RESEARCH ARTICLE

SABRAO Journal
of Breeding and Genetics
50 (1) 25-35, 2018

ENHANCEMENT OF ROSMARINIC ACID PRODUCTION BY *Satureja khuzistanica* CELL SUSPENSIONS: EFFECTS OF PHENYLALANINE AND SUCROSE

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SUMMARY

Rosmarinic acid (RA) is one of the most common and important caffeic esters in plant material. The effects of precursor feeding and carbon source concentration on biosynthesis of rosmarinic acid by *S. khuzistanica* cell suspension culture were evaluated. Different concentrations of sucrose (30, 45 and 60 g/l) and L-phenylalanine (0, 0.5, 1.5, 3 and 4.5 mM) were examined in two independent experiments. Feeding phenylalanine reduced growth of cultures and enhanced rosmarinic acid content of cells. Maximum rosmarinic acid accumulation (227.76 ± 3.36 mg RA g/DW) was observed with 3.0 mM phenylalanine at day 7th. Different concentrations of sucrose also influence growth and RA production of *S. khuzistanica* cell suspension culture. The highest concentration of sucrose (60 g/l) resulted in maximum DW (25.28 ± 0.07 g DW/l) after 21 days. A very high rosmarinic acid yield (about 4350 mg/l RA) was achieved when 45 or 60 g/l sucrose was added to the culture media. Results of this investigation revealed that altering of culture media components (carbohydrate and precursors) can improve RA yield in *S. khuzistanica* cell cultures and it can represent a promising biotechnological platform for commercial RA production. In general, 3 mM phenylalanine and 60 g/l sucrose concentrations are suggested to be used in the *S. khuzistanica* cell culture media for higher RA production.

Key words: Cell culture, medicinal plants, precursor feeding, secondary metabolites, media

Key findings: By supplementing Phe and altering sucrose concentrations in the culture media, RA production improved in *S. khuzistanica* cell suspension. Phe at 3 mM concentration not only increased RA contents of cell dry weight, but also advanced the time require for achieving the highest concentration of this medicinal important substance.

Manuscript received: December 15, 2017; Decision on manuscript: December 23, 2017; Accepted: February 7, 2018.

Communicating Editor: Dr. Sobir

INTRODUCTION

Rosmarinic acid (RA), an important phenolic compound, is synthesized from phenylalanine (Phe) through the esterification of caffeic acid and from tyrosine through 3,4-dihydroxyphenyllactic acid (Ellis and Towers 1970). RA is widely distributed in the plant kingdom and its presence has been reported in 39 families of plants, ranging from hornworts and pteridophytes, to species of the monocotyledonous and dicotyledonous plants (Petersen and Simmonds 2003). RA is usually isolated from species of Boraginaceae and the subfamily Nepetoideae (family Lamiaceae) and was first detected as a pure compound from *Rosmarinus officinalis* (Scarpati and Oriente 1958).

Numerous pharmacological effects have been attributed to RA and its derivatives which include anti-inflammatory, antioxidant, antitumor, anti-viral, and anti-microbial properties (Bulgakov *et al*., 2012; Wu and Wang 2012). Due to these properties, RA and its derived compounds have been investigated for use in pharmaceutical or nutritional additives to improve human health; therapeutic targets include Alzheimer's disease (Vladimir-Knežević *et al*., 2014), cardiovascular disease (Karthik *et al*., 2011), atopic dermatitis (AD)-like symptoms (Jang *et al*., 2011) and allergic inflammation (Sanbongi *et al*., 2004). The molecular mechanisms underlying clinical efficacy have been revised recently (Kim *et al*., 2015). Taking into consideration the RA pharmacological activities and low toxicity, its possible application in processed foods as a natural antioxidant has been reached a new interest levels in recent years (Nunes *et al*., 2015).

Based on the increased demand of RA, different biotechnological approaches to enhance its production have been undertaken by using cell cultures derived from several plant species, such as *Anchusa officinalis*, *Eritrichium sericeum*, *Lithospermum erythrorhizon* (Boraginaceae), *Coleus blumei*, *Lavandula vera*, *Ocimum basilicum*, *Salvia officinalis*, *Salvia miltiorrhiza*, *Zataria multiflora* (Lamiaceae) and *Anthoceros agrestis* (Anthocerotaceae) (Georgiev *et al*., 2004; Matkowski, 2008; Nasiri-Bezenjani *et al*., 2014; Petersen and Simmonds 2003). To the list of plant cell cultures for production included in the revision of Bulgakov *et al*., (2012) it has recently been added another plant species as a source of RA: *Satureja khuzistanica*.

