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SHORT COMMUNICATION

Effect of signal molecules for enhanced production of boeravinone B in shoot cultures of *Boerhaavia diffusa* (L.)

Savita Sharma[1], Jyotsna Sharma[1], Deepika Singh[2], Vandana Sharma[2] and Sharada Mallubhotla[1]*

[1]School of Biotechnology, Shri Mata Vaishno Devi University, Kakryal, Katra, J&K-182320, India, [2]Quality Assurance and CMC Division, Council of Scientific and Industrial Research - Indian Institute of Integrative Medicine, Jammu, J&K-180001, India

ABSTRACT

High performance thin layer chromatography (HPTLC) analysis was used to analyze boeravinone B production in shoot cultures of *Boerhaavia diffusa* under the influence of different biotic [yeast extract (YE), cellulase (CL)] and abiotic [salicylic acid (SA), jasmonic acid (JA)] signal molecules at different concentrations. Biomass accumulation and boeravinone B production in shoot cultures raised on agar solidified medium were analysed for a period of 30 days to optimize the suitable age of culture for treatment with signal molecules. A maximum yield of boeravinone B (5.74 %) was obtained after 7 days and therefore treatments were performed at a gap of 3, 6 and 9 days. Signal molecules used at varied concentrations differentially influenced the shoot cultures for biomass regeneration and culture growth. Cellulase treatment (0.5 mgl^{-1}) resulted in maximizing biomass (1.30gm) and boeravinone B content (22.7 %) after 6 days of exposure time as compared to other treatments used in the study. Thus the current study can be exploited further for enhancement of boeravinone B from shoot cultures of *Boerhaavia diffusa*.

Keywords: Boeravinone B; *Boerhaavia diffusa*; Elicitors; HPTLC profiling

INTRODUCTION

Boerhaavia diffusa L. (Nyctaginaceae) most popularly known as Punarnava has been used for the treatment of blood pressure, rheumatism, piles, liver disorders, seminal weakness, men infertility, leucorrhea and stomach ache (Kumar and Dora, 2012). Rotenoids known as boeravinones *viz.* boeravinone A, B, C, D, E and F have been isolated from the roots of the herb and studied in detail for their pharmacological properties. Among them boeravinone B is the most potent and interesting metabolite from a therapeutic point of view and is responsible for the anticancer, antiageing and antiinflammatory properties (Bairwa et al., 2013; Bairwa and Jachak, 2015; Biradar et al., 2018; Huang et al., 2018; Prathapan and Raghu 2018; Mathais et al., 2020). More recently, phytochemicals isolated from *B. diffusa* like boerhavisterol, bioquercetin and biorobin have shown a potential to inhibit viral protease of SARS-CoV-2, the causative agent of COVID-19 (Surya and Praveen, 2021). Phytochemical production in plants is mostly low being dependent upon the stage of plant development and environmental conditions (Thakur et al., 2013). Although these metabolites are produced by plants mostly in low concentration during specific phases within the life cycle of the plant for defence purposes, however, under *in vitro* conditions, there remains no such limitation as the production becomes independent of time and age of the plant culture (Tripathi et al., 2019). Keen interest has been devoted to the enhancement of their production especially utilizing media manipulation, culture condition optimization and elicitor/signal molecule feeding techniques under *in vitro* conditions (Sathuluri and Gokare, 2002). Among them elicitation (biotic or abiotic) of plant cells is a very important technique (Sharma et al., 2015; Ahammed et al., 2020a; Ahammed et al., 2020b; Nabi et al., 2021). Previous studies have clearly indicated that yeast extract, salicylic acid, methyl jasmonate, chitosan, cellulase, fungal carbohydrates and heavy metals are the most commonly used signal molecules for overproduction of phytoconstituents in plant cultures (Mialoundama et al.,

***Corresponding author:**
Sharada Mallubhotla, School of Biotechnology, Shri Mata Vaishno Devi University, Kakryal, Katra, J&K-182320, India.
E-mail: sharda.p@smvdu.ac.in, babasharada@gmail.com

