Influence of organic supplements on production of shoot and callus biomass and accumulation of bacoside in *Bacopa monniera* (L.) Pennell.

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ABSTRACT

Production of valuable secondary metabolites through plant cell or organ culture is the best suited alternative to extraction of whole plant material and to increase production of secondary metabolites in *in-vitro* systems, feeding precursor or intermediate metabolites is an obvious and popular approach. The present investigation was aimed to study the influence of feeding of organic supplements, glycine (0-125 μ M), ferulic acid (0-200 μ M), phenylalanine (0-200 μ M), α -ketoglutaric acid (0-200 μ M) and pyruvic acid (0-200 μ M) on production of bacoside-A (a triterpenoid type secondary metabolite responsible for cognition effects) in shoot and callus biomass of *Bacopa monniera* (L.) Pennell. The shoots were raised in liquid Murashige and Skoog's (MS) medium fortified with 5 μ M 6-benzyladenine (BA) and callus biomass on agar solidified MS medium containing 1 μ M 2,4-dichlorophenoxyacetic acid (2,4 -D) in conjunction with 5 μ M 1-napthaleneacetic acid (NAA). Among the organic supplements used, 100 μ M pyruvic acid effectively enhanced the production of bacoside-A in shoot as well as callus biomass. The bacoside-A content in *in-vitro* raised shoot biomass was 4.0 and 1.2 times higher as compared to control and shoot biomass of naturally grown plants respectively. Inclusion of pyruvic acid in MS medium for *in-vitro* shoot cultures of *B. monniera*, can be adapted for enhanced production of bacoside-A. [Physiol. Mol. Biol. Plants 2010; 16(2) : 167-175]
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INTRODUCTION

Bacopa monniera (L.) Pennell (Brahmi, Scrophulariaceae) is a creeper widely distributed in the warmer parts of Asia, Australia and America (Rahman *et al.*, 2002). It is a source of bacosides - the triterpene glycosides (saponins) - responsible for the memory enhancing property that has resulted in extensive use of this plant in several commercial preparations and placed the plant in high demand (Rahman *et al.*, 2002). *Bacopa*'s cognitive effect is attributed to a triterpenoid saponin glycoside, bacoside-A. The saponin, bacoside-B generally co-occurs with bacoside-A and is considered as an artefact produced during the process of isolating bacoside-A (Rastogi, 1990) or probably, it is an optical

isomer of bacoside-A (Mathur *et al.*, 2002). In addition, other secondary metabolites possess anti-inflammatory, analgesic, antipyretic, anti-epilepsy, anti-insanity, anticancer and antioxidant activities (Binita *et al.*, 2005). It is also used in the treatment of asthma, hoarseness, water retention, and blood purification. Moreover, leaf juice of Brahmi is given to children for relief in bronchitis and diarrhea (Binita *et al.*, 2005).

Propagation of this species through seed is unreliable due to poor seed setting, low seed viability and frequent seedling death at two leaf stage. However, it can be easily propagated through stem cuttings. Since, the natural populations of this species are found in marshy places, water content in the plant biomass is very high and dry biomass produced is therefore insufficient for utilisation. Moreover, the biomass generated through cultivated populations is also insufficient. Though protocols for organogenesis, somatic embryogenesis

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(Shrivastava *et al.*, 1999; Tiwari *et al.*, 1998, 2000, 2001; Binita *et al.*, 2005), cell cultures (Rahman *et al.*, 2002) and shoot cultures (Praveen *et al.*, 2009) producing bacoside have been reported; procedures for enhanced production of bacoside are inadequately explored in this species.

Secondary metabolites have physiological effects on animals and in many cases, they have pharmacological importance. Chemical synthesis of many secondary metabolites is not possible due to their complex structure. Therefore, the plant species that are sources of these metabolites are intensely exploited for commercial reasons, which puts pressure on the natural populations, threatens the survival of species and such species become rare and endangered. Therefore, several studies were carried out on different classes of secondary metabolites with a view to enhance their production in the intact plants as well as in cultured tissues. (Such studies form the basis for commercial exploitation of *in-vitro* systems). Abiotic and biotic stresses, variations in plant growth regulators, incorporation of metabolic intermediates, and nutritional alterations can intensify the production of secondary metabolites (Mulabagal *et al.*, 2004). Glycine, phenylalanine, ferulic acid, α -ketoglutaric acid and pyruvic acid are cheaper organic supplements and their incorporation in the culture media has resulted in improved production/accumulation of different types of secondary metabolites like diterpen alkaloids (Fett-Neto *et al.*, 1994; Palenius and Alejo, 2005); anthocyanin pigments (Piovan and Raffaella, 2007); steroids (El-Sharabasy, 2004) and anthraquinone (Zenk *et al.*, 1975).