S. khuzistanica is an Iranian endemism belonging to the Lamiaceae family whose RA contents in methanol (MeOH) extracts range from 0.59 to 1.81% dry weight (DW) (Hadian *et al*.,

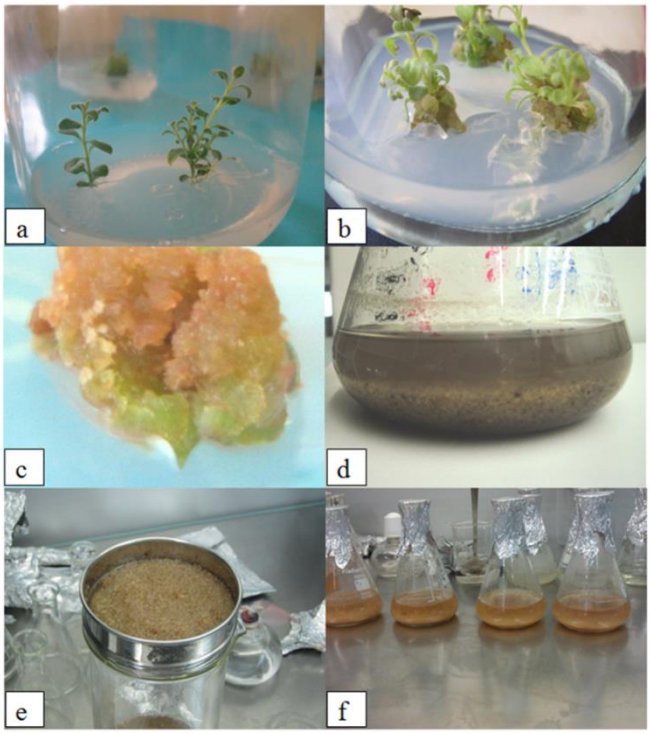

Figure 1. Preparing of cell suspension culture of *S. khuzistanica* for phenylalanine and sucrose treatments: **a**) establishing of in vitro-grown plantlets, **b**) callus induction by using micropropagated plantlets, **c**) subculturing and proliferation of calli, **d**) cell suspension culture was done in Erlenmeyer flask, **e**) filtered cells and cell clumps for **f**) subculturing, treatments and subsequent analysis.

2011). Successful protocols for in vitro cultures were established by Sahraroo *et al*., in 2014 and 7.5% basal levels of RA, based on DW, were reported. Production raised to 18% dry cell weight in suspension cultures (Sahraroo *et al*., 2016) and a maximum production of 3.1 gl^{-1} RA and productivity of 18.7 g $l^{-1}d^{-1}$ biomass was achieved in wave-mixed bioreactor under methyl jasmonate elicitation (Khojasteh *et al*., 2016).

S. khuzistanica cell suspensions can thus be considered as a suitable platform for production of RA by biotechnological ways. The efforts made to improve the productivity of RA in *S. khuzistanica* cultures have been scarce up to now and it is conceivable that significant benefits can be gained by identifying the optimum culture conditions for growth and RA production.

Some of the most useful modifications made in the growth medium to promote secondary metabolite production, among others, are related to the source of energy. The most suitable carbon source and its optimal concentration for growth and secondary metabolite production are plant species and products dependent (Misawa, 1994). Precursor feeding is also one of the most common approaches to increase secondary metabolite accumulation in plant cell cultures. According to Rao and Ravishankar (2002), any

intermediary compound of a secondary metabolite biosynthetic pathway, can play a key role for increasing the yield of the final product (Ramachandra Rao and Ravishankar 2002). Due to importance of phenylalanine (Phe) on phenylpropanoid pathway some studies were conducted to demonstrate the effect of this precursor for synthesizing considerable amounts of secondary metabolite (Hippolyte *et al*., 1992; Karam *et al*., 2003; Pavlov and Ilieva 1999).

Therefore, the effects of Phe feeding and sucrose levels on RA production have been investigated in cell suspension culture of *S. khuzistanica*.

