ARTICLE

Effect of sugars on production of β-sitosterol from in vitro callus culture of *Boerhaavia diffusa* L.

Kapil S. Patil, Sanjivani R. Bhalsing*

Department of Biotechnology, School of Life Sciences, North Maharashtra University, Jalgaon-425 001, Maharashtra, India

ABSTRACT   The establishment of friable callus was achieved on Murashige and Skoog (MS) medium fortified with 18 µM of 2,4-dichlorophenoxyacetic acid in combination with 2 µM of 6-benzylaminopurine (BAP). The effect of different sugars such as 3% sucrose, 3% glucose and 3% galactose on the growth of callus tissue and β-sitosterol accumulation was tested. Quantification of β-sitosterol was carried out with the help of high performance liquid chromatography. High growth of callus tissue was observed on MS medium, containing sucrose and glucose as carbon sources and maximum β-sitosterol content was observed in 60 days old callus tissue with 3% glucose as carbon source.   **Acta Biol Szeged 60(2):99-104 (2016)**

Abbreviations

MS - Murashige and Skoog, 2,4-D - (2,4-dichlorophenoxy acetic acid), TLC - Thin Layer Chromatography.

Introduction

Boerhaavia diffusa is a creeping, perennial herbaceous plant that belongs to the family Nyctaginaceae (Four o'clock Family). Although it is native to India and Brazil, but it has been traditionally used as a medicine in many different parts of the world (Patil and Bhalsing 2016). Out of the 40 species of the genus *Boerhaavia*, five were found in India (*B. diffusa*, *B. chinensis*, *B. erecta*, *B. rependa*, and *B. rubicund*) (Chopra et al. 1969). In Ayurvedic and Unani medicine, *B. diffusa* L plant is mentioned to cure 22 different types of ailments. In Brazilian pharmacopeia, 23 traditional uses have been described for this plant, while in Africa and Middle East the plant is prescribed for 14 ailments (Apu et al. 2012). In many other traditional medicines, root and aerial parts of this plant were reported for the treatment of diabetes, jaundice, infectious diseases and urinary problems (Patil and Bhalsing 2016).

  B. diffusa is among the 46 medicinal plant species in high trade sourced mainly from the wastelands (Ved and Goraya 2010). Beside this, the plant is an exclusive source or used

in polyherbal formulations, including Himalaya, Dibecon, Dabur Chyawanprash, etc.

  The plant possesses coccineone B, coccineone E, boeravinone C, boerhavinone D, boerhavinone F, boerhavinone G, boerhavinone H, boerhavinone I, boerhavinone J (Borelli et al. 2010), boerhavinone P, boerhavinone Q, boerhavinone R, boerhavinone S, boerhavinone M (Bairwa et al. 2013a and b), β-sitosterol (Patil and Bhalsing 2016), and eupalitin (Pandey et al. 2010).

  Phytosterols have many useful effects on human health,which has increased demand and consumption of phytosterol-enriched foods. More than 40 patents on phytosterol products and more than 15 commercial plant sterol products were incorporated in the market (Ana et al. 2013). Among 200 different types of phytosterols, β-sitosterol (65%) and stigmasterol (30%) are most widely consumed. This requires huge quantity of plant materials for extractions, which can have serious impact on the environment (Lagarda et al. 2006; Ana et al. 2013). Also, due to the effects of different environmental conditions like pollutants and pathogens, the naturally grown plants used for drug preparation can affect productivity (Nikam et al. 2009). The commercial bulk of *B. diffusa* possesses a heterogeneous population, which results in poor quality roots and biomass (Saini et al. 2011; Patil and Bhalsing 2015). Plant tissue culture technique can serve as an ideal system to circumvent the above said problems. Moreover, a uniform quality of secondary metabolites can be obtained from tissue culture technology, due to clones or less genetic variations. The β-sitosterol was isolated from tissue cultures of plants such as *Balanites aegyptiaca* (Bidawat 2006), *Citullus colocynthis* (Meena et al. 2011) and

Terminalia chebula (Archana et al. 2011). There is continued research on finding the new sources. There are few reports of tissue cultures including *in vitro* regeneration and callus induction in *B. diffusa* (Patil and Bhalsing 2015; Jain et al. 2003; Wesely et al. 2011). Srivastava et al. (1995) reported alkaloid content of *in vitro* regenerated roots of *Boerhaavia diffusa*. However, there seems no any report of isolation of secondary metabolite from *in vitro* callus culture of *B. diffusa* L. Here, we established the protocol for isolation and estimation of β-sitosterol from friable callus of *B. diffusa* L and further demonstrated the effect of different sugars on accumulation of β-sitosterol in callus cultures.