Triterpenoids are biosynthesized from acetyl CoA or glycolytic intermediates through mevalonic acid pathway (MAP) or methylerythritol phosphate (MEP) pathway (Taiz and Zeiger, 2006). Though bacosides are triterpenoids, the route of their biosynthesis in *B. monniera* is not known and chemical synthesis has not become feasible so far. The species *monniera* under the genus *Bacopa* is the only major source of bacosides. Overexploitation of this species by pharmaceutical companies has resulted in a rapid exhaustion of the wild stock to such an extent that it is included in the list of endangered rare species (Pandey *et al.*, 1993).

Incorporation of intermediates and precursors in the nutrient medium is one of the best strategies to enhance the production of secondary metabolites. Such study is a prerequisite for optimal biomass production as well as accumulation of bacoside-A in *B. monniera*. Therefore in this study we investigated the effect of

some organic supplements viz., glycine, phenylalanine, ferulic acid, α -ketoglutaric acid and pyruvic acid through nutrient medium on the production of shoot and callus biomass and accumulation of bacoside-A in *B. monniera*.

MATERIALS AND METHODS

Plant material and culture conditions

The healthy *B. monniera* plants procured from 'Jawaharlal Nehru Centre for Medicinal and Aromatic Plant' and authenticated by the Botanical Survey of India, Regional Office, Western Circle, Pune- 411 007, were used as source of explant.

The leaf explants from healthy plants were excised and washed five times with sterile distilled water; surface sterilised for five minutes with 0.1 % (w/v) aqueous solution of mercuric chloride (HgCl_2) and rinsed five times with sterile distilled water. The disinfected leaves (0.75 cm^2) were inoculated in 300 ml screw-capped glass bottles containing 30 ml MS medium (Murashige and Skoog, 1962).

The type of plant growth regulator and its concentration and the nature of medium (liquid or semisolid) used was based on preliminary experiments and earlier studies (Parale and Nikam, 2009). Liquid MS medium supplemented with $5 \mu\text{M}$ 6-benzylaminopurine (BA) was used for inducing shoot regeneration and multiplication of shoots, whereas MS medium gelled with 0.8 % agar and fortified with $5 \mu\text{M}$ naphthaleneacetic acid (NAA) + $1 \mu\text{M}$ 2,4-Dichlorophenoxyacetic acid (2,4-D) was used for callus induction and proliferation. The pH of the medium was adjusted to 5.8 with 1 N sodium hydroxide (NaOH) or 1 N hydrochloric acid (HCl) before it was autoclaved at 121°C and 104 KPa for 15 min.

All the cultures were incubated at $25 \pm 2^\circ\text{C}$ and 60–70 % relative humidity. The cultures were illuminated for 8 h photoperiod with $40 \mu\text{mol m}^{-2} \text{ s}^{-1}$ light from white cool florescent tubes (Champion 40 W, Philips Electronics Ltd., India). The shoots as well as callus were subcultured at the interval of 28 days in 300 ml bottle containing 30 ml fresh parental medium.

Production of shoot and callus biomass in media supplemented with organic supplements

The MS basal liquid medium supplemented with $5 \mu\text{M}$ BA, and MS medium gelled with 0.8 % agar and fortified with $5 \mu\text{M}$ NAA + $1 \mu\text{M}$ 2,4-D was used as control for shoot culture and callus culture respectively.

These media were supplemented either with 0–125 μM glycine or 0–200 μM of phenylalanine, ferulic acid, α -ketoglutaric acid, and pyruvic acid separately. The shoot and callus biomass was separated from the medium after four weeks from initiation of cultures. Growth of shoots and callus was expressed in terms of dry weight that was determined by oven drying the biomass at 60 $^{\circ}\text{C}$ till constant weight was obtained.

