (Table 1). Under *in vitro* conditions PGR's are highly significant as they control apical dormancy as well as help in maintaining a proper balance between organic and inorganic nutrients required for growing tissues. Synergetic effect of cytokinins and auxins in shoot proliferation has also been reported by various scientists in different plant species (Roy 1998; Madhulatha *et al*. 2004).

***In vitro* root induction:** Successful rooting of *in vitro* regenerated shoots is important for their establishment in soil. For rooting, elongated shoots were inoculated on MS basal medium containing various concentrations of auxins along with 3 % sucrose and 0.7 % agar. Among the various auxins tested, maximum *in vitro* rooting of individual shoots was observed in MS medium supplemented with 0.5 mg/L NAA (Table 2, Figure 3 A, D) wherein maximum root number (11.3 ± 0.39) and the longest roots (7.8 ± 0.25) were recorded after three weeks of culture. Moreover, rooting was also observed when NAA was used along with Zn but it took longer time period of eight weeks of culture. Results obtained for rooting are in correspondence with previous findings (Sudarshana *et al*. 2008), wherein maximum rooting was observed on MS medium fortified with 0.5 mg/L NAA. The rooted plantlets were transferred to plastic pots and kept inside the growth chamber for hardening and acclimatization. After 21 days of culture, well rooted hardened plants were acclimatized under green house conditions with a 95% survival rate. Growth parameters like shoot length, mean number of leaves and number of nodes were determined on zero day and after 21 days of transfer and there was an increase in the growth parameters recorded for the transferred plants. The regenerated plantlets showed normal plant morphology when compared to field plants (Figure 3 E, H).

Effect of CNP ratio on proliferation and boeravinone B production in *Boerhaavia diffusa:* When the effect of different ratio of media major nutrients was examined in comparison to control i.e. 1:1:1 ratio of carbon, nitrogen and phosphate; culture morphology was significantly influenced but no major influence on production of boeravinone B was observed. One half reduction in concentration of nitrogen (CNP3) and phosphate (CNP4) leads to reduction in culture growth whereas when concentration of nitrogen (CNP6) and phosphate (CNP7) were increased to its two fold, culture showed normal morphology with that of control (CNP1). Conversely, when concentration of carbon (CNP2) was reduced to half, large internodal distance was observed and when the concentration (CNP5) was increased to two fold, plantlets showed stunted morphology (Supplementary Table S2, Figure 4). Existing corpus of study confirmed that an appropriate concentration of nitrogen and phosphorus is required for growth and proliferation of plant species under *in vitro* conditions (Vidal and Guitierrez 2008). Moreover, when concentration of phosphate salt was reduced to half of its normal concentration, there was a decrease in boeravinone B production (9.43 %) as compared to that of control (13.78 %) and when concentration of carbon source was increased to its two fold, highest boeravinone B content was recorded (16.78 %) (Figure 4 A). In case of culture morphology of CNP5 (two fold increase in carbon source), it was observed that increase in concentration of carbon source is not suited for culture growth and it leads to stress condition in plantlets. As a result, the plantlet showed stunted morphology. But from higher yield of boeravinone B under these culture conditions, it may be concluded that stress conditions are stimulatory for production of this secondary metabolite and further studies can be attempted to ascertain the fact. From previous finding it was also reported that type and concentration of carbon source in nutrient significantly influenced the metabolite production (Ramachandra and Ravishanker 2002).

Figure 3: *In vitro* rooting and hardening of *Boerhaavia diffusa* plantlets (A-B) Initiation of roots from isolated shoots in MS medium fortified with NAA (0.5 mg/L); (C-D) Shoots showing roots; (E-F) Hardening and acclimatization of *in vitro* rooted plantlets in growth chamber; (G) Transfer of hardened regenerated plantlet to net house conditions (3 wks old); (H) hardened plant in field conditions (8 wks old)

Table 2:Effect of auxins (IBA, NAA, IAA) on *in vitro* root induction in *Boerhaavia diffusa* after 30 days of culture.

