J. Basic. Appl. Sci. Res., 7 (9) 27-35, 2017
© 2017, TextRoad Publication
ISSN 2090-4304
Journal of Basic and Applied Scientific Research
www.textroad.com

Enhancing *In Vitro* Production of Bacoside A from *Bacopa monnieri* Using Precursor and Elicitors Feeding

Ghada Abd El-Moneim Hegazi*[1], Hussein Sayed Taha[2], Abdel Monem Mohamed Sharaf[3], Saad Ramzy Elaish[1]

[1]Genetic Resources, Desert Research Center, El-Matareya, Cairo, Egypt
[2]Department of Plant Biotechnology and Genetic Engineering, National Research Center, Giza, Egypt
[3]Departmentof Botany and Microbiology, Faculty of Science, Al-Azhar University, Cairo, Egypt

Received: June 10, 2017
Accepted: August 19, 2017

ABSTRACT

A successful protocol for *in vitro* production of bacoside A in callus and suspension cultures of *Bacopa monnieri* (L.) Pennell was described. *B. monnieri* is a rare medicinal herb containing bacoside A, one of the active principles responsible for improving memory. Leaf explants of *B. monnieri* were collected from mature plants grown in damp and marshy places in the Eastern Mediterranean coastal region of Egypt (North Sinai). They induced 100% of yellowish white friable callus on all tested Murashige and Skoog (MS) media supplemented with 2,4-dichloro-phenoxyacetic acid (2,4-D), either individually or in combination with 6-benzylaminopurine (BAP) or N6-furfuryladenine (Kinetin; KIN). MS medium supplemented with 9 µM 2,4-D and 2.3 µM KIN was the best medium for callus induction and proliferation from leaf segments of *B. monnieri*, it gave the maximum mean fresh weight of callus and percentage of increase in fresh weight after two subcultures, comparing to the other tested media. The effect of mevalonic acid, as a precursor of bacoside A and the elicitors; chitosan and methyl jasmonate, on the stimulation of biomass growth and bacoside A production in callus cultures of *B. monnieri* was also investigated. The greatest biomass was achieved when callus was elicited by chitosan at 100 mg/L. Addition of 10 mM mevalonic acid induced the highest accumulation of bacoside A in callus cultures of *B. monnieri* after six weeks of culture, representing a 9.13- and 10.51-fold increase as compared with that in the control cultures and intact plant, respectively. The effect of elicitation with 100 mg/L chitosan on biomass and bacoside A accumulation in shake-flask culture during different durations of incubation was studied. The maximum accumulation of bacoside A was observed after 30 days of culture, it reached 30.76-fold increase compared to the intact plant. This paper reports a promising procedure for enhancing production of bacoside A in *B. monnieri* callus and suspension cultures.
KEYWORDS: brahmi, callus, suspension culture, mevanolic acid, chitosan, methyl jasmonate

INTRODUCTION

Medicinal plants produce a great number of secondary metabolites that are not only necessary for plant growth, but also have a crucial role in defense and adaptation to the environmental conditions (Ramirez-Estrada *et al.*, 2016). Secondary metabolites produced in plants, including terpenes, steroids, phenolics and alkaloids, have a wide range of biological activities and various applications in the pharmaceutical industries (Gupta *et al.*, 2010 and Cusidó *et al.*, 2014).

Bacopa monnieri (L.) Pennell, commonly known as 'Brahmi', is a rare medically important herb, from family Scrophulariaceae. The plant grows naturally in damp and marshy places of the Eastern Mediterranean coastal region in North Sinai, Egypt. It is mainly used to treat neurological disorders. The plant contains different saponins such as bacosides A, B, C, and D, which are the active triterpenoid principles in the plant and known as "memory chemicals" (Sivaramakrishna *et al.*, 2005). Abundance of these medicinally important triterpenoid saponins in *B. monnieri* is very low, which prevents the optimal utilization of these valuable compounds.

The *in vitro* production of secondary metabolites in plant cell and organ cultures can act as successfull alternative methods to their extraction from the whole plants, thereby conserving the natural plant resources (Skrzypczak-Pietraszek *et al.*, 2014). The faster proliferation rate and shorter biosynthetic cycle of cell and organ cultures causes higher rates of metabolism when compared to the field grown plants (Rao and Ravishankar, 2002). Furthermore, plant cell and organ cultures proliferates at their optimum rates under controlled conditions, when compared to the field grown plants, which are facing climate change and the variations of the ecological conditions.

Corresponding Author: Ghada Abd El-Moneim Hegazi, Genetic Resources, Desert Research Center, El-Matareya, Cairo, Egypt. Email:ghada.hegazi1211@gmail.com, (+20122)7550486

Many plant cell cultures are used to convert precursors into products by utilizing enzyme systems present in them (Murthy *et al.*, 2014). The addition of MVA enhanced the production of triterpenoids. Triterpoinds are biosynthesized *via* MVA pathway and regulated by several enzymes (Sawai and Saito, 2011).