MATERIAL AND METHODS

Chemicals and reagents

Plant growth regulators (PGRs), vitamins, basal media salts, sucrose, and a RA standard were purchased from Merck (Darmstadt, Germany) and Sigma (Sigma-Aldrich Corporation, Spruce Street, St. Louis, MO, USA). Methanol in 99.5% purity was obtained from Merck (Darmstadt, Germany). HPLC grade water was used throughout the analysis.

Cell culture

Callus induction of *Satureja khuzistanica* was done by using different plant growth regulators and six-month-old in vitro-grown plantlets. A stem cutting of such plantlets was used (Figures 1a and b) (Sahraroo *et al*., 2014). Afterwards the best treatment [liquid B5 medium (Gamborg *et al*., 1968) supplemented with 30 g/l sucrose, 20 mg/l L-glutamine, 200 mg/l casein hydrolysate, 5 mg/l benzyladenine (BA) and 1 mg/l indole-3-butyric acid (IBA)] was used for callus sub culturing and establishing of cell suspension culture. Cultures were subculture every 21 days (Figures 1c, d, e and f) (Sahraroo *et al*., 2016). The pH of the medium was adjusted to 5.8 with NaOH or HCl before autoclaving for 20 min at 121°C. Cell cultures were kept in the dark at 23 ± 2 ºC and shaken at 75 rpm. Cultures were routinely maintained by periodical subcultures every 3 weeks.

PHE and sucrose treatments

Approximately 1.5 g fresh cells and cell clumps (from 21-days-old cultures) as the initial inoculum was transferred to 100 mL Erlenmeyer flasks containing 20 mL of the abovementioned liquid medium. Different concentrations of L-phenylalanine (0, 0.5, 1.5, 3 and 4.5 mM) were added to the culture medium prior to autoclaving. The keeping conditions described before and data were collected 2, 7 and 14 days after treatment. Three flasks were considered for every concentration at each time and all cells were filtered, weighted, dried and used for further measurements. In order to study the effect of sucrose, three concentrations of sucrose (30, 45 and 60 g/l) were used on *S. khuzistanica* cultures and cells were harvested every 7 days until day 21st (the end of exponential phase). Cells and cell clumps were separated from the medium by filtration and weighed as fresh weight (Figure 1e). The dry weight of the cells was recorded after drying them to a constant weight at 35-40 °C for 5 days. The experiment

was done as a completely randomized design (CRD) with three replication (3 Erlenmeyer flasks) per time. All treatments were done at the initial stage of experiment, so cells were cultured on media containing phenylalanine or sucrose and then they were maintained at Phe and sucrose for 7, 14 or 21 days.

Data were recorded for RA content (mg rosmarinic acid per g dry weight), fresh and dry weight (g fresh and dried cells per litter), dry matter allocation (dry weight/fresh weight × 100), RA yield (mg rosmarinic acid per litter) as well as RA productivity (mg RA produced by mM phenylalanine which added to the media).

Extraction and RA analysis

RA was extracted according to the method previously described (Sahraroo *et al*., 2014, 2016). Briefly the procedure was as follow: Nine mL of methanol were added to the 20 mg of dried and grounded cells of *Saturia khuzistanica.* This mixture was incubated in an ultrasonic bath containing two mixing for 10 min. After that, the mixture pass through Whatman papers and evaporated using a vacuum evaporator at 40-45 ºC, then 1 ml of methanol was added to the residues. A 0.2 µm, Millipore filter syringe (Bedford, MA, USA) was used to filter the solution. For analysis of RA, 20 µL of the filtrate were injected into HPLC instrument containing Spherisorb ODS-2 (5 µm) reversed phase 4.6 mm × 250 mm column. A flow rate of 1 mL/min and wavelength of 330 nm was used for elution and detection, respectively. Solution (0.1%) of formic acid in water (A) and acetonitrile (B) were used as two mobile phases in this investigation. During the experiment, the following program was applied: 30 min 88% A and 12% B, 15 min 80% A and 20% B and 15 min 70% A and 30% B. Retention time of a reference standard was used to compare and confirm the chromatographic peak of RA. Solutions of working standards were injected into the HPLC instrument and standard graphs were depicted according to concentration vs. area. Quantification was performed from integrated peak areas of the samples using the respective standard diagram. Means of three replications were used to depict respective graphs in Excel software. Error bars were calculated from subtracting of standard deviation on three replicates.