Materials and Methods

Plant material and chemicals

The plants of *B. diffusa* were obtained from the campus of North Maharashtra University, Jalgaon and used as a source of explants for experiment. The plant specimen was authenticated and deposited at the Botanical Survey of India Regional Office, Western Circle, Pune, India (specimen voucher number KSP-01). Standard β-sitosterol was purchased from Sigma Aldrich, India. All other solvents used for extraction and chromatography conditions were HPLC-grade and obtained from Fisher Scientific (PA, USA).

Surface sterilization, media preparation, establishment and maintenance of callus culture

The mature leaf explants of *B. diffusa* were thoroughly washed under running tap water for 10-15 min accompanied with 2-3 drops of Tween 20 to ensure removal of dirt. Then the explants were treated with 0.4 g/l of antifungal agent, *i.e.* bavistin (BASF, India) and washed 3-4 times with sterile distilled water. These antifungal treated explants were surface sterilized with 0.1% $HgCl_2$ for 2 min and washed 3-4 times with sterile distilled to remove any traces of $HgCl_2$. All these surface sterilization steps were carried out aseptically in laminar airflow hood (Laphosp™). MS basal medium was used for the experimental part with 3% (w/v) sucrose as carbon source and 0.8% (w/v) agar. The pH was adjusted to 5.6±0.2 using 1 N HCl or 1 N NaOH before adding 0.8% (w/v) of agar. The medium was autoclaved under 105 kPa at a temperature of 121 °C for 15 min. All growth regulators were filter-sterilized (0.22 μm, Millipore) and added after the autoclave. The above trimmed surface sterilized leaf segments of *B. diffusa* L were inoculated aseptically on this sterile MS medium, fortified with 18 μM 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 2 μM of 6-benzylaminopurine

(BAP) for callus induction. Cultures were incubated at 25±2 °C in 16/8 h photoperiod with light intensity of 2000 lux provided by white, fluorescent tubes (Philips™) and 55±5% relative humidity.

Effect of different sugars on callus induction and accumulation of β-sitosterol

Different sugars such as 3% glucose and 3% galactose were incorporated in MS medium, supplemented with 18 μM 2,4-D+2 μM BAP for callus induction and β-sitosterol accumulation. A negative control was set in the experiments, which do not contain any carbon source and 3% sucrose served as a positive control.

Determination of Growth index

The proliferated calli were subcultured at 30 days intervals on fresh culture medium. Growth index was determined after initial subculturing for 20, 45, 60, and 80 days old calli using following formula.

$$\text{Growth Index} = \frac{\text{Final fresh weight of callus} - \text{Initial fresh weight of callus}}{\text{Initial fresh weight of callus}}$$

Extraction of β-sitosterol from in vivo plant parts and callus cultures

In vivo plant parts and harvested calli were air dried and powdered. Each powdered material (2 g) was hydrolysed with 25 ml of 30% (v/v) hydrochloric acid for 4 h in a water bath at 60 °C. The hydrolysed extract was filtered and washed with distilled water through Whatman filter paper no. 1 until the filtrate reaches around pH 7. The obtained residue was dried at 60 °C for 8 h and Soxhlet extracted with benzene (200 ml) for 24 h. The benzene extracts were dried and reconstituted in chloroform and used for further analysis (Archana et al. 2011; Meena et al. 2011).

Thin layer chromatography(TLC)

The crude extracts along with a standard reference sample of sterols (β-sitosterol) were applied separately on silica gel 'G' (Merck Ltd., Mumbai, India) coated plates (20 × 20 cm; wet thickness 0.2-0.4 mm), which were activated at ~60 °C in an oven for 30 min and brought to room temperature before use. The plates were developed in organic solvent mixtures of hexane:acetone (8:2, V/V). The developed plates were air dried, sprayed with 50% sulphuric acid and subsequently heated at 100 °C for 10 min (Meena et al. 2011).
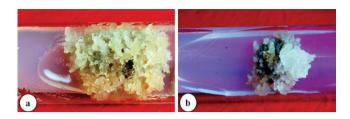
Figure 1. Callus formation on MS medium supplemented with 18 μM of 2,4-D+2 μM BAP. (a) callus obtained on 3% sucrose (b) callus obtained on 3% glucose.

Figure 2. The β-sitosterol detection in leaf derived callus extract of *Boerhaavia diffusa* L TLC plate. C=callus extract; S=standard (β-sitosterol).