Elicitation is a complex process and depends on many factors; such as elicitor concentration, growth stage of the culture and the exposure time with the elicitor (Bourgaud *et al.*, 2001; Zhao *et al.*, 2005 and Namedo, 2007). Enhanced production of many valuable secondary metabolites using various elicitors has been reported in various plant species (Liu *et al.*, 2007; Kamonwannasit *et al.*, 2008; Shabani *et al.*, 2009; Putalun *et al.*, 2010; Coste *et al.*, 2011; Zayed 2011; Siddiqui and Mujib, 2012; and Sivanandhan *et al.*, 2012 and Hegazi, 2017). Chitosan (CH) is an elicitor and a natural polysaccharide derived from partial deacetylation of chitin (Cheung *et al.*, 2015). Chitin is the structural element in the exoskeleton of insects, crustaceans (mainly shrimps and crabs) and fungi cell walls. Methyl jasmonate (MJ) is a volatile organic compound used in plant defense and many diverse developmental pathways. MJ and its derivatives are key signal compounds widely used as elicitors for eliciting secondary metabolism of several plant species (Yuan *et al.*, 2002 and Qian *et al.*, 2005).

The aim of the present research work was to standardize parameters for maximum biomass production and enhancing bacoside A accumulation in callus and shake-flask suspension cultures of *B. monnieri* using MVA as a precursor and CH and MJ as elicitors.

MATERIALS AND METHODS

The experiments of the present study was carried out in Tissue Culture Unit, Desert Research Center, El-Matareya, Cairo, Egypt.

1. Induction and Proliferation of Callus Culture

Explants of *B. monnieri* were collected from damp and marshy places in the Eastern Mediterranean coastal region, North Sinai, Egypt. Leaves from field grown *B. monnieri* plant were dissected out of the cuttings and cut into approximately 1 cm^2 segments. Then, they were washed under running tap water for 2-3 hours. Surface sterilization was carried out using 2% aqueous solution of sodium hypochlorite for five minutes followed by washing in sterilized distilled water for three times. Subsequently, explants were treated with 0.1% (w/v) mercuric chloride for two minutes, followed by washing in sterile distilled water for three times.

Explants were cultured on the surface of Murashige and Skoog (1962) medium (MS medium) supplemented with different concentrations and combinations of an auxin; 2,4-dichloro-phenoxyacetic acid (2,4-D) (2.25, 4.5 and 9 µM) or α-naphthaleneacetic acid (NAA) (2.7, 5.4 and 10.8 µM) and a cytokinin; 6-benzylaminopurine (BAP) (2.2 µM) or N6-furfuryladenine (Kinetin; KIN) (2.3 µM). MS medium without growth regulators was served as a control. Culture medium and additives were supplied by Duchefa Biochemie Chemical Co., Netherlands. The pH of the medium was adjusted to 5.7-5.8 and solidified with 0.7% w/v agar. Media (40-45 ml volumes) were dispensed into glass jars. Jars were sealed with autoclavable polypropylene caps and autoclaved at 1.1 kg/cm^2 for 15 minutes at 121°C. Cultures were maintained under a 16-hour photoperiod provided by cool-white fluorescent tubes (F 140t9d/38, Toshiba) at a temperature of 25±2ºC and 70±10% relative humidity. Percentage of callus induction and mean fresh weight of callus (g/jar) were recorded after eight weeks of culture.

Callus proliferation was carried out using different concentrations of 2,4-D (2.25, 4.5 and 9 µM), either individually or in combination with BAP (2.2 µM) or KIN (2.3 µM). The mean fresh weight of callus (g/jar) and the percentage of its increase were recorded after six weeks of culture. Callus cultures thus raised were subcultured to fresh media every six weeks for proliferation.

2. Precursor Feeding

An amount of the produced callus (5 g) was cultured on MS medium supplemented with the best growth regulators combination for callus proliferation of 9 µM 2,4-D and 2.3 µM KIN, and augmented with the precursor; mevalonic acid (MVA) at 0, 2.5, 5.0, 7.5 and 10 mM. Aqueous solution of MVA (Sigma-Aldrich, United Kingdom) was prepared by adding 50 ml distilled water to 1 g of MVA. The solution was filter sterilized by filtration in Millex syringe driven filter unit (0.22 µm).

3. Elicitation

Five grams of the produced callus was cultured also on MS medium supplemented with 9 µM 2,4-D and 2.3 µM KIN, and augmented with the elicitors; CH (Fluka, Japan) at 0, 50, 100, 200 and 250 mg/L and MJ (Fluka, Japan) at 0, 25, 50, 100 and 200 µM. CH was prepared by dissolving 500 mg in about 30 ml of glacial acetic acid and the solution was titrated with 1 N NaOH to give a final pH of 5.7

before autoclaving. MJ was filter sterilized by filtration in Millex syringe driven filter unit (0.22 µm). The mean fresh weight of callus (g/jar), percentage of increase in fresh weight and bacoside A content (µg/g dry weight) were recorded after six weeks of culture.