RESULTS AND DISCUSSION

Precursor feeding

The effects of Phe application on growth and rosmarinic acid production were examined in *S. khuzistanica* cell suspensions. As shown in Figures 2a and 2b, the different Phe concentrations reduced culture growth in a dose-dependent manner, being the effect more acute during the second week of culture, probably due to a toxic effect, as feeding of feedback-competent amino acids to the culture media disrupts homeostasis of amino acid by inhibiting related enzymes and finally causes deficiencies of other amino acids resulting from these biosynthetic pathways (Voll *et al*., 2004).

The evolution of RA accumulation in cultures over two weeks is shown in Figure 2c. In control cultures, RA content increased during the second week of the culture cycle, reaching the maximum at day 14 of the studied period. Inclusion of Phe, at

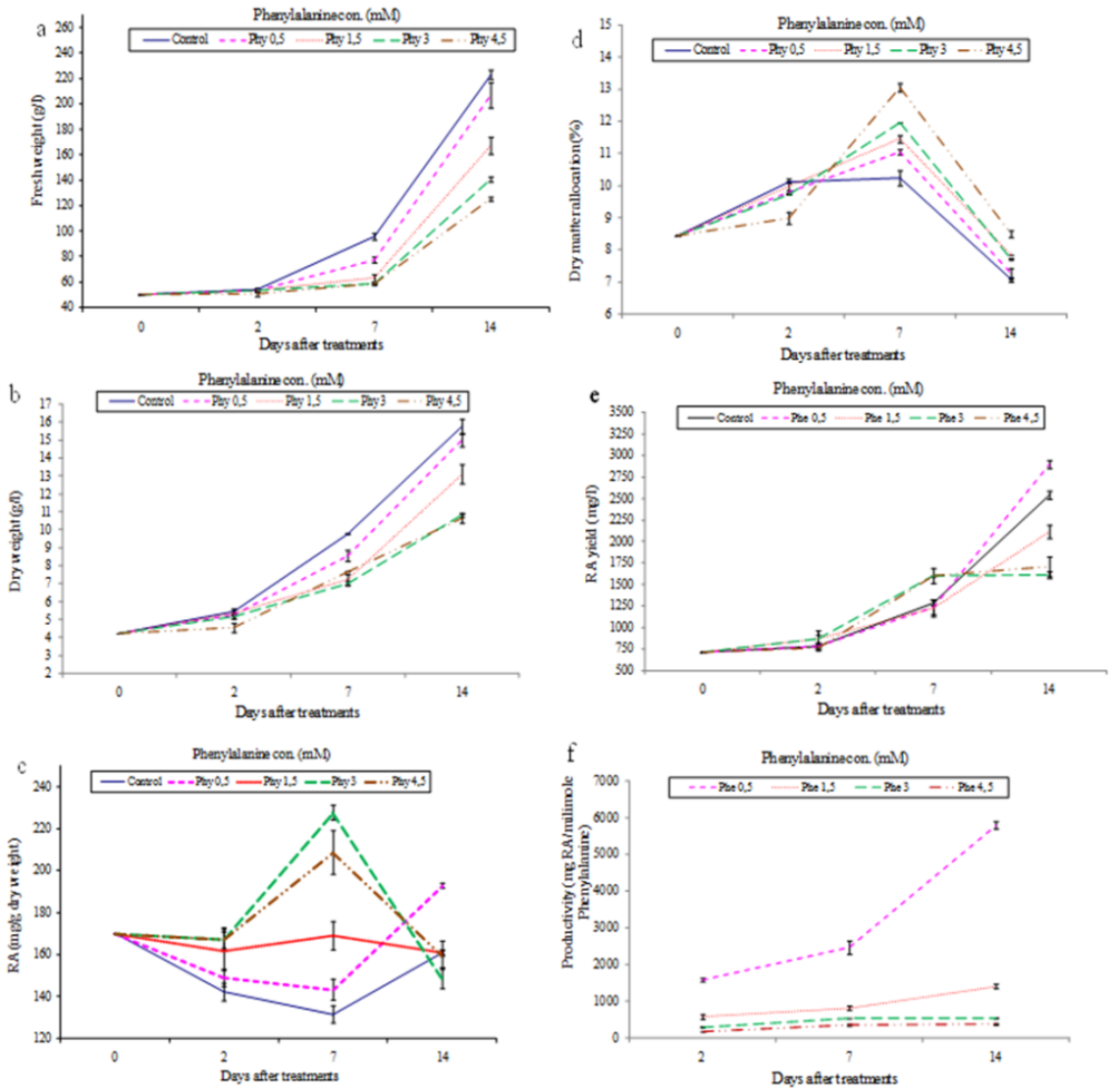

Figure 2. Effects of precursor feeding on cell suspension culture of *S. khuzistanica*: a- fresh weight, b- dry weight, c- RA content of cells, d- dry matter allocation, e- RA yield and f- cell productivity.

the four concentrations tested, was beneficial for production of RA (expressed as mg RA per g cell DW) during the first week of experimentation. Optimum RA increase (227.76 ± 3.36 mg RA per g DW) was attained with 3 mM Phe at the end of first week. During the second week, the trend was reversed, and Phe at concentrations higher than 1.5 mM strongly reduced the RA content. One possible reason for the decline in RA seen after a week may have been the shortage of the other internal precursor of RA, tyrosine, thus becoming limiting for RA synthesis. In addition, it should also be noted that the effect of external precursor Phe on RA content was noticeable after 2 days. This finding can be helpful for further studies like injecting Phe during exponential phase or at the end

of the cell culture cycle to synthesize more RA by cells, thus avoiding the unfavorable sub culturing condition for RA biosynthesis that occurs during the first days of culture.

In order to assess whether fresh growth alteration were due to the changes in the cell water content or the increase in DW, dry matter allocation (DW/fresh weight percentage) or percentage of DW, was calculated over the cell culture cycle. The maximum dry matter allocation in control and Phe supplemented cultures was achieved at day 7th of experiment (Figure 2d). Concerning RA accumulation, in control cultures, both the highest RA content and the lowest dry matter allocation were simultaneously seen at day 14 (Figures 2c and d). Therefore, from results of this investigation suggests that the biosynthesis of RA occurs when cell growth becomes limiting. The same finding was observed by Gertlowski and Petersen (1993) who reported that the maximum RA production by *Coleus blumei* was seen when cell growth was reduced.