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2009; Koul and Mallubhotla, 2020). Some important factors like concentration of elicitor, exposure time and age of culture at the time of treatment greatly influences the accumulation of metabolites and regeneration of biomass (Ma, 2008; Sharma et al., 2021a). For instance, effect of jasmonic acid at different concentrations on production of ginsenoside in ginseng *in vitro* root cultures showed that an increase in the concentration of elicitor caused a decrease in both biomass as precise exact concentration of signal molecule to be used and suitable harvesting time for maximizing yield of metabolites. Moreover, from various studies an increase in various antioxidant enzyme activity like dehydroascorbate reductase, monodehydroascorbate reductase, ascorbate peroxidase, glutathione reductase, peroxidase and superoxidase dismutase was observed when methyl jasmonate was used as an elicitor (Ho et al., 2020; Yosefi et al., 2020). Methyl jasmonate also leads to an increase in the production of alkaloids sanguinarine and chelerythrine (used as substitutes for antibiotics) in *Macleaya cordata* (Huang et al., 2021). The present study involves modification of cell metabolism for enhancement of useful secondary metabolites (boeravinone B) under *in vitro* culture conditions using biotic and abiotic signal molecules exhibits the influence of these elicitors on production of boeravinone B in shoot cultures of *Boerhaavia diffusa* using HPTLC analysis.

MATERIAL AND METHODS

Plant material collection and kinetics of boeravinone B production

Boerhavia diffusa (3 month old net house grown) plant material was collected from vegetatively propagated plants habituated at "Trikuta Hill Herbal Garden" of Shri Mata Vaishno Devi University, Katra, (J&K) and a reference specimen of the same has been deposited at Janaki Ammal Herbarium, CSIR- Indian Institute of Integrative Medicine (CSIR-IIIM), Jammu, J&K with Accession No.RRLH23492. *In vitro* regenerated shoot tips (1.5-2 cm length) were regenerated on Murashige and Skoog (MS) medium with Zeatin (1.0 mg l^{-1}) and Naphthalene acetic acid (NAA) (0.5 mg l^{-1}) as earlier reported (Sharma et al., 2021b). Biomass accumulation and boeravinone B production in agar solidified medium were analysed for a period of 30 days to determine the optimum culture age to be used for treatments with signal molecules.

Signal molecule feeding

On the basis of results of above study, the effect of various signal molecules like cellulase (CL), yeast extract (YE), jasmonic acid (JA) and salicylic acid (SA) at various concentrations (0.1, 0.5, 1.0 mgl^{-1}) were analyzed to check their influence on biomass accumulation and boeravinone B content at 3, 6, 9 day intervals. SA and YE were dissolved in sterile distilled water to prepare stock solutions of 1.0 $mgml^{-1}$ (w/v) while CL and JA were dissolved in pure sterile ethanol at a concentration of 1.0 $mgml^{-1}$ (w/v). These stock solutions were sterilized further by using a disposable polytetrafluoroethylene (PTFE) syringe filter (Millipore, USA) of 0.2 μm pore size and finally added into autoclaved molten medium. Medium devoid of any signal molecules but with appropriate volume of water/ethanol were used as control for the experimentations. Growth expressed as growth index (GI) was calculated for all treatments on the basis of fresh weight (FW) and dry weight (DW) in order to assess biomass increment.

Extract preparation

Using standardized protocols process samples were independently harvested and extracted by soxhletation method. Filtered extracts [using 0.45 μm pore size PVDF (polyvinylidene fluoride) filters (Millipore, USA)] were air dried and dissolved in HPLC grade methanol prior to HPTLC (high performance thin layer chromatography) analysis against standard marker boeravinone B.

Preparation of boeravinone B standard

100 μg ml^{-1} working solution of boeravinone B (standard marker) of 97.0% purity (Natural Remedies Private Limited, Bangalore) was prepared as reported earlier (Sharma et al., 2021b).

Boeravinone B quantification using HPTLC

Quantitative analysis was performed using a CAMAG HPTLC system (Muttenz, Switzerland). Details of the procedure employed were as reported earlier (Sharma et al., 2021b). An eight point calibration curve was plotted using standard boeravinone B solutions ranging from concentrations 100 - 800 ng. Boeravinone B content in different samples was analysed by utilizing the linear regression equation of calibration curves and content is expressed in percentage.