4. Suspension Culture

Fine suspension cultures were established from the produced friable callus using the best medium formulation, which gave the highest callus biomass, in liquid state from the previous experiment (9 µM 2,4-D and 2.3 µM KIN in addition to 100 mg/L CH). The suspensions were grown in 250 ml Erlenmeyer flasks containing 7 g of friable callus in 50 ml medium and incubated at 25±2°C with continuous shaking (110 rpm), in the dark. Data were taken at different durations of 0, 1, 10, 20, 30, 40 and 50 days. Samples (50 ml) were taken periodically in triplicates and their mean fresh weights (g/flask) and bacoside A content (µg/g dry weight) were recorded after each period of incubation.

5. Determination of Bacoside A

5.1. Extraction of Bacoside A

Oven-dried plant tissues (at 40°C for three days until constant weights were obtained) from mother plant; callus and cell cultures, which were subjected to precursor and elicitors feeding; were finely powdered and extracted with 10 ml of 80% methanol for 24 hours at room temperature. The extracts were filtered through Whatmann no. 1 filter paper, kept in the vacuum desiccator for one week and the residue was then dissolved in 1 ml of methanol and filtered through 0.22 µM membrane filters (Largia *et al*., 2015).

5.2. Estimation of Bacoside A Content

Bacoside A content was determined in the samples by Dionex UltMate 3000 HPLC system equipped with quaternary pump LPG3400SD, a WPS 3000 SL analytical autosampler, and a DAD – 3000 photodiode array detector (Thermo Dionex, Germany). Samples were run on an analytical column C18 using gradient elution. The mobile phase was a mixture of Milli Q water containing 0.2% phosphoric acid and acetonitrile (65:35, v/v; pH 3) at a flow rate of 1 ml/min and column temperature was maintained at 30°C. The detection wavelength was set at 205 nm. The injection volume was 20 µl and the chromatography system was equilibrated by the mobile phase. Data were analyzed and integrated by Chromeleon 7 software (Largia *et al*., 2015).

6. Experimental Design and Statistical Analysis

The experiments were subjected to completely randomized design. Each experiment was repeated twice and treatments consisted of at least 10 replicates. Variance analysis of data was carried out using ANOVA program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan´s multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

1. Induction and Proliferation of Callus Culture

The leaf segments of *B. monnieri* induced 100% of yellowish white friable callus on all MS media supplemented with 2,4-D, either individually or in combination with BAP or KIN (Table 1). On the other hand, MS medium supplemented with NAA, individually or in combinations with BAP or KIN failed to induce any callus formation from the leaf segments. Also, the control medium without PGRs gave a negative response. The highest mean fresh weight of callus was 3.22 g/jar on MS medium supplemented with 9 µM 2,4-D and 2.3 µM KIN (Fig. 1), followed by MS medium supplemented with 4.5 µM 2,4-D and 2.3 µM KIN (2.98 g/jar), then MS medium supplemented with 9 µM 2,4-D and 2.2 µM BAP (2.81 g/jar) and MS medium supplemented with 9 µM 2,4-D individually (2.60 g/jar). All these treatments gave insignificantly different results. It is noticed from the obtained data that when using MS medium supplemented with 9 µM 2,4-D, either individually or in combination with 2.3 µM KIN or 2.2 µM BAP, it gave the highest fresh weight of callus, comparing to the other tested 2,4-D concentrations.

Concerning callus proliferation, Table 2 show that the highest percentage of increase in fresh weight of callus (34.47%) was observed on MS medium supplemented with 9 µM 2,4-D and 2.3 µM KIN reaching 4.33 g/jar. Followed by MS medium supplemented with 9 µM 2,4-D individually, which gave 3.24 g/jar with percentage of increase in callus fresh weight of 24.61%, then MS medium supplemented with 9 µM 2,4-D and 2.2 µM BAP (3.44 g/jar), that gave 22.41% of increase in callus fresh weight. Data represented in Table 2 show that fresh weight of callus and percentage of increase in

fresh weight attained their maximum values at 9 µM 2,4-D, either individually or in combination with BAP or KIN. These values decreased with the decrease in 2,4-D concentration.

It could be concluded from the obtained data that MS medium supplemented with 9 µM 2,4-D and 2.3 µM KIN was the best medium for callus induction and proliferation from leaf segments of *B. monnieri*, it gave the maximum mean fresh weight of callus and percentage of increase in fresh weight, comparing to the other tested media (Fig. 2).

Table 1. Effect of MS medium supplemented with 2,4-D individually or in combination with BAP or KIN on callus induction from leaf segments of *B. monnieri.* Data were taken after eight weeks of culture.