PGR concentration mg/L	Root number ± SE	Root length ± SE (cm)
Control	No root induction	No root induction
IBA		
0.5	4.3 ± 0.27^{b}	$3.0 \pm 0.19^{e,f}$
1.0	3.8 ± 0.18^{b}	2.5 ± 0.12^{f}
1.5	3.3 ± 0.21^{b}	2.6 ± 0.18^{f}
2.0	2.7 ± 0.12^{b}	2.5 ± 0.12^{f}

NAA		
0.5	11.3 ± 0.39[a]	7.8 ± 0.25[a]
1.0	5.0 ± 0.19[b]	5.0 ± 0.19[b]
1.5	5.0 ± 0.27[b]	4.8 ± 0.19[b]
2.0	5.1 ± 0.25[b]	3.8 ± 0.16[c,d]
IAA		
0.5	2.2 ± 0.12[b]	2.6 ± 0.11[f]
1.0	3.9 ± 0.18[b]	3.5 ± 0.12[d,e]
1.5	4.8 ± 0.26[b]	4.3 ± 0.26[c]
2.0	3.8 ± 0.20[b]	4.0 ± 0.19[c,d]

Data represent Mean ± SE of 18 replicates of each treatment; Means followed by a different superscript in each column are significantly ($p \leq 0.05$) different from each other using DMRT

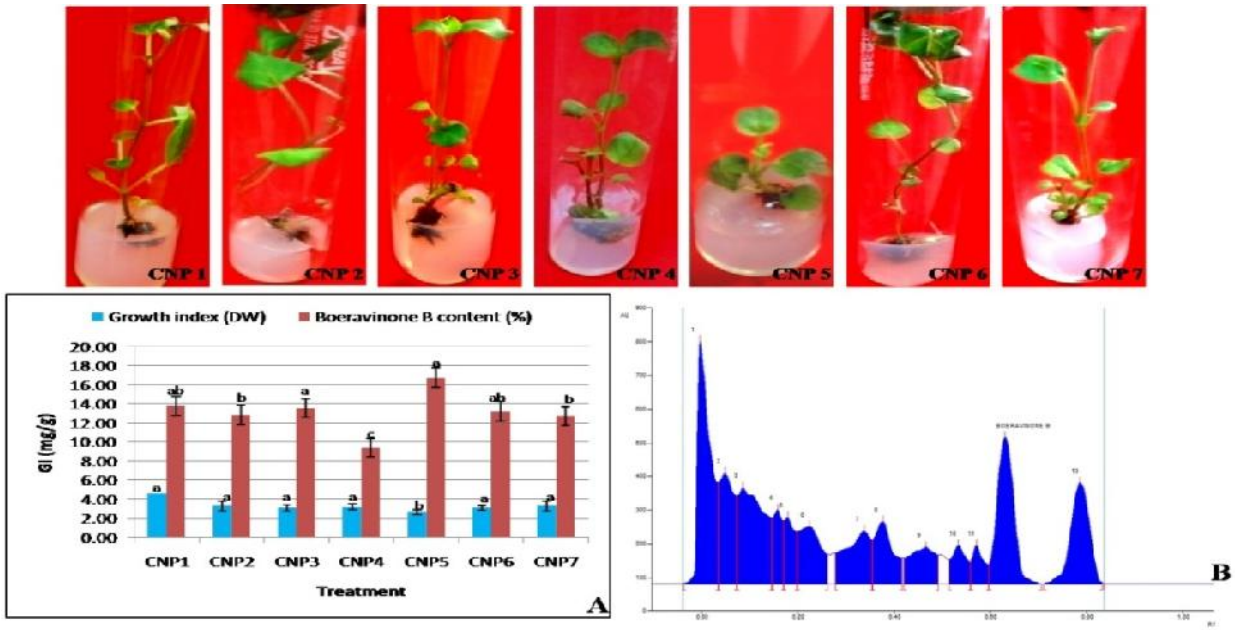

Figure 4: Effect of carbon (C), nitrogen (N) and phosphate (P) ratio in MS medium containing Zn (1 mg L^{-1}) and NAA (0.5 mg L^{-1}) on *in vitro* regeneration and boeravinone B production in *Boerhaavia diffusa*: CNP1 (control - where normal concentration of carbon, nitrogen and phosphate as per MS medium was present) (1:1:1), CNP2 (0.5:1:1), CNP3 (1:0.5:1), CNP4 (1:1:0.5), CNP5 (2:1:1), CNP6 (1:2:1), CNP7 (1:1:2) (A) Comparative graphical representation of GI (DW) and boeravinone B production and bars with same letter are not significantly different from each other at p value ≤ 0.05 using DMRT (B) HPTLC profile of culture extract from MS medium fortified with CNP ratio of 2:1:1.