Statistical study

One way analysis of variance **(**ANOVA) was used (SPSS version 17.0) (SPSS Inc., Chicago, USA) to analyze the differences in means of data recorded and significance of difference is determined by Duncan's multiple range test (DMRT) at p value ≤ 0.05. Each sample was quantified in triplicates in order to maintain consistency.

RESULTS AND DISCUSSION

Shoot culture growth was highly influenced by treatments with various signal molecules. A maximum biomass was observed at 30th day of the culture period while the maximum boeravinone B content (5.74 %) was obtained

after 7 days of incubation time which gradually decreased with further increase in culture period and therefore further elicitation experiments were performed at a gap interval of 3, 6 and 9 days (Table 1).

Different signal molecules (*viz.* jasmonic acid, salicylic acid, yeast extract, cellulase) at varied concentrations differently influenced the growth of shoot cultures and boeravinone B content (Table 2, Figure 1). Enhancement of secondary metabolite through the treatments is a complex process and its response varies from species to species depending upon certain factors like elicitor concentration, age of plant at the time of elicitation and exposure time of plant for elicitation. In the current study, it has been observed that cellulase at a concentration of 0.5 mgl^{-1} after 6 days of exposure time resulted in highest biomass regeneration as well as it is effective in induction of highest content of boeravinone B (22.7 %) under *in vitro* conditions (Figure 1, e) while, yeast extract at a concentration of 1 mgl^{-1} gave maximum yield (20.79 %) of this secondary metabolite after exposure time of 9 days (Figure 1, f). In case of abiotic molecules higher content of boeravinone B were found in the treatment with jasmonic acid (JA) (1 mgl^{-1}) and salicylic acid (SA) (0.1 mgl^{-1}) of 18.48 % and 19.62 % respectively after 9 days of exposure time. (Fig. 1 c & d). However amongst all the treatments; the best response was obtained with cellulase (0.5 mg l-1).

Cellulase is produced by a number of plant pathogenic fungi but less attention has been paid to the role of cellulase as a signal molecule and only a few studies have reported its activity (Ma, 2008; Mialoundama et al., 2009). From previous studies it was recorded that cellulase in culture medium leads to a 1.6 fold increase in solasodine and alpha solanine content in *Solanum khasianum* (Srivastava et al., 2016). Moreover, a further increase in cellulase concentration caused a decrease in metabolite production. Similar results have also been observed in Ginseng *in vitro* root culture where an increased concentration of jasmonic acid lead to a decrease in ginsenoside production (Yu et al., 2002). On the basis of current research work and previous studies including the use of various signal

Table 1: Timeline studies on biomass regeneration and boeravinone B production in *B. diffusa* mother culture.

Parameter	Culture period (number of days)				
	7	14	21	28	35
Leaf number	2.1±0.51^{c}	2.6±0.58^{bc}	3.2±0.65^{b}	4.4±0.4^{ab}	5.0±0.71^{a}
Shoot number	0.58±0.16^{c}	0.64±0.21^{bc}	0.96±0.18^{b}	1.24±0.33^{ab}	2.1±0.58^{a}
Length of shoots (cm)	0.62±0.16^{c}	0.78±0.24^{bc}	0.82±0.33^{b}	1.08±0.11^{ab}	1.12±0.09^{a}
Fresh weight	1.68±0.12^{c}	2.21±0.18^{bc}	2.32±0.21^{b}	3.98±0.12^{ab}	4.83±0.11^{a}
Dry weight	0.72±0.21^{c}	1.12±0.23^{bc}	1.24±0.18^{b}	2.52±0.13^{ab}	3.96±0.24^{a}
Boeravinone B content %	5.04±0.79	4.42±0.71	4.38±0.77	3.61±0.14	3.53±0.40

Data represents Mean ± SE from triplicate experiment; Means followed by a different superscript in each column are significantly ($p \leq 0.05$) different from each other using DMRT.