PGRs concentration (µM)			% of callus induction	Mean fresh weight of callus (g/jar)
2,4-D	BAP	KIN		
0.00	0.00	0.00	0	0.00^{d}
2.25	0.00	0.00	100	1.45^{c}
4.50	0.00	0.00	100	2.01^{bc}
9.00	0.00	0.00	100	2.60^{ab}
2.25	2.20	0.00	100	1.61^{c}
4.50	2.20	0.00	100	2.11^{bc}
9.00	2.20	0.00	100	2.81^{a}
2.25	0.00	2.30	100	2.05^{bc}
4.50	0.00	2.30	100	2.98^{a}
9.00	0.00	2.30	100	3.22^{a}

Means followed by the same letter within a column are insignificantly different at $P \leq 0.05$.

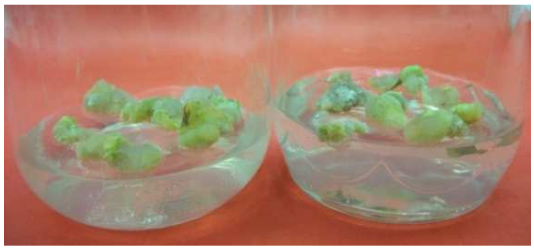

Fig. 1. Callus of *B. monnieri* induced on MS medium supplemented with 9 µM 2,4-D + 2.3 µM KIN.

Table 2. Effect of MS medium supplemented with 2,4-D individually or in combination with BAP or KIN on callus proliferation of *B. monnieri*. Data were taken after six weeks of culture.

PGRs concentration (µM)			Mean fresh weight of callus (g/jar)	Increase in mean fresh weight of callus (%)
2,4-D	BAP	KIN		
0.00	0.00	0.00	0.00^{g}	0.00^{i}
2.25	0.00	0.00	1.69^{f}	16.89^{g}
4.50	0.00	0.00	2.45^{e}	21.51^{e}
9.00	0.00	0.00	3.24^{d}	24.61^{b}
2.25	2.20	0.00	1.75^{f}	8.69^{h}
4.50	2.20	0.00	2.49^{e}	18.00^{f}
9.00	2.20	0.00	3.44^{c}	22.41^{c}
2.25	0.00	2.30	2.45^{e}	19.51^{e}
4.50	0.00	2.30	3.62^{b}	21.47^{d}
9.00	0.00	2.30	4.33^{a}	34.47^{a}

Means followed by the same letter within a column are insignificantly different at $P \leq 0.05$.

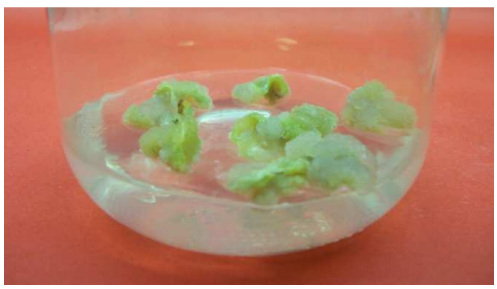

Fig. 2. Proliferated callus of *B. monnieri* on MS medium supplemented with 9 µM 2,4-D + 2.3 µM KIN.

From the obtained results; it could be concluded that 2,4-D played an important role in callus induction and proliferation. The supplementation of the culture medium with the three concentrations (2.25, 4.5 and 9 µM) of 2,4-D, individually or in combination with the two tested cytokinins (BAP or KIN) generally led to 100% of yellowish white friable callus production. However, the mean fresh weight of callus and percentage of increase in callus fresh weight varied depending on the PGRs concentrations used. Similar results were obtained by several authors; Alam *et al.* (2011) maintained successfully leaf callus of *B. monnieri* on MS medium supplemented with IAA (5.7 µM), 2,4-D (8.8 µM) and KIN (18.4 µM). Begum and Mathur (2014) used leaf petiole explants for the purpose of callus induction of *B. monnieri*. The best growth was observed on MS medium supplemented with 0.25 mg/L (1.125 µM) 2,4-D and 2.3 µM KIN or 1.125 µM 2,4-D and 0.44 µM BAP.

It is well known that auxins play an important role in the callus induction and the different types of auxins had various effects as reported by Gang *et al*. (2003). The superiority of 2,4-D is supported by Yang *et al*. (2008), who found that it is superior to NAA in callus induction of *Leonurus heterophylus*. Moreover, Mendhulkar *et al.* (2011) used the leaf explants of *B. monnieri* to induce callus on MS medium supplemented with various concentrations and combinations of IAA, NAA, 2,4-D, BAP and KIN. Whereas, BAP (0.5 mg/L; 2.2 µM) and 2,4-D (1 mg/L; 4.5 µM) showed the maximum percentage of callus induction. Also, Talukdar (2014) induced callus from shoot tips of *B. monnieri* on MS medium supplemented with IAA, NAA and 2,4-D. Degree of callus induction was the highest when using 2 mg/L (9 µM) 2,4-D. The positive effect of 2,4-D as an auxin in combination with a cytokinin is supported also by Perez-Frances *et al*. (1995), Nowak and Miczynski (2002) and Gang *et al*. (2003), who mentioned that in order to induce callus development, explants from *Artichum mosses* are cultured on MS medium supplemented with auxins and cytokinins.