HPLC analysis

Different concentrations of standard β-sitosterol were prepared and subjected to HPLC to obtain the calibration curve of β-sitosterol. The purified sample of β-sitosterol from callus tissue and *in vivo* parts of plant *viz*., leaves, root and stem were subjected to HPLC for obtaining the chromatograms. HPLC analysis was carried out on a Young Lin (S.K) Gradientcratic System provided with an autosampler (injection volume 20 μl). The separation was made on a reversed phase C18 column (Agilent Technologies, USA) (4.6 x 150 mm) of 5 μm packed size particles in conjunction with Autochro-3000 software. The HPLC was equipped with SP 930 D isocratic pump and UV 730 D detector (Young Lin, Korea). The detection wavelength was set at 220 nm. The samples were eluted at a flow rate of 0.5 ml/min using mobile phase acetonitrile:water (90:10, V/V) (mix pH 3).

Statistical analysis

Each experiment was carried out in triplicates and data from all experiments were used to calculate the mean and standard deviations in Microsoft Excel 2013.

Results and Discussion

Growth index and morphology of callus

Here, we have established the friable callus on MS medium supplemented with 18 μM of 2,4-D + 2 μM BAP (Fig. 1). A pale yellow, friable callus was obtained on MS medium supplemented with 3% sucrose. The well-proliferated calli harvested after 20, 40, 60, and 80 days showed an increase in the Growth index up to 60 days and slightly declined thereafter (Fig. 5). Similar patterns of growth with maximum growth index on 60 days old callus was observed with other sugars incorporated in MS medium. A necrosis with almost no growth was observed in negative control, which was lacking any carbon source.

Effect of different sugars on growth of the callus

The development of callus was observed in each of the sugar *viz*., 3% sucrose, 3% glucose and 3% galactose incorporated in MS medium supplemented with 18 μM of 2,4-D+2 μM BAP. However, in our study, the growth of the callus was found to be high in MS medium with 3% sucrose and 3% glucose as carbon sources as compared to MS medium containing 3% galactose (Fig. 6). A brownish callus with least growth, was observed in medium containing galactose.

Thin layer chromatography

When the developed plates were sprayed with 50% sulphuric acid, the purple colored spot appeared of the callus extracts applied, which are coincided with those of standard compound samples of β-sitosterol. Rf value (0.55) of β-sitosterol isolated from the samples equal to the Rf value of reference compound β-sitosterol (Fig. 2). Similar results were observed by Sen and co-workers (2012) by identifying β-sitosterol in leaves of *Momordica charantia*.

Effect of different ages on β-sitosterol accumulation.

HPLC chromatogram was developed for each *in vivo* plant parts and calli harvested after 20, 40, 60 and 80 days. Quantification was carried out using linear regression equation obtained from the HPLC calibration curve of standard β-sitosterol. HPLC chromatogram of standard β-sitosterol and callus (60 days old) showed the peak of β-sitosterol in Fig. 3 A-C. Among the different plant parts *viz*., roots, stems and leaves of *Boerhaavia diffusa* L, maximum content of
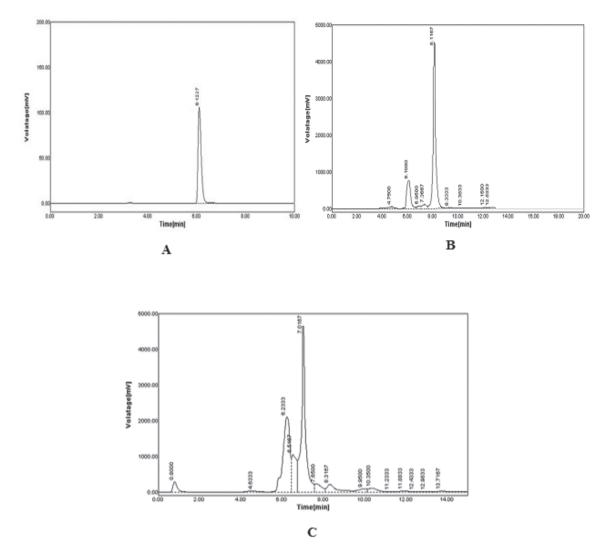
Figure 3. (A) HPLC chromatogram of standard β-sitosterol. (B) HPLC chromatogram of callus extract cultivated with 3% sucrose. (C) HPLC chromatogram of callus extract cultivated with 3% glucose.

