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Elicitation of Bacoside Content Using Plant Growth Regulators in Cell Suspension Culture of *Bacopa monnieri* (L.) Wettst.

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Abstract

Cell suspension culture is used for the optimization of plant growth regulators used for the elicitation of bacoside content of *Bacopa monnieri* (L.) Wettst. Callus induction was carried out on MS supplemented with 2, 4-D (0.5 mg/l) by using leaf explants. For suspension culture MS liquid medium was supplemented with constant α-naphthalene acetic acid (0.5 mg/l) and casein hydrolysate (1 g/l) as well as the varied concentrations of 6-benzyleadenine, kinetin, thidiazuaron and chitosan. It was observed that bacoside production was increased by 6-benzyleadenine (0.5 mg/l), Kn (0.5 mg/l) and thidiazuaron (0.25 mg/l) was 3.07, 3.79 and 3.97 mg/g, respectively. This indicates that lower concentration of growth regulator favor the bacoside enhancement under *in vitro* conditions, while chitosan affects the bacoside production in suspension culture. In this study thidiazuaron at 0.25 mg/l induces maximum elicitation of bacoside content i.e. 3.97 mg/g.

Introduction

Bacopa monnieri L. Wettst is a medicinally important plant belongs to the family Plantaginaceae commonly known as Brahmi, found across the Indian subcontinent (Praveen et al. 2009). It grows in the humid climate, mainly distributed in damp and marshy tracts in the subtropical region of the Indian subcontinent. It requires a well drained, moist, sandy loam soil, rich in organic matter and grows well at a temperature from 30 to 40°C (Talukdar 2014). It is

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used as neuropharmacological drug against insomnia, insanity, depression, psychosis, epilepsy and stress (Elangovan et al. 1995, Tripathi et al. 1996 and Russo and Borrelli 2005). Brahmi is also used as brain tonic and blood purifier (Kala et al. 2006). The active ingredient found in *Bacopa monnieri* is triterpenoid saponin called bacoside, which is a complex mixture of glycosides of jujubogenin and pseudojujubogenin (Naik et al. 2010).

Brahmi has a good market demand due to its medicinal properties (Pravina et al. 2007). Estimated consumption of this drug in India is 1000 tons per year (Tripathi et al. 2012). More than 90% of plant material used by the industry is collected from the wild sources of which 70% involves unorganized harvesting. Natural regeneration requires specific habitat and also hampered by death of plant at two leaf stage (Chaplot et al. 2005). These factors possess a serious threat to the genetic stock and the biodiversity of medicinal plant. Due to the high demand and short supply of *Bacopa monnieri*, it is mostly adulterated species in Ayurvedic formulations. *Bacopa monnieri* is one of the important plant species in 32 prioritized medicinal plants for research and development by NMPB (Kala et al. 2006). The National Medicinal Plant Board (NMPB), Government of India and Technology Information Forecasting and Assessment Council (TIFAC) recommended immediate attention to seven medicinal plants, among which *Bacopa monnieri* prominently features and included in the category of highly endangered medicinal plants in India (Tripathi et al. 2012). Recent reports showed that there are variations in the bacoside content in the different parts of Brahmi plant and also the accessions collected from the various geographical locations (Naik et al. 2012), as well as there are seasonal variations in the bacoside content (Jain et al. 2013). Due to these reasons, there is an urgent need to find out the alternatives for the production of bacoside content of Brahmi to meet the huge market demands.

In present scenario plant cell culture is an attractive alternative for the production of secondary metabolites (Rao and Ravishankar 2002). Suspension culture offers an efficient mechanism for the enhancement of secondary metabolite production. It facilitates the incorporation of precursors in the media which provides the easy uptake of the nutrients by submerged cells than the other methods. It is also easy to harvest and scaling up the production of bioactive components in suspension culture. The possibilities of secondary metabolite production through plant cell culture have been demonstrated by various researchers (Alfermann et al. 1995, Fowler and Scragg 1998). Rahman et al. (2002) established cell suspension culture of *Bacopa monnieri* for first time, while Jain et al. (2013) reported enhancement of bacoside content in cell suspension culture in *B. monnieri*. Various strategies have been reported for the

enhancement of secondary metabolite as well as biomass accumulation under *in vitro* conditions such as optimization of the medium and culture environment, selection of high secondary metabolite producing cell lines, use of organic supplements, elicitors and precursors in the medium (Parale et al. 2010 and Murthy et al. 2014). In plants secondary metabolite production influenced by various biotic and abiotic factors are called as 'elicitors'. Elicitation is a complex process and depends on many factors such as elicitor concentration, growth stage of the culture at the time of elicitor addition and exposure time with the elicitor (Sharma et al. 2014). Plant growth regulators are used as abiotic elicitors and play an important role in the enhancement of biomass and bacoside content under *in vitro* conditions.