2. Effect of Precursor and Elicitors Feeding on Callus Culture

The produced callus of *B. monnieri* was cultured on MS medium supplemented with the best PGRs combination for callus proliferation of 9 µM 2,4-D and 2.3 µM KIN, and augmented with different concentrations of the precursor; MVA, and the elicitors; CH and MJ. Data in Table 3 show that the highest mean fresh weight of callus and the percentage of its increase were obtained on MS medium supplemented with CH at its different concentrations. The maximum mean fresh weight of callus and percentage of its increase were observed at the concentration of 100 mg/L CH, followed by 200 mg/L CH, and their values reached 9.50 g/jar, 90% and 8.20 g/jar, 64%, respectively. On the other hand, the lowest callus formation was observed on MS medium supplemented with MVA at all tested concentrations. Concerning MJ, its addition to the medium increased the mean fresh weight of callus and the percentage of its increase as compared to the control, and the concentration of 200 µM MJ gave the highest values comparing to its other concentrations (Fig. 3). The same effect of CH on mean fresh weight of callus of *Ocimum basilicum* were obtained by Mathew and Sankar (2012), the highest accumulation of biomass of callus cultures was observed at 200 mg/L. Also, Hegazi (2017) increased callus biomass of *Salvadora persica* by elicitation with CH at 100 mg/L.

Table 3. Effect of precursor feeding and elicitation on biomass of *B. monnieri* callus and bacoside A content on MS medium supplemented with 9 µM 2,4-D and 2.3 µM KIN. Data were taken after six weeks of culture.

Precursor	Elicitors		Mean fresh weight of callus (g/jar)	Increase in fresh weight of callus (%)	Bacoside A content (µg/g dry weight)	Bacoside A increase (fold) compared to intact plant (1.13 µg/g dry weight)
MVA (mM)	CH (mg/L)	MJ (µM)				
0.0	0	0	6.09^{bc}	21.18^{j}	1.30	1.15
2.5	0	0	6.30^{c}	26.00^{i}	7.37	6.52
5.0	0	0	6.20^{c}	24.00^{i}	8.46	7.48
7.5	0	0	6.30^{c}	26.00^{i}	9.76	8.63
10.0	0	0	6.30^{c}	26.00^{i}	11.88	10.51
0.0	50	0	6.90^{bc}	38.00^{f}	2.25	1.99
0.0	100	0	9.50^{a}	90.00^{a}	3.92	3.47
0.0	150	0	7.80^{bc}	56.00^{c}	4.05	3.58
0.0	200	0	8.20^{ab}	64.00^{b}	2.19	1.94
0.0	250	0	7.40^{bc}	48.00^{d}	1.67	1.48
0.0	0	25	6.80^{bc}	36.00^{fg}	4.45	3.93
0.0	0	50	6.60^{bc}	32.00^{h}	4.54	4.02
0.0	0	100	6.70^{bc}	34.00^{gh}	4.80	4.24
0.0	0	200	7.10^{bc}	42.00^{e}	4.46	3.94

Means followed by the same letter within a column are insignificantly different at $P \leq 0.05$.

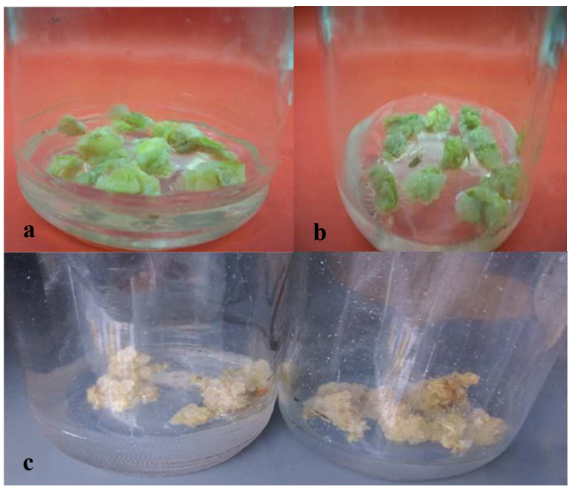

Fig. 3. Callus of *B. monnieri* on MS medium supplemented with 9 µM 2,4-D + 2.3 µM KIN, and augmented with **a.** MVA, **b.** MJ and **c.** CH.

Data in Table 3 show also the effect of various concentrations of MVA, CH and MJ on bacoside A accumulation in callus cultures of *B. monnieri* after six weeks of culture. All concentrations of precursor and elicitors enhanced bacoside A production. The maximum bacoside A accumulation was achieved in callus cultures of *B. monnieri* treated with MVA. Addition of 10 mM MVA induced the highest accumulation of bacoside A (11.88 µg/g dry weight) in callus cultures of *B. monnieri* after six weeks of MVA feeding, representing a 9.13- and 10.51-fold increase as compared with that in the control cultures and intact plant, respectively.