Quantification of Bacoside-A

Bacoside-A was extracted as per the modified procedure of Watoo *et al.* (2007). In brief, finely powdered dry biomass (1 g) was soaked in 4 ml distilled water for 2 h. The residual biomass was extracted thrice with 20 ml of 95 % (v/v) ethanol. The extracts were pooled, filtered and evaporated under vacuum. For HPTLC analysis, 1 mg dried residue was dissolved in 1 ml HPLC grade methanol. By using automatic CAMAG sample applicator (Anchrom Enterprises, Mumbai, India), 10 μl extract was loaded on pre-coated silica gel TLC 60F254 plate (Merck, India). The mobile phase of toluene: ethanol: methanol: glacial acetic acid (4:3:3:1, v/v) was used for the development of chromatogram. The TLC plate was dried and sprayed with 5 % methanolic sulphuric acid. The densitometric scanning at 366 nm was done on CAMAG TLC Scanner-III. Bacoside-A peak from the sample was confirmed by spiking 5 μg standard bacoside-A. The peak areas of sample and standard were compared to quantify bacoside-A in the sample (Anonymous, 1998; Prakash *et al.*, 2008).

Statistical analysis

All the experiments were repeated at least three times, with 20 replicates and five explants per replicate. One hundred explants were allotted to each treatment. Results on biomass production and accumulation of bacoside-A were subjected to ANOVA ($P=0.05$). Differences among means were weighed with Duncan's Multiple Range Test (DMRT) at $P=0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

The active principle in *B. monniera* is bacoside-A which is characterized as triterpenoid saponin (Chatterji *et al.*, 1965; Basu *et al.*, 1967). Though biosynthesis of triterpenoids occur through mevalonic acid pathway (MAP) or methylerythritol phosphate (MEP) pathway, very little is known about the enzymes and biochemical pathways involved in biosynthesis of saponins. Moreover, the genetic machinery required for the

elaboration of this important family of plant secondary metabolites is yet largely uncharacterised, despite the considerable commercial interest in this group of natural products. This is likely to be due to the complexity of the molecules and unknown pathway intermediates for biochemical studies (Haralampidis *et al.*, 2002).

The amino acid glycine is commonly used in plant tissue culture media possibly due to its role in transamination reaction for the synthesis of other amino acids (Salisbury and Ross, 2007) and its easier availability at an affordable cost. The MS medium contains about 27 μM glycine. In the present investigation, non incorporation of glycine in the MS medium slightly affected the caulogenic as well as callogenic response of the explant as compared to observed on MS medium containing 25 μM glycine (Fig. 1a, 3a). Increasing the glycine level up to 100 μM improved production of shoot as well as callus biomass on dry weight basis (Fig. 1a, 3a) and maximum of 13.4 % and 10 % higher shoot and callus biomass was obtained on such medium as compared to MS medium lacking glycine. At higher concentration (125 μM), the biomass production was reduced but was still higher than the control. However, inclusion of 75 μM glycine was ideal for enhancing accumulation of bacoside-A that was 1.1 and 1.9 times higher in shoot and callus biomass respectively (Fig. 2a, 4a). The enhancement in bacoside-A content can be correlated to the involvement of glycine in biosynthesis of terpenes in the fungus *Cochiobolus miyabeanus* (Bose *et al.*, 1971). At higher concentration ($>100 \mu\text{M}$), glycine appeared to inhibit bacoside-A accumulation (Fig. 2a, 4a). This may be due to increase in the concentrations of intermediates regulating the pathway via feedback control mechanisms at high glycine levels.

L-Phenylalanine is an initial compound of anthocyanin biosynthesis via phenylpropanoid and flavonoid metabolic pathways. Inclusion of L-phenylalanine in the medium has induced accumulation of anthocyanin in *Catharanthus roseus* (Piovan and Raffaella, 2007) and *Vitis* cell cultures (Kakegawa *et al.*, 1995). In the present study, incorporation of phenylalanine at low concentration inhibited production of shoot and callus biomass. With increase in the level of phenylalanine, a gradual decline in the production of shoot and callus biomass was observed, and at highest concentration (200 μM) used, the reduction was 43 % and 25 % respectively (Fig. 1b, 2b). Similar observation was reported by Bauer *et al.* (2004), where suppression of *Coleus blumei* transformed callus growth was observed on medium containing 1mg/l phenylalanine.

Fig. 1. Effect of glycine (a), phenylalanine (b), ferulic acid (c), α -ketoglutaric acid (d) and pyruvic acid (e) on shoot biomass production.