Effect of different type and concentration of carbon sources on regeneration and boeravinone B production in *Boerhaavia diffusa:* Exogenous carbon source added to the culture medium highly influenced culture growth and acted as an osmotic agent and energy source (Haque *et al*. 2013). In most of the plant species like *Centella asiatica* (Hossain *et al*. 2005) *Prunus persica* (Ahmed *et al*. 2007), *Pogostemon cablin* (Kuamara *et al*. 2010) normally 3% sucrose is used as carbon source. Variance in shoot response is a consequence of the differential ability of plant species within the plant kingdom to metabolize different types of carbohydrates. In the present study, 3% sucrose (control) and 3% table sugar showed similar results (Figure 5 A, B) and had the highest response rate. Therefore, the finding indicates a possible way of using table sugar (a cheaper source of carbon) in place of sucrose for *in vitro* regeneration of *B. diffusa* to reduce the production cost without significant loss in plant quality and growth. Maltose was found to be a poor source of carbon for plant regeneration and MS medium supplemented with dextrose 1% produced maximum leaf number (14.1 ± 0.32) followed by incorporation of dextrose 3% (12.1±0.25). Proliferative shoots were produced in dextrose supplemented medium; however the regenerants were weak and light green in color. Additionally, the formation of callus in dextrose containing medium reflects that table sugar is a better carbon source for *in*

vitro growth of this plant species. Negative effect of fructose on plant growth was also observed, probably due to its inefficient metabolization by the cells of plant. The rate of culture growth expressed as GI was highly influenced by different sources of carbon at different concentrations and highest biomass regeneration was observed in dextrose containing medium (6.32 DW) at different concentrations. 3% sucrose (5.58 DW) and 3% table sugar (5.63 DW) showed almost similar results for biomass increment (Fig 5 b). Most remarkable observation in this experiment was a significant increase (~3.9 fold) in boeravinone B production (19.15%) in medium containing table sugar (5%) as compared to field grown mother plant (4.83%) and yield was 1.3 fold more than that of control treatment (14.18%), wherein 3% sucrose was used as carbon source. It is also remarkable that in both the media manipulation experiments, increase in the concentration of carbon source highly influences the production of boeravinone B. Likewise, fructose and maltose sugar which showed least response for biomass regeneration at different concentrations also contributed to an increase in production of boeravinone B and dextrose sugar showed least results for its production as compared to control (Supplementary Table S3, Figure5).The study explicitly hinges upon development of a cost effective and reproducible method for conservation of *Boerhaavia diffusa* by utilizing a minimal quantity of cytokinin in combination with auxin along with 3% table sugar. It also establishes an efficient protocol for enhancement of boeravinone B (medicinally valuable metabolite) production by simply manipulating the media components. Enhanced boeravinone B production by the regenerated tissues emphasises on the fact that the activity of enzymes related to its biosynthesis are significantly increased by altering the media components. Moreover, its production is not bound to any specific season and is not dependent upon the age of the plant material as is usually the case in field grown plants. In cue with the above, the study serves as a basis for pharmaceutical companies to utilize this medicinally important pharmaceutical component of *B. diffusa* without posing threats to its natural population. Further, it assists in selection of high metabolite yielding tissues which can be utilized for enhanced metabolite production in cultured tissues.