Chaplot et al. (2005) reported the positive interactions of cytokinin (BA) and auxin (IAA) in Brahmi on biomass and bacoside production under *in vitro* conditions. Praveen et al. (2009) reported the use of various plant growth regulators viz. 6-benzyleadenine, Kn and thidiazuaron and Kamonwannasit et al. (2008) used 6-benzyleadenine, thidiazuron and chitosan for the enhancement of biomass and bacoside content in Brahmi.

Hence the present investigation is carried out with major objective to study the effect of plant growth regulators on the elicitation of bacoside content in Brahmi in cell suspension culture.

Materials and Methods

Bacopa monnieri (L.) Wettst. plants were collected from Paithan (19°28´33.9852´´N and 75°22´45.0948´´E), Aurangabad district in Maharashtra (India). Young leaf explants were selected from the healthy plantlets grown in the nursery for the experiment (Shrivastava and Rajani 1999). Excised leaf samples were washed and surface sterilized with 70% ethanol for 2 - 3 sec then rinsed 3 - 4 times with sterile distilled water under aseptic conditions. Further antifungal treatment of 0.1 % (w/v) mercuric chloride was given for five min (Parale et al. 2010) followed by 4 - 5 repeated washing with sterile distilled water. The surface sterilized leaf explants were incised to form wound using sterile surgical blades and used for the further experiment.

Surface sterilized leaf explants were inoculated on MS supplemented with 2, 4-D (0.5 mg/l) (Jain et al. 2013) in the glass culture tubes (25 × 150 mm, Borosil). The pH of the medium was adjusted to 5.8 before autoclaving. Then cultures were incubated at 25 ± 2°C with 16 hrs light and 8 hrs dark cycles, respectively. Induced callus was repeatedly sub cultured at every 21 days interval to produce friable callus. Friable callus was transferred to MS liquid medium (Bilore et al. 2016) supplemented with 2, 4-D (0.5 mg/l) to establish the suspension culture.

Fine suspension culture was established by repeated sub culturing with continuous agitation. Suspension cultures were maintained in 50 ml medium of 250 ml conical flask and kept at 110 rpm in dark conditions at 25 ± 2°C on rotary incubating shaker (Rahaman et al. 2002 and Jain et al. 2013).

Media modifications were done with the aim to elicit bacoside production in cell suspension culture by using plant growth regulators as abiotic elicitors. The MS was supplemented with α-naphthalene acetic acid (0.5 mg/l) (Kamonwannasit et al. 2008) and casein hydrolysate (1 g/l) (Rahaman et al. 2002 and Jain et al. 2013) as constant, while the varied concentrations of elicitors such as 6-benzyleadenine (0, 0.5, 1.0, 1.5 and 2.0 mg/l), Kn (0, 0.5, 1.0, 1.5 and 2.0 mg/l), thidiazuaron (0, 0.25, 0.5, 0.75 and 1.0 mg/l) and chitosan (0, 25, 50, 75 and 100 mg/l). The pH of the suspension culture medium was adjusted to 6.0 before autoclaving (Naik et al. 2010). Suspension cultures of 6-benzyladenine and kinetin were incubated for 21 days, while chitosan and thidiazuaron for 7 days (Kamonwannasit et al. 2008).

After completion of incubation period cell suspensions were filtered by using Whatman filter paper (Grade No.1) to harvest the cells biomass from the culture. Filtered cells were washed with double distilled water to remove the traces of culture medium and the fresh cell weight (FCW) was recorded. The cells were dried in hot air oven at 60°C to achieve a constant dry cell weight (DCW). Dried cells were crushed and powdered with mortar and pestle. Powdered samples were soaked with water for 24 hrs and squeezed out, followed by extraction with 95% (v/v) ethanol (Phrompittayarat et al. 2007). Standard curve was plotted with known quantities of standard bacoside (Sigma Aldrich®) and detected at 278 nm using UV spectrophotometer (Saini et al. 2012). The samples were similarly treated and quantified by using standard curve.