Fig. 2. Effect of glycine (a), phenylalanine (b), ferulic acid (c), α -ketoglutaric acid (d) and pyruvic acid (e) on bacoside-A accumulation in shoot biomass.

However, increasing levels of phenylalanine in the medium improved accumulation of bacoside-A. The shoot biomass grown on MS medium containing 100 μM phenylalanine accumulated 14.86 ± 0.2 mg bacoside-A per gram of dry biomass which was 1.7 times higher than that of the control. In case of callus, the accumulation of bacoside-A was 2.8 times more as compared to control (Fig. 2b, 4b). The results obtained in the present study are in agreement with the previous reports on *in-vitro* production of other phytochemicals (Boitel-Conti *et al.*, 2000; Premjet *et al.*, 2002; Ionkova *et al.*, 2007). Fortification with phenylalanine has found to be effective for inducing or increasing the production of different secondary metabolites. Addition of phenylalanine to *Salvia officinalis* cell suspension cultures stimulated the production of rosmarinic acid (Ellis and Towers, 1970). It was found to assist taxol production in *Taxus cuspidate* cultures (Fett-Neto *et al.*, 1994), significantly increased yield of capsaicin in immobilized as well as freely suspended cells of *Capsicum frutescens* (Lindsey and Yeoman, 1984) and cell culture of *Capsicum annum* (Palenius and Alejo, 2005). Feeding of phenylalanine in cell suspension cultures of *Linum flavum* has resulted in a 3–5-fold increase in 5-methoxypodophyllotoxin levels (Uden *et al.*, 1990). Thus, results of the present investigation

and earlier reports are indicative of direct or indirect participation of phenylalanine in the biosynthesis of secondary metabolites.

Ferulic acid exists as secondary metabolite in many plant species and is classified as monophenolic acid. At low concentrations, it inhibits enzymatic oxidation of IAA and this result in cell elongation and cell division and subsequent plant growth and development (Volpert *et al.*, 1995; Arnaldos *et al.*, 2001). In the present study, fortification of MS medium with ferulic acid (25–150 μM) had positive effect on culture growth as well as accumulation of bacoside-A. At 100 μM , it improved shoot biomass production by about 29.6 % (Fig. 1c, 5a) and accumulated 1.4 times more bacoside-A than control (Fig. 2c), whereas the production of callus biomass was 25.6 % higher (Fig. 3c, 5b) and the callus contained 2.7 times more bacoside-A (Fig. 4c). On the same line, *Vanilla planifolia* cultures treated with ferulic acid accumulated more vanillin (Romagnoli *et al.*, 1988) and treatment of ferulic acid to the immobilized cell cultures of *Capsicum frutescens* resulted in maximum production (315 $\mu\text{g}/\text{culture}$) of vanillin (Sudhaker *et al.*, 1991). However, there was not much difference in capsaicinoid content in *Capsicum annum* cell cultures treated with ferulic acid (Palenius and Alejo, 2005).

Fig. 3. Effect of glycine (a), phenylalanine (b), ferulic acid (c), α -ketoglutaric acid (d) and pyruvic acid (e) on callus biomass production.

α -ketoglutaric acid is an intermediate of shikimic acid pathway. Enhancement in anthraquinone biosynthesis in α -ketoglutaric acid treated *Morinda citrifolia* cells has been reported by Zenk et al. (1975). In case of *Rubia cordifolia* callus culture (Shin and Chi, 1989), α -ketoglutaric acid enhanced the total pigment biosynthesis. In the present study, incorporation of α -ketoglutaric acid at low concentration was ineffectual for production of shoot and callus biomass. A significant decline in the shoot and callus biomass was observed at higher levels of α -ketoglutaric acid (Fig. 1d, 3d). Compared to control, the bacoside-A accumulation in shoot biomass grown in the presence of 25 and 50 μ M α -ketoglutaric acid was not significantly different (Fig. 2d). At higher level (100 μ M), 1.3 times more accumulation of bacoside-A was observed. Likewise, bacoside A accumulation in the callus biomass grown in the presence of 100 μ M α -ketoglutaric acid was 3.3 times more as compared to the control (Fig. 4d).