The effect of MJ on bacoside A accumulation was less than that of MVA. Among the tested concentrations, the highest accumulation of bacoside A (4.80 µg/g dry weight) was obtained with 100 µM MJ, representing a 3.69-fold increase as compared with that in the control and 4.24-fold as compared with that in the intact plant. The lowest increase in accumulation of bacoside A was achieved with the elicitation with CH. Comparing the different concentrations of CH, it was found that the maximum production of bacoside A in callus of *B. monnieri* after six weeks of culture was shown at 150 mg/L CH (4.05 µg/g dry weight), representing a 3.11-fold increase as compared with that in the control and 3.58-fold as compared with that in the intact plant. The results proved that MVA as a precursor of bacoside A enhanced its production in *B. monnieri* callus cultures more than elicitated callus cultures. This result was also reported by Abdul Rahman *et al.* (2003), who stated that exogenous supply of a biosynthetic precursor (MVA), to the callus culture medium may improve the production of limonene (mono-terpene) in *Citrus grandis* cell cultures. Similar pattern was observed also by Baldi and Dixit (2008), who studied on MVA feeding effects on artemisinin (sesquiterpene lactone) biosynthesis in callus culture of *Artemisia annua*. Moreover, it was reported that the addition of MVA enhanced the production of triterpenoids (Murthy *et al.*, 2014).

3. Suspension Culture

Fine suspension cultures were established from the produced callus of *B. monnieri* using the best medium composition in liquid state, which gave the highest callus biomass accumulation from the previous experiment (MS medium supplemented with 9 µM 2,4-D and 2.3 µM KIN in addition to 100 mg/L CH). Table 4 shows the effect of elicitation with CH on mean fresh weight of callus and bacoside A accumulation in shake-flask suspension culture during different durations of incubation. It was observed that the mean fresh weight of callus in shake-flask suspension culture was positively affected by the increase in the duration of incubation until 40 days of incubation, then declined by increasing the duration of culture (Fig. 4). This is in agreement with Rahman *et al*. (2002), who reported that suspension cultures of *B. monnieri*, grown in modified MS medium, gave 5-6-fold increase in the mean weight of callus after 40 days.

Concerning the accumulation of the target compound; bacoside A, in suspension cultures of *B. monnieri*, it was observed that the maximum accumulation of bacoside A (34.77 µg/g dry weight) was recorded after 30 days of culture, it reached 30.76-fold increase compared to the intact plant. Bacoside A accumulation increased with the increase in the duration of incubation time, until 30 days, which was the optimum time of incubation. After that that accumulation of bacoside A decreased gradually by increasing the duration of incubation. However they resulted in higher accumulation of bacoside A as

compared to the short durations (1, 10 and 20 days). The minimum accumulation of bacoside A (6.97 µg/g dry weight) was noticed after one day of culture. It did not reveal remarkable impact on product accumulation when compared to intact plant (6.17-fold). The results are in agreement with Rahman *et al*. (2002), who reported the potential of the suspension cultures of *B. monnieri* for production of bacoside A. Also, the duration of cell cultures with the elicitor was important for the maximum bacoside A accumulation. This agrees with Karwasara *et al*. (2011) and Ahmed and Baig (2014), who stated that the amount of metabolite production varied with the duration of incubation with elicitors.

Table 4. Effect of different culture durations of *B. monnieri* suspension cultures grown in MS medium supplemented with 9 µM 2,4-D + 2.3 µM KIN, in addition to100 mg/L CH on biomass and bacoside A accumulation.

Culture duration (days)	Mean fresh weight of callus (g/flask)	Bacoside A content (µg/g dry weight)	Bacoside A content (fold) compared to intact plant (1.13 µg/g dry weight)
0	9.5	3.92	3.47
1	10.5	6.97	6.17
10	17.0	18.37	16.20
20	19.0	20.25	17.90
30	32.0	34.77	30.76
40	60.0	26.67	23.60
50	53.0	24.37	21.56

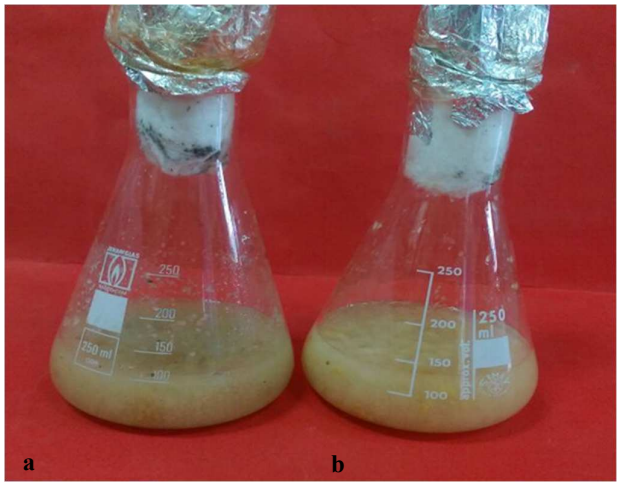

Fig. 4. Suspension cultures of *B. monnieri* grown in MS medium supplemented with 9 µM 2,4-D + 2.3 µM KIN augmented by 100 mg/L CH for **a.** 40 days and **b.** 50 days.