All the experiments were arranged in completely randomized design. Each experiment consists of five treatments of different concentrations of plant growth regulators with four replications of each treatment (Panse and Sukhathme 1967). The results were analyzed by WASP 1.0 (www.ccari.res.in), using analysis of variance at $p = 0.05$.

Results and Discussion

Bacopa monnieri leaf explants were injured and cultured on MS supplemented with 2, 4-D (0.5 mg/l); were observed for the callus induction. A week after inoculation swelling was observed at the cuts and leaf attachment site followed by the callus initiation (Fig. 1a) and further covered the whole leaf explants (Fig. 1b). Similar pattern of callus initiation was reported in *Arabidopsis thaliana* (Ikeuchi et al. 2013), *Bacopa monnieri* (Kharde et al. 2017) and *Celastrus paniculatus*

(Bilore et al. 2016). Callus proliferation was initiated due to the wound tissue produced in response to injury, by the progression of the mitotic cell cycle and the reacquisition of the cell proliferation ability of the differentiated plant cells (Ikeuchi et al. 2013). Friable callus was produced by repeated subcultures (Fig. 1c) and used for the development of suspension culture.

Fig. 1. Callus culture of *Bacopa monnieri.* (a) callus initiation from leaf, (b) proliferated callus after 4 weeks and (c) friable callus at the outer surface.

Present study on cell suspension culture of *Bacopa monnieri* was the first report in relation to the changes in color of the culture media, cell shape, biomass production and elicitation of bacoside content using plant growth regulators. Suspension cultures showed color changes from light white to pale yellow followed by yellowish brown at 7, 15 and 21 days after incubation, respectively (Fig. 2a-c). Similar type of color changes in cell suspension culture was reported by Ali et al. (2013) in *Artemisia absinthium* at different growth stages of cell culture, these changes in suspension might be due to the changes in pH of the medium, secretion of the phenols and other compounds, increased cell density

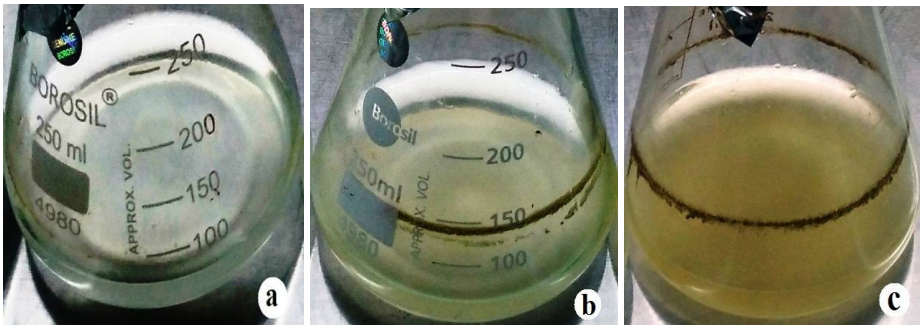

Fig. 2. Suspension culture of *B. monnieri* by suspending friable callus in MS liquid medium supplemented with 2, 4-D (0.5 mg/l) on rotary incubating shaker at 110 rpm in dark at 25 ± 2°C, (a) Cell suspension 7 days after sub culturing, (b) 15 days after subculturing and (c) 21 days after subculturing.

and inadequate nutrients availability and due to the oxidation (Mustafa et al. 2011). In the present investigation the morphological variations were also observed in the *Bacopa monnieri* cells at different growth periods. In which, early stages of culture the clumped cells showed round cells, while elongated in later stages (Fig. 3a-c), such results were observed by Maraschin et al. (2002) in *Mandevilla velutina* cell culture and reported that the meristematic cells showed typical round shape in initial growth phases, whereas elongated cells in exponential phases.

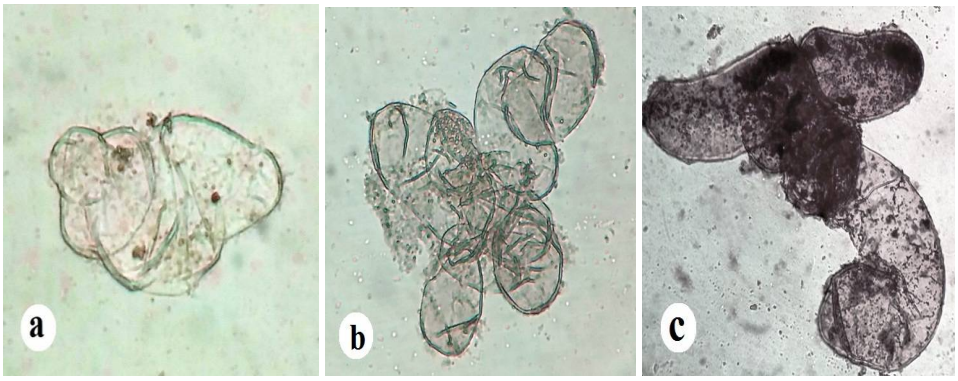

Fig. 3. Microscopic observations of *B. monnieri* cells in suspension culture (a-b), round cells in 7, 15 days and (c) elongated cells in 21 days after subculturing.