In cultures fed with Phe, the RA biosynthesis took place 7 days earlier. In contrast to the control cultures, at high Phe concentrations dry matter allocation and RA accumulation was directly related at day 7th, and only at 0.5 mM Phe RA production and dry matter allocation followed the same trend as the control cultures (Figures 2d and 1c). Hippolyte *et al*., (1992) also showed that growth and production of RA in *Salvia officinalis* cultures changed by Phe application (Hippolyte *et al*., 1992). Therefore, these findings show that the availability of the precursor is one of the main factors that limit RA production in the cultures.

RA yield (mg RA per liter culture media) and productivity (mg RA per mmol precursor) are useful parameters that should also be estimated for industrial production. The highest RA yield (2900 ± 50 mg RA per liter) was attained by feeding 0.5 mM Phe to the culture media after 14 days of culture (Figure 2e). It was about 1.8 times higher than those observed with 3 and 4.5 mM Phe. Concerning productivity, maximum RA production per mmol external precursor was also achieved at 0.5 mM Phe (Figure 2f); increasing precursor levels lead to low RA productivity.

Sucrose concentration

Carbohydrates exert several roles in the *in vitro* culture conditions such as carbon source and osmotic agent. Among carbon source alternatives, sucrose is widely used for cell, tissue and organ cultures. Therefore, many researchers have pointed out the effects of sucrose concentration on secondary metabolite biosynthesis (Gertlowski and Petersen 1993; Hippolyte *et al*., 1992; Ilieva and Pavlov 1997; Karam *et al*., 2003; Mulabagal and Tsay 2004; Pavlov *et al*., 2000). In *S. khuzistanica* cultures, fresh and dry weight, RA content and RA yield were significantly affected at different sucrose levels. The highest sucrose concentration tested (60 g/l sucrose) probably caused an osmotic effect seen by the lower level of fresh biomass; however, the highest DW (25.28 ± 0.07 g DW per liter) was measured at this concentration after 21 days (Figures 3a and b). Figure 3c also shows that percentages of dry matter at 60 g/l sucrose were higher than with the other tested concentrations during experiment