CONCLUSION

To the best of our knowledge, this is the first report in Egypt on enhancing production of bacoside A from callus and suspension cultures of *B. monnieri*. Addition of 10 mM MVA induced the highest accumulation of bacoside A (11.88 µg/g dry weight) in callus cultures, representing a 9.13- and 10.51-fold increase as compared with that in the control cultures and intact plant, respectively. The maximum accumulation of bacoside A (34.77 µg/g dry weight) was observed after 30 days of culture in shake-flask suspension cultures elicitated with CH (100 mg/L), it reached 30.79-fold increase compared to the intact plant. The results obtained from the present study can act as a stepping-stone for further studies regarding large-scale production of bacoside A from callus and suspension cultures of *B. monnieri*.

REFERENCES

Abdul Rahman, N. N. N., Z. Zakaria and M. O. Abd Kadir, 2003. Influence of elicitor availability on limonene and linalool accumulation from *Citrus grandis* Cell cultures. Malays. J. Pharmaceut. Sci., 1: 39-50.

Ahmed. S. A. and V. M. M. Baig, 2014. Biotic elicitor enhanced production of psoralen in suspension cultures of *Psoralea corylifolia* L. Saudi J. Biol. Sci., 21 (5): 499-504.

Alam, K., N. Parvez, S. Yadav, K. Molvi, N. Hwisa, S. M. Al Sharif, D. Pathak, Y. Murti and R. Zafar, 2011. Antimicrobial activity of leaf callus of *Bacopa monnieri* L. Der Pharma. Lett., 3 (1): 287-291.

Baldi, A. and V. K. Dixit, 2008. Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. Bioresource Technol., 99: 4609-4614.

Begum, T. and M. Mathur, 2014. *In vitro* regeneration of *Catharanthus roseus* and *Bacopa monnieri* and their survey around Jaipur District. Int. J. Pure App. Bio. Sci., 2 (4): 210-221.

Bourgaud, F., A. Gravot, S. Milesi and E. Gontier, 2001. Production of plant secondary metabolites: a historical perspective. Plant Sci., 161: 839-851.

Cheung, R. C. F., T. B. Ng, J. H. Wong and W. Y. Chan, 2015. Chitosan: an update on potential biomedical and pharmaceutical applications. Mar. Drugs, 13: 5156-5186.

Coste, A., L. Vlase, A. Halmagyi, C. Deliu and G. Coldea, 2011. Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum*. Plant Cell Tiss. Org. Cult., 106: 279-288.

Cusidó, R. M., M. Onrubia, A. B. Sabater-Jara, E. Moyano, M. Bonfill and A. Goossens, 2014. A rational approach to improving the biotechnological production of taxanes in plant cell cultures of *Taxus* spp. Biotechnol. Adv., 32: 1157-1167.

Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics, 11: 1-42.

Gang, Y. Y., G. S. Dh, D. J. Shi, M. Z. Weng and D. Lix, 2003. Establisement of *in vitro* regeneration system of the *Atrichum mosses*. Acta Bot. Sin., 45 (12): 1475-1480.

Gupta, V. K., C. Shukla, G. R. S. Bisht, D. Saikia, S. Kumar and R. L. Thakur, 2010. Detection of anti-tuberculosis activity in some folklore plants by radiometric BACTEC assay. Lett. App. Microbiol., 52: 33-40.

Hegazi, Gh. A. 2017. Establishment of suspension cultures of *Salvadora persica* L. for benzyl isothiocyanate production. J. Pharm. Bio. Sci., 12 (1): 72-78.

Kamonwannasit, S., W. Phrompittayarat, K. Ingkaninan, H. Tanaka and W. Putalun, 2008. Improvement of pseudojujubogenin glycosides production from regenerated *Bacopa monnieri* (L.) Wettst. and enhanced yield by elicitors. Z. Naturforsch. C., 63: 879-883.

Karwasara, V.S., P. Tomar and V. K. Dixit, 2011. Influence of fungal elicitation on glycyrrhizin production in transformed cell cultures of *Abrus precatorius* L. Pharmacogn. Mag., 7 (28): 307-313.

Largia, M. J. V., G. Pothiraj, J. Shilpha and M. Ramesh, 2015. Methyl jasmonate and salicylic acid synergism enhances bacoside A content in shoot cultures of *Bacopa monnieri* (L.). Plant Cell Tiss. Org. Cult., 122 (1): 9-20.

Liu, D. H., H. B. Jin, Y. H. Chen, L. J. Cui, W. W. Ren, Y. F. Gong and K. X. Tang, 2007. Terpenoidindole alkaloids biosynthesis and metabolic engineering in *Catharanthus roseus*. J. Integr. Plant Biol., 49: 961-974.

Mathew, R. and P. D. Sankar, 2012. Effect of methyl jasmonate and chitosan on growth characteristics of *Ocimum basilicum* L., *Ocimum sanctum* L. and *Ocimum gratissimum* L. cell suspension cultures. Afr. J. Biotechnol., 11 (21): 4759-4766.