Cell biomass was influenced by the different concentrations of plant growth regulators in suspension culture. Present investigation reported enhancement in biomass of the cells i.e. 6-benzyleadenine at 0.5 mg/l, kinetin at 1.5 mg/l, thidiazuaron at 0.75 mg/l and chitosan at 100 mg/l produces significantly higher dry cell weight i.e. 0.56, 0.32, 0.54 and 0.47 g/l, respectively (Fig. 4a-d). Previous reports by Praveen et al. (2009) showed the enhancement production of biomass as well as bacoside in *in vitro* regenerated shoots of Brahmi using different growth regulators in liquid medium over solid media. Bacoside elicitation was successfully achieved at lower concentrations of the growth regulators i.e. 6-benzyleadenine and Kn at 0.5 mg/l and thidiazuaron at 0.25 mg/l produces 3.07, 3.79 and 3.97 mg/g on dry weight basis, respectively (Fig. 4a-c) and significantly higher than the other levels of these growth regulators. While chitosan showed decrease in bacoside contents in suspension culture (Fig. 4d), Kamonwannasit et al. (2008) observed increased in the contents of bacoside in micropropagated plants. This might be due to the interactive effects of chitosan, NAA, caesin hydrolysate, pH and type of the culture used.

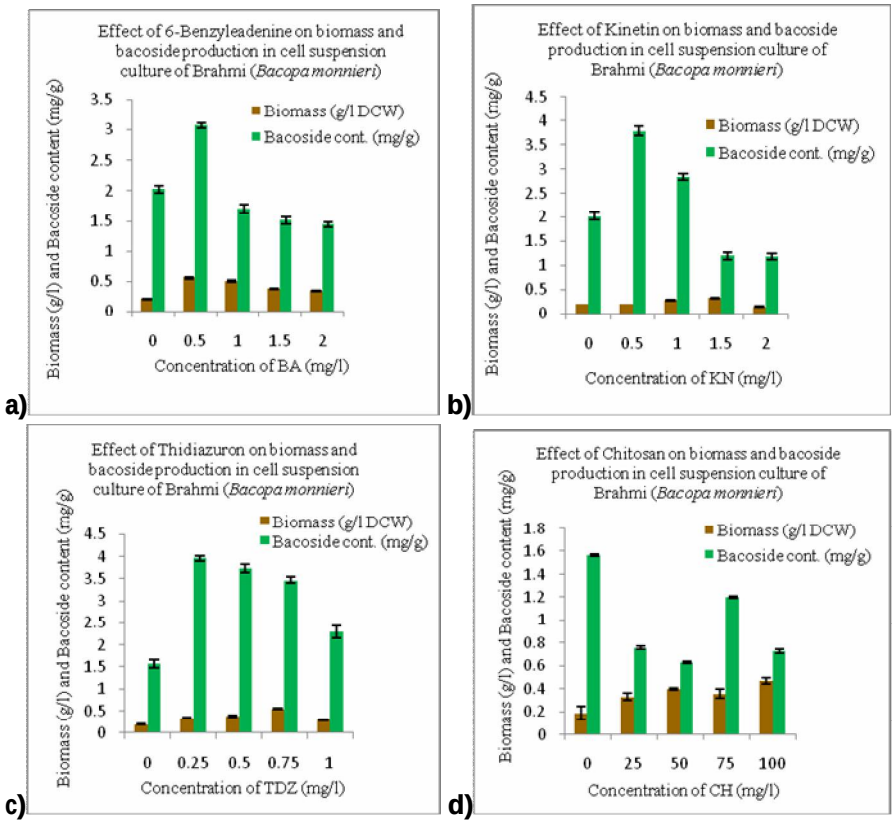

Fig. 4. Effect of 6-benzyleadenine (a), Kn (b), thidiazuaron (c) and chitosan (d) on biomass and bacoside production in Brahmi.

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