Pyruvic acid, also known as 2-oxopropanoic acid, α -ketopropionic acid or acetylformic acid, is the most important α -oxocarboxylic acid. It plays a central role

in energy metabolism of organisms. It has been reported as a precursor for biosynthesis of steroids, a major group of terpenoid (El-Sharabasy, 2004). In the present study, pyruvic acid was the most effective organic supplement for accumulation of bacoside-A. Inclusion of 25–75 μ M pyruvic acid in the culture medium gradually improved production of shoot biomass as well as accumulation of bacoside-A (Fig. 1e, 2e). The shoots obtained on MS medium containing 100 μ M pyruvic acid accumulated maximum bacoside-A (35.2 ± 1.4 mg/g D.W.) which was 4.0 times more than the control. Likewise, the callus biomass on the MS medium containing 100 μ M pyruvic acid produced 3.8 times more bacoside-A (12.8 ± 0.5 mg/g D.W.) than the control (Fig. 4e). A stimulatory effect of pyruvic acid on steroid production has been reported by El-Sharabasy (2004) wherein incorporation of 0.01 mg/l pyruvic acid in culture medium was best for steroid production in date palm.

Synthesis and accumulation of a specific compound in *in-vitro* cultures depend on several factors like state of cell differentiation, organized or unorganized nature of cell biomass, composition of the culture medium, environmental conditions, and presence of precursor and

Fig. 4. Effect of glycine (a), phenylalanine (b), ferulic acid (c), α -ketoglutaric acid (d) and pyruvic acid (e) on bacoside-A accumulation in callus biomass.

Fig. 5. *In-vitro* biomass production in *Bacopa monniera*. (a) Multiple shoot culture in liquid MS medium containing 5 µM BA and 100 µM ferulic acid. (b) Callus culture on MS medium fortified with 5 µM NAA + 1 µM 2,4-D + 100 µM ferulic acid

intermediates at an appropriate level in the culture medium (Palenius and Alejo, 2005). Present investigation indicates that the selected organic supplements influenced the production of biomass and bacoside-A accumulation in organized as well as unorganized cultures of *B. monniera* (Fig. 6a). The intact plants (field grown) of *B. monniera* possessed 28.7 ± 1.0 mg/g D.W. bacoside-A. Compared to this, an impressive increase in bacoside-A accumulation was observed in *in-vitro* shoot biomass obtained on nutrient media fortified with 100 µM pyruvic acid (Fig. 6 b).

CONCLUSION

In the light of memory boosting property of bacoside-A, *B. monniera* is in great market demand, which has consequently lead to its rapid depletion from natural system. Heavy demand and less supply made this species as most adulterated component of ayurvedic formulations. The present study reveals the possible

Fig. 6. (a) HPTLC fingerprint profile of Shoot and callus biomass of *B. monniera*. (The sample chromatograms Lane 1- 5 were shoot biomass derived on, Lane 1 MS + 5 µM BA + 100 µM Phenylalanine; Lane 2 MS + 5 µM BA + 100 µM Pyruvic acid; Lane 3 MS + 5µM BA + 0.0 Glycine; Lane 4 MS + 5 µM BA + 100 µM α-ketoglutaric acid; Lane 5 MS + 5 µM BA + 100 µM Ferulic acid. The sample chromatograms Lane 6-7 were standard bacoside-A. The sample chromatograms Lane 8-12 were callus biomass derived on, Lane 8 MS + 5 µM NAA + 1 µM 2,4-D + 100 µM Pyruvic acid; Lane 9 MS + 5 µM NAA + 1 µM 2,4-D + 100 µM Phenylalanine; Lane 10 MS + 5 µM NAA + 1 µM 2,4-D + 100 µM Glycine; Lane 11 MS + 5 µM NAA + 1 µM 2,4-D + 100 µM α-ketoglutaric acid; Lane 12 MS + 5 µM NAA + 1 µM 2,4-D + 100 µM Ferulic acid). (b) HPTLC chromatogram of bacoside-A from shoot biomass raised on liquid MS medium containing 5 µM BA and 100 µM Pyruvic acid.

involvement of glycine, phenylalanine, ferulic acid, α-ketoglutaric acid and pyruvic acid in the biosynthesis of bacoside-A. Among these, inclusion of 100 µM pyruvic acid in culture medium was most effective for bacoside-A accumulation. The results obtained in the present study may be useful for perceiving additional knowledge of biosynthetic pathway(s) involved in bacoside synthesis in *Bacopa monniera* (L.) Pennell.

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