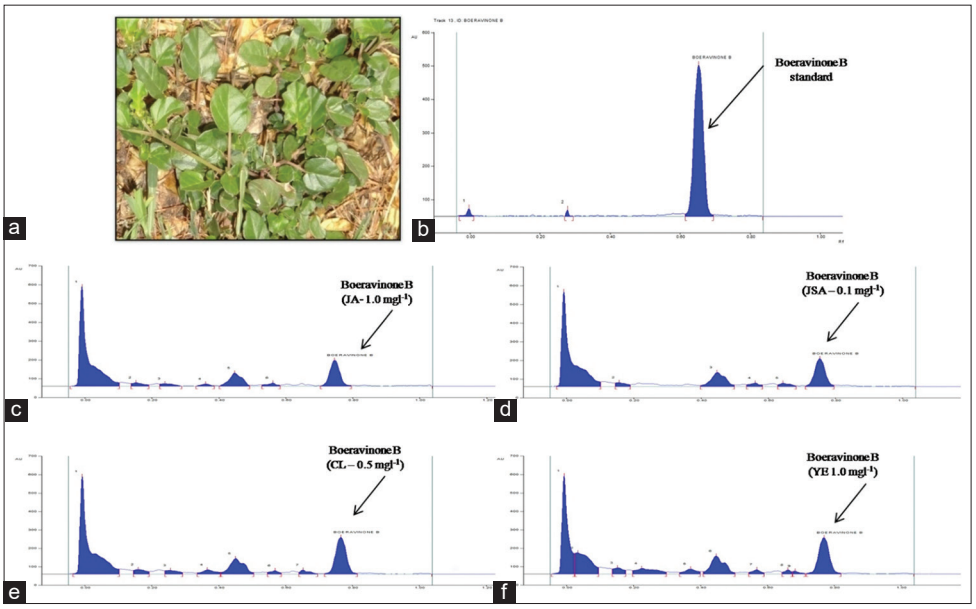

Fig 1. Boeravinone B estimation in shoot cultures subjected to various signal molecules (JA, SA, CL, YE) under in vitro culture conditions at different exposure time (a) B. diffusa mother plant at herbal garden, SMVDU. HPTLC chromatogram of (b) Standard marker boeravinone B, (c) Jasmonic acid (1.0 mgl^{-1}) after 9th day, (d) Salicylic acid (0.1 mgl^{-1}) after 9th day, (e) Cellulase (0.5 mgl^{-1}) after 6th day, (f) Yeast extract (1.0 mgl^{-1}) after 9th day.

Table 2: Effect of signal molecule treatment on biomass regeneration and production of boeravinone B in *B. diffusa.*