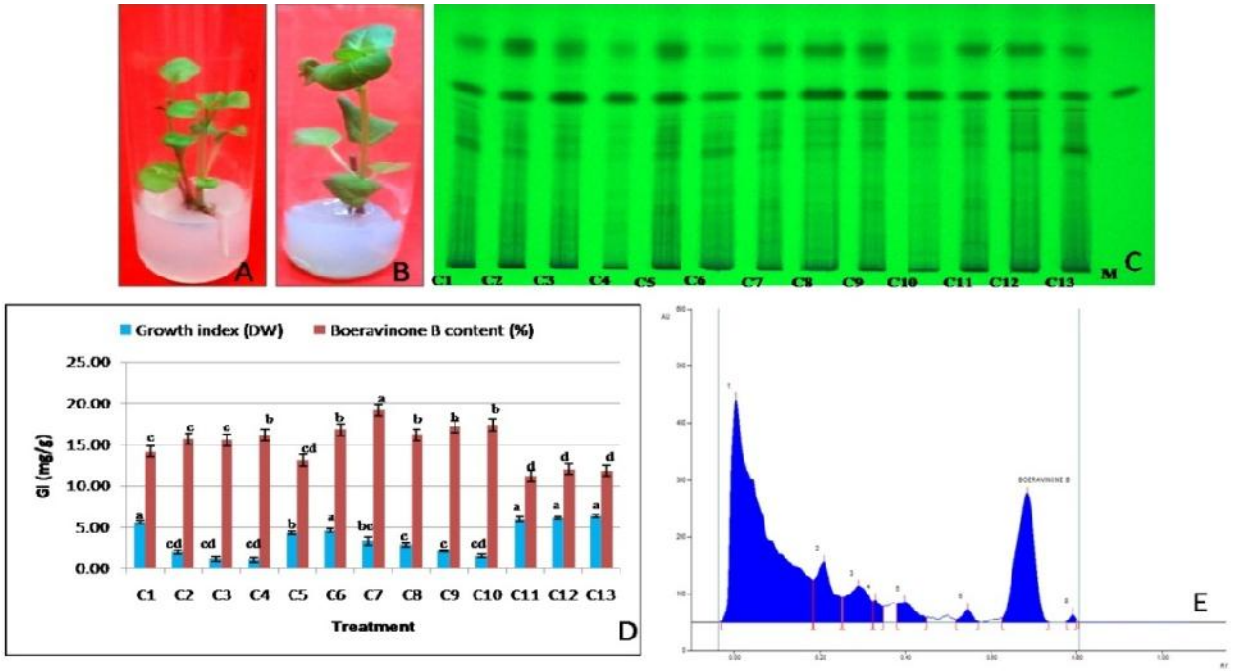

Figure 5: Effect of different carbon sources at different concentration in MS medium fortified with Zn (1 mg L^{-1}) and NAA (0.5 mg L^{-1}) on *in vitro* regeneration and boeravinone B production in *Boerhaavia diffusa*: C1 (control – 3% sucrose), C2 (fructose 1%), C3 (fructose 3%), C4 (fructose 5%), C5 (table sugar 1%), C6 (table sugar 3%), C7 (table sugar 5%), C8 (maltose 1%), C9 (maltose 3%), C10 (maltose 5%), C11 (dextrose 1%), C12 (dextrose 3%), C13 (dextrose 5%) (A) proliferated shoot at 3% sucrose; (B) proliferated shoot at 3% table sugar; (C) HPTLC plate at 254 nm under fluorescent light showing the presence of boeravinone B in all treatment as compared to standard marker; (D) Comparative graphical representation of GI (DW) and boeravinone B production and bars with same letter are not significantly different from each other at p value ≤ 0.05 using DMRT (E) HPTLC chromatogram of culture extract from MS medium fortified with 5% table sugar

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Supplementary Material

Table S1: Effect of different strengths of MS medium on shoot regeneration in *Boerhaavia diffusa.*

*Medium concentration	No. of leaves ± SE	No. of Shoots ± SE	Shoot Length ± SE (cm)	Culture morphology
Quarter	5.8 ± 0.17^{c}	1.5 ± 0.12^{b}	4.3 ± 0.10^{c}	Stunted shoot
Half	6.5 ± 0.24^{b}	1.6 ± 0.18^{b}	4.7 ± 0.11^{b}	Normal shoot
Full	9.94 ± 0.25^{a}	2.1 ± 0.17^{a}	4.9 ± 0.12^{a}	Healthy and elongated shoot
Double	3.0 ± 0.19^{d}	1.6 ± 0.17^{c}	3.3 ± 0.07^{d}	Stunted shoot

Data represent Mean ± SE of 18 replicates of each treatment; Means followed by a different superscript in each column are significantly ($p \leq 0.05$) different from each other using Duncan multi range test (DMRT); *Murashige and Skoog medium

Table S2:Effect of different concentration of CNP on biomass production of *Boerhaavia diffusa.*