Mendhulkar, V. D., P. S. Patade and S. N. Singh, 2011. DMSO induced product recovery of bacoside A in cell suspension culture of *Bacopa monnieri* Linn. Int. J. Pharm. Sci. Res., 2 (11): 3006-3009.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and biossays with tobacco tissue cultures. Physiol. Plant., 15: 497–573.

Murthy, H. N., E. J. Lee and K.Y. Paek, 2014. Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. Plant Cell Tiss. Org. Cult., 118: 1-16.

Namedo, A. G. 2007. Plant cell elicitation for production of secondary metabolites: a review. Pharma. Rev., 1 (1): 69-79.

Nowak, B. and K. Miczynski, 2002. The course and efficiency of organogenesis on leaf explants of plum (*Prunus domestica* L.) induced by cytokinin. Electronic Journal of Polish Agricultural Universities, Biotechnol., 5 (1).

Perez-Frances, J. F., F. Valdes and R. Msrtin, 1995. Callus induction and culture from explants of *Erysimum scoparium* in a growth regulator-free medium. Plant Cell Tiss. Org. Cult., 43 (3): 223-228.

Putalun, W., O. Udomsin, G. Yusakul, T. Juengwatanatrakul, S. Sakamoto and H. Tanaka, 2010. Enhanced plumbagin production from *in vitro* cultures of *Drosera burmanii* using elicitation. Biotechnol. Lett., 32: 721-724.

Qian, Z. G., Z. J. Zhao, Y. F. Xu, X. H. Qian and J. J. Zhong, 2005. A novel synthetic fluoro-containing jasmonate derivative acts as a chemical inducing signal for plant secondary metabolism. Appl. Microbiol. Biotechnol., 68: 98-103.

Rahman, L. Q., P.C. Verma, D. Singh, M. M. Gupta and S. Banerjee, 2002. Bacoside production by suspension cultures of *Bacopa monnieri* (L.) Pennell. Biotechnol. Lett., 24: 1427-1429.

Ramirez-Estrada, K., H. Vidal-Limon, D. Hidalgo, E. Moyano, M. Golenioswki, R. M. Cusidó and J. Palazon, 2016. Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. Molecules, 21: 182.

Rao, S. R. and G. A. Ravishankar, 2002. Plant cell cultures: chemical factories of secondary metabolites. Biotechnol. Adv., 20: 101-153.

Sawai, S. and K. Saito (2011). Triterpenoid biosynthesis and engineering in plants. Front. Plant Sci., 2: 25.

Shabani, L. A. A., E. G. Asghari and J. Emami, 2009. Glycyrrhizin production by *in vitro* cultured *Glycyrrhiza glabra* elicited by methyl jasmonate and salicylic Acid. Rus. J. Plant Physiol., 56 (5): 688–694.

Siddiqui, Z. H. and A. Mujib, 2012. Accumulation of vincristine in calcium chloride elicitated *Catharanthus roseus* Cultures. Nat. Prod. J., 2: 307–315.

Sivanandhan, G., M. Arun, S. Mayavan, M. Rajesh, M. Jeyaraj and G. K. Dev, 2012. Increased production of withanolide A, withanone, and withaferin A in hairy root cultures of *Withania somnifera* (L.) Dunal elicited with methyl jasmonate and salicylic acid. Appl. Biochem. Biotechnol., 168 (3): 681-96.

Sivaramakrishna, C., C.V. Rao, G.Trimurtulu, M. Vanisree and G. V. Subbaraju, 2005. Triterpenoid glycosides from *Bacopa monnieri*. Phytochem., 66 (23): 2719-2728.

Skrzypczak-Pietraszek, E., J. Słota and J. Pietraszek, 2014. The influence of L-phenylalanine, methyl jasmonate and sucrose concentration on the accumulation of phenolic acids in *Exacum affine*. Balf. f. Ex Regel shoot culture. Acta Biochim. Pol., 61 (1):47–53.

Talukdar, A. 2014. Biosynthesis of total bacosides in the callus culture of *Bacopa monnieri*. L. Pennel from North-east India. Int. J. Curr. Microbiol. App. Sci., 3 (3): 140-145.

Yang, J., Z. C. Gong and X. Tan, 2008. Induction of callus and extraction of alkaloids from Yi Mu Cao (*Leonurus heterophylus* Sw.) culture. African J. Biotech., 7 (8): 1157-1162.

Yuan, Y. J., C. Li, Z. D. Hu and J. C. Wu, 2002. A double oxidative burst for taxol production in suspension cultures of *Taxus chinensis* var. mairei induced by oligosaccharide from *Fusarium oxysprum*. Enzyme Microb.Technol., 30: 774–778.

Zayed, R. 2011. Efficient *in vitro* elicitation of b-carboline alkaloids in transformed root cultures of *Peganum harmala*. Bull. Faculty Pharm. Cairo Univ., 49: 7–11.

Zhao, J., L. C. Davis and R. Verpoorte, 2005. Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol. Adv., 23: 283–333.