Signal molecule treatment	Exposuretime (days)	Growth index (GI)		Boeravinone B content (%)
		Fresh weight (FW)	Dry weight (DW)	
Jasmonic acid (JA) (mgl^{-1})				
*Control (ethanol)	3	0.66±0.33	0.53±0.12	1.58±0.58
	6	0.76±0.21	0.63±0.21	0.59±0.93
	9	0.83±0.19	0.66±0.11	1.75±0.66
0.1	3	0.56±0.31	0.38±0.17	12.17±0.14
	6	0.84±0.29	0.57±0.13	13.19±0.05
	9	0.87±0.20	0.69±0.12	12.05±0.09
0.5	3	0.86±0.14	0.69±0.29	12.16±0.06
	6	0.85±0.31	0.63±0.31	15.03±0.13
	9	0.58±0.44	0.40±0.13	16.13±0.17
1.0	3	0.60±0.10	0.39±0.25	12.25±0.08
	6	0.91±0.18	0.73±0.20	14.08±0.03
	9	0.86±0.13	0.61±0.16	18.48±0.11
Salicylic acid (SA) (mgl^{-1})				
Control (water)	3	0.35±0.24	0.24±0.11	0.64±0.23
	6	0.55±0.11	0.30±0.19	0.95±0.12
	9	0.66±0.26	0.49±0.15	1.55±0.08
0.1	3	0.54±0.28	0.33±0.16	14.06±0.12
	6	0.84±0.29	0.63±0.13	15.08±0.07
	9	0.89±0.30	0.72±0.14	19.62±0.04
0.5	3	0.54±0.30	0.37±0.30	15.06±0.08
	6	0.82±0.40	0.66±0.51	17.09±0.02
	9	0.92±0.10	0.71±0.36	16.07±0.11
1.0	3	0.49±0.37	0.28±0.23	16.46±0.07
	6	0.74±0.16	0.50±0.16	16.75±0.02
	9	0.69±0.09	0.49±0.21	17.08±0.09
Cellulase (CL) (mgl^{-1})				
Control (ethanol)	3	0.37±0.11	0.09±0.08	0.96±0.08
	6	0.36±0.18	0.29±0.21	0.82±0.11
	9	0.46±0.09	0.19±0.11	1.24±0.15
0.1	3	0.53±0.18	0.37±0.12	15.18±0.22
	6	0.66±0.21	0.42±0.36	16.34±0.14
	9	0.48±0.29	0.22±0.10	16.13±0.24
0.5	3	1.23±0.35	0.71±0.33	19.43±0.15
	6	2.15±0.15	1.30±0.20	22.70±0.07
	9	1.06±0.11	0.92±0.12	16.64±0.03
1.0	3	1.50±0.44	0.97±0.41	17.26±0.17
	6	1.70±0.13	0.94±0.36	15.34±0.06
	9	2.06±0.17	1.03±0.19	15.11±0.04
Yeast extract (YE) (mgl^{-1})				
Control (water)	3	0.37±0.21	0.20±0.11	1.12±0.10
	6	0.49±0.10	0.32±0.21	0.78±0.06
	9	0.58±0.09	0.40±0.17	1.21±0.13
0.1	3	1.12±0.33	0.90±0.10	13.18±0.07
	6	1.44±0.14	1.10±0.20	13.11±0.15
	9	1.19±0.21	1.00±0.23	14.09±0.04
0.5	3	1.02±0.16	0.93±0.11	13.16±0.08
	6	0.89±0.10	0.66±0.10	14.18±0.11
	9	0.86±0.21	0.62±0.08	14.09±0.08
1.0	3	0.74±0.23	0.41±0.06	13.17±0.06
	6	0.66±0.12	0.35±0.12	17.48±0.12
	9	0.40±0.10	0.21±0.13	20.79±0.09

Data represents Mean±SE from triplicate experiment; Means followed by a different superscript in each column are significantly ($p \leq 0.05$) different from each other using DMRT**;** Growth index (GI) = Dry weight (DW) – Fresh weight (FW)/Fresh weight; * 1.0 ml l^{-1}

molecules i.e. elicitors to enhance the production of secondary metabolites in *in vitro* cultures of medicinal plant species, one is more likely to develop *in vitro* system to enhance production of useful secondary metabolites and also conclude that the type of signal molecule at a specific concentration, exposure time and age of culture at the time of treatment influence the production of metabolites under *in vitro* culture conditions (Koul and Mallubhotla, 2020; Ali, 2021; Guru et al., 2021).

CONCLUSIONS

Biotic and abiotic signal molecules assisted in enhancing the production of this particular metabolite - boeravinone B under *in vitro* conditions and this technique could pave way for commercialization by pharmaceutical industries for large scale production using bioreactors for cultivating shoot cultures of *Boerhaavia diffusa*. Enhanced yield of boeravinone B content under *in vitro* conditions also emphasises the fact that the activity of enzymes related to its biosynthesis were significantly affected by the treatment, which has a direct correlation to plant growth and changes in biomass regeneration.

Author's contributions

Sharada Mallubhotla was the brainchild behind this research work, provided the resources for the study, designed the experiments and also helped in improvement of formulating the article in writing. Experimental work, sub-culturing, maintenance of cultures in lab conditions, data acquisition, data recording, compilation of results and writing of the manuscript was done by Savita Sharma. Jyotsna Sharma helped in collection of plant material and data recording. Deepika Singh and Vandana Sharma helped in acquiring the HPTLC data and provided its interpretation.

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