*MS + varied ratio of CNP (w/v)	No. of leaves ± SE	No. of Shoots ± SE	Shoot Length ± SE (cm)	Culture morphology
CNP1	9.1 ± 0.74^{a}	1.5 ± 0.22^{a}	6.5 ± 0.82^{a}	Healthy shoot
CNP2	7.6 ± 1.11^{b}	1.5 ± 0.34^{a}	5.3 ± 0.69^{b}	Normal shoot
CNP3	7 ± 1.99^{bc}	1 ± 0.16^{b}	4.1 ± 0.53^{bc}	Large internodal distance
CNP4	7 ± 1.52^{bc}	1 ± 0.16^{b}	4.2 ± 0.29^{bc}	Normal shoot, small leaf size
CNP5	6 ± 1.15^{cd}	1.1 ± 0.16^{b}	2.6 ± 0.21^{d}	Stunted shoot
CNP6	9 ± 1.36^{a}	1.5 ± 0.34^{a}	5.8 ± 0.57^{b}	Normal shoot
CNP7	8.3 ± 0.84^{ab}	1.5 ± 0.34^{a}	5.3 ± 0.63^{b}	Normal shoot

Data represent Mean ± SE of 18 replicates of each treatment; CNP1 (control - where normal concentration of carbon, nitrogen and phosphate as per MS medium was present) (1:1:1), CNP2 (0.5:1:1), CNP3 (1:0.5:1), CNP4 (1:1:0.5), CNP5 (2:1:1), CNP6 (1:2:1), CNP7 (1:1:2; Means followed by a different superscript in each column are significantly ($p \leq 0.05$) different from each other using DMRT; *Murashige and Skoog medium + BAP (1mg/L) and NAA (0.5 mg/L)

Table S3: Effect of different carbon sources at different concentrations on biomass production of *Boerhaavia diffusa.*

*MS + carbon source (w/v)	No. of leaves ± SE	No. of Shoots ± SE	Shoot Length ± SE (cm)	Culture morphology
C1	7.4 ± 0.20^{d}	3.5 ± 0.23^{a}	6.9 ± 0.21^{a}	Healthy shoot
C2	6.0 ± 0.19^{e}	1.6 ± 0.18^{cde}	5.3 ± 0.14^{c}	Unhealthy shoot with small callus at base
C3	5 ± 0.19^{f}	1.5 ± 0.18^{def}	4.4 ± 0.10^{e}	Unhealthy shoot
C4	2.4 ± 0.12^{i}	1 ± 0^{f}	1.5 ± 0.1^{g}	Stunted shoot
C5	4.0 ± 0.19^{gh}	2.1 ± 0.25^{c}	4.1 ± 0.17^{e}	Large internodal distance
C6	7.5 ± 0.23^{d}	3.6 ± 0.18^{a}	7.0 ± 0.29^{a}	Healthy shoot
C7	4.3 ± 0.26^{g}	1.9 ± 0.20^{cd}	3.6 ± 0.09^{f}	Normal shoot with small callus at base
C8	3.5 ± 0.23^{h}	1.3 ± 0.11^{ef}	4.9 ± 0.17^{d}	Normal shoot with small callus at base
C9	4.0 ± 0.19^{gh}	1.3 ± 0.12^{ef}	4.2 ± 0.15^{e}	Normal shoot with small callus at base
C10	2.3 ± 0.22^{i}	1 ± 0^{f}	1.5 ± 0.1^{g}	Unhealthy shoot
C11	14.1 ± 0.32^{a}	2.8 ± 0.16^{b}	5.6 ± 0.13^{c}	Highly proliferated shoot with small callus at base
C12	12.1 ± 0.25^{b}	2.6 ± 0.21^{b}	6.1 ± 0.16^{b}	Proliferated shoot with small callus at base
C13	9.1 ± 0.25^{c}	1.8 ± 0.16^{cde}	5.7 ± 0.11^{bc}	Proliferated shoot with small callus at base

Data represent Mean ± SE of 18 replicates of each treatment;C1 (control - 3 % sucrose), C2 (fructose 1 %), C3 (fructose 3 %), C4 (fructose 5 %), C5 (table sugar 1 %), C6 (table sugar 3 %), C7 (table sugar 5 %), C8 (maltose 1 %), C9 (maltose 3 %), C10 (maltose 5 %), C11 (dextrose 1 %), C12 (dextrose 3 %), C13 (dextrose 5 %; Means followed by a different superscript in each column are significantly ($p \leq 0.05$) different from each other using DMRT; *Murashige and Skoog medium + BAP (1mg/L) and NAA (0.5 mg/L)