β-sitosterol was observed in roots followed by leaves (Fig. 4). However, compared to *in vivo* leaves, the *in vitro* developed leaf derived calli (specifically 60 days old) showed higher accumulation of β-sitosterol (Fig. 4, 5). Chawla and Bansal (2014) also observed more percentage of β-sitosterol in *in vitro* regenerated bark and callus compare to *in vivo* bark of *Helicteres isora* L. They further concluded that phytohormones used for *in vitro* propagated plants, may have favorable role in accumulation of higher β-sitosterol. In our present study, the content of β-sitosterol was increased with an increase in the growth index of callus. Maximum β-sitosterol (456.01±9.2 μg/g dw) was accumulated in 60 days old callus culture, which decreased in the culture collected on 80 days (443.2±11μg/g dw) (Fig. 5). Many workers have also observed the higher percentage of β-sitosterol in

6 weeks and decrease in percentage thereafter (8 weeks) in callus tissue of *Citullus colocynthis* (Meena et al. 2011) and *Terminalia chebula* (Archana et al. 2011). They also observed more accumulation of β-sitosterol in callus culture than their *in vivo* plant parts. Plant hormones such as 2,4-D and BA was also favorably accumulated the harmala alkaloids in calli of *Tribulus terrestris* (Nikam et al. 2009). This suggest that plant growth hormones, beside growth and differentiation, may have influence on accumulation of secondary metabolites. Production of sterols from *in vivo* and *in vitro* tissue culture, their structure, composition, effects, biochemistry, identification and their function has also been reported in several plants since last few years (Clifton et al. 2004). Our study is significant in the establishment of callus as an alternative source of metabolites in *Boerhaavia diffusa* L.
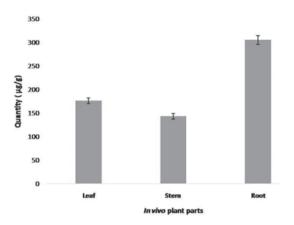
Figure 4. The β-sitosterol content in different *in vivo* plant parts of *Boerhaavia diffusa* L.

Figure 5. Effect of different ages of callus on accumulation of β-sitosterol in *Boerhaavia diffusa* L. under the influence of 18 μM of 2,4-D+2 μM BAP with 3% sucrose. Bars represent the quantity of β-sitosterol with mean±SD of three independent experiments and line represents the Growth index.

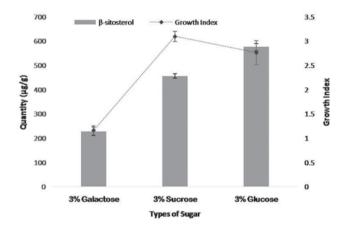### Effect of sugars on β-sitosterol accumulation

Besides the phytohormones, the types of sugar also influenced the production of β-sitosterol. In callus culture produced on MS medium containing 3% glucose as carbon source has profound effect on production of β-sitosterol. The β-sitosterol accumulated in this case was 575.27±15.0 μg/g dw, while least accumulation of β-sitosterol was observed in callus produced on MS medium containing 3% galactose (Fig. 6).

Conclusion

Present study concluded that different ages of the callus tissue have influences on the secondary metabolite production. Also, the culture conditions, including phytohormones can also affect the β-sitosterol accumulation. In the present protocol, different sugars have a profound effect on β-sitosterol productions in callus tissue. As there is continual establishment of β-sitosterol potentials in different pharmaceutical drugs, the present protocol is of considerable interest as the β-sitosterol accumulation is found to be more in *in vitro* culture than *in vivo* plant parts.

Figure 6. Effect of different sugars on accumulation of β-sitosterol in callus cultures of *Boerhaavia diffusa* L. Bars represent the quantity of β-sitosterol with mean±SD of three independent experiments and line represents the Growth index of 60 days old callus.

References

Ana BS, María AP. (2013) Use of β-cyclodextrins to enhance phytosterol production in cell suspension cultures of carrot (*Daucus carota* L.). Plant Cell Tiss Organ Cult 114 (2) doi: 10.1007/s11240-013-0320-4.

Apu AS, Liza SM, Jamaluddin ATM, Md. AH, Saha RK, Rizwan F, Nasrin N (2012) Phytochemical screening and *in vitro* bioactivities of the extracts of aerial part of *Boerhaavia diffusa* Linn. Asian Pac J Trop Biomed

2(9):673-678.

Archana S, Suchitra M, Anirudha R (2011) Quantitative estimation of β-sitosterol and stigmasterol *in vivo* and *in vitro Terminalia chebula* Ritz. Int Res J Pharm 2(3):115-116.

Bairwa K, Singh IN, Roy SK, Grover J, Srivastava A, Jachak SM (2013a) Rotenoids from *Boerhaavia diffusa* as potential anti-inflammatory agents. J Nat Prod 76:1393-1398.

Bairwa K, Singh IN, Roy SK, Grover J, Srivastava A, Jachak SM (2013b) Correction to rotenoids from *Boerhaavia diffusa* as potential anti-inflammatory agents. J Nat Prod 76:2364.

Bidawat S (2006) Evaluation of *Balanites aegyptiaca* (Hingota) an arid zone medicinal plant for phytochemically important metabolites. Ph.D Thesis, M.D.S. University, Ajmer, India.

Borrelli F, Ascione V, Capasso R, Izzo AA, Fattorusso E, Taglialatela-Scafati O (2006) Spasmolytic effects of nonprenylatedrotenoid constituents of *Boerhaavia diffusa* roots. J Nat Prod 69(6):903-906.

Chawla S, Bansal YK (2014) Qualitative and quantitative estimation of β-sitosterol from *in vitro* regenerated stem bark and callus of *Helicteres isora* L. Asian J Pharm Clin Res 7(3):178-180.

Chopra GL (1969) Angiosperms: Systematics and Life Cycle. S.Nagin&Co., Jalandhar, Punjab, India, pp:361-365.

Clifton PM, Noakes M, Ross D, Fassoulakis A, Cehun M, Nestel P (2004) High dietary intake of phytosterols esters decreases carotenoids and increase plasma sterol level with no additional cholesterol lowering. J Lipid Res 45:1493-1495.

Jain V, Singh D, Saraf S (2003) *In vitro* culture studies for callus and root generation of *Boerhaavia diffusa* Linn. Ancient Sci Life XXII (4):134-139.

Lagarda MJ, García-Llatas G, Farre R (2006) Analysis of phytosterols in foods. J Pharmaceut Biomed Anal 41:1486-1496.

Marimuthu M, Gurumoorthi P (2013) Phytochemical screening and FT-IR studies on wild and common South Indian legumes. Asian J Pharm Clin Res 6(2):141-144.

Meena MK, Meena MC, Patni V (2011) Isolation, identification and quantification of ß-sitosterol from *in vivo* and *in vitro* samples of *Citrullus colocynthis* (Linn.) Schrad J Pharm Res 4(12):4515-4517.

Murashige T, Skoog FA (1962) Revised medium for rapid growth and bioassays with Tobacco tissue culture. Physiol Plant 15:473-479.

Nikam TD, Mohammad Ali Ebrahimi, Patil VA (2009) Embryogenic callus culture of *Tribulus terrestris* L. a potential source of harmaline, harmine and diosgenin. Plant Biotech Rep 3:243-250.

Pandey R, Maurya R, Singh G, Sathiamoorthy B, Naik S (2005) Immunosuppressive properties of flavonoids isolated from *Boerhaavia diffusa* (Linn). Int Immunopharmacol 5(3):541-553.

Patil KS, Bhalsing SR (2015) Efficient micropropagation and assessment of genetic fidelity of *Boerhaavia diffusa* L- High trade medicinal plant. Physiol Mol Biol Plants 21(3):425-432.

Patil KS, Bhalsing SR (2016) Ethnomedicinal uses, phytochemistry and pharmacological properties of the genus *Boerhaavia*. J Ethnopharm182:200-220.

Saini R, Sharma M, Sharma A, Batra A (2011) Impact of phytohormones in micropropagation of medicinally potent plant: *Boerhaavia diffusa* L. Int J Pharm Sci Rev Res 8(1):85-88.

Sen A, Dhavan P, Shukla KK, Singh S, Tejovathi G (2012) Analysis of IR, NMR and antimicrobial activity of β-sitosterol isolated from *Momordica charantia*. Sci Sec J Biotechnol 1(1):9-13.

Sharma S, Meena S, Rishi A (2011) Quantification of β-sitosterol and stigmasterol in vivo and in vitro *Terminalia belerica*. Agric Biol J North Amer 1(5):827-833.

Srivastava N, Padhya MA (1995) 'Punarnavine' profile in the regenerated roots of *Boerhaavia diffusa* L. from leaf segments. Curr Sci 68:653-656.

Sudarshana MS, Niranjan MH, Girisha ST (2008) *In vitro* flowering, somatic embryogenesis and regeneration in *Boerhaavia diffusa Linn*.-A medicinal plant. Global J Biotech Biochem 3(2):83-86.

Ved DK, Goraya GS (2007) Demand and supply of medicinal plants in India. NMPB, New Delhi&FRLHT, Bangalore, India, pp:15.

Wesely EG, Johnson M, Regha P, Kavitha MS (2010) *In vitro* propagation of *Boerhaavia diffusa* L through direct and indirect organogenesis. J Chem Pharm Res 2(5):339-347.