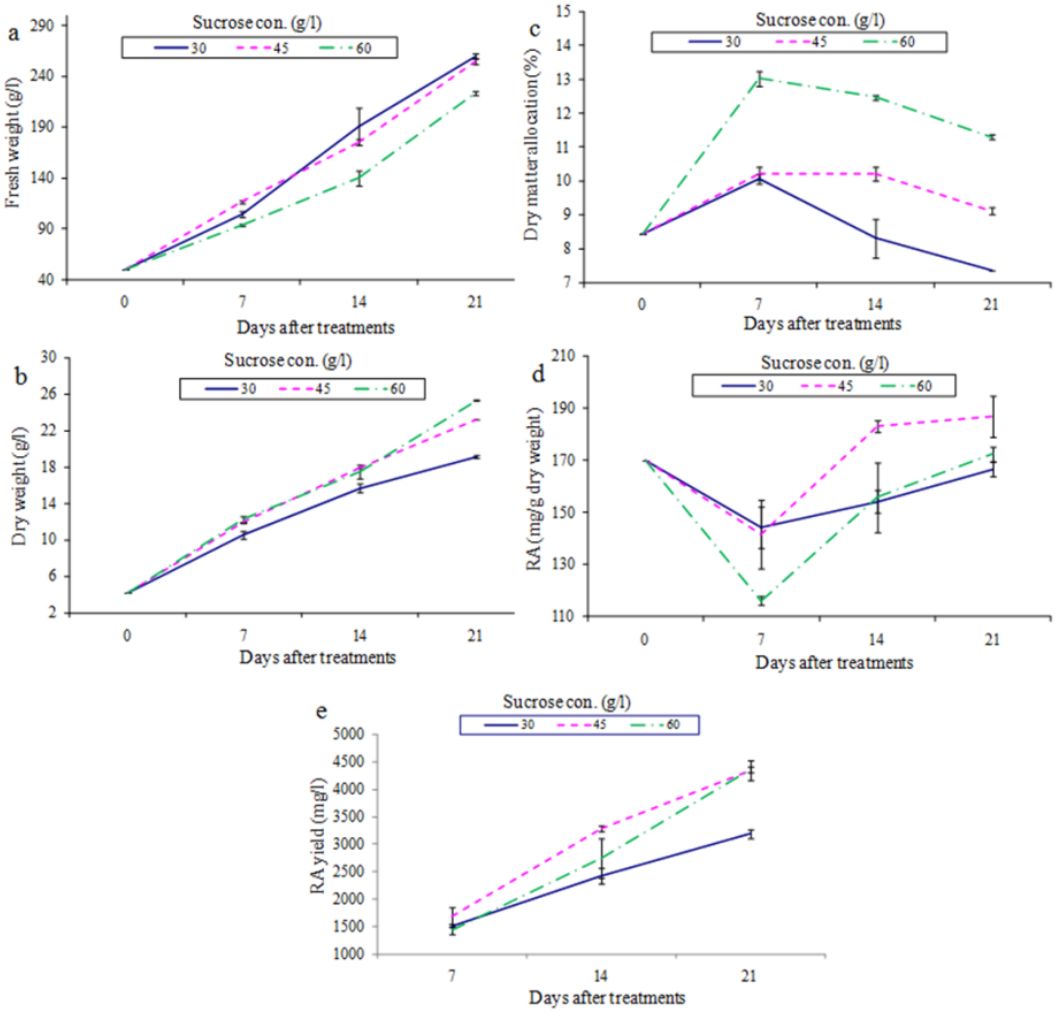

Figure 3. Effects of sucrose concentrations on cell suspension culture of *S. khuzistanica*: a- fresh weight, b- dry weight, c- dry matter allocation, d- RA content of cells and e- RA yield.

(13.03 ± 0.21, 12.47 ± 0.07 and 11.3 ± 0.08%); dry biomass and dry matter allocation was higher at this concentration which could be explained by both the nutritional and osmotic effects of sucrose.

Contradictory findings were reported about the effects of sucrose concentration on plant cell and tissue cultures. In some cases media supplemented with sucrose concentrations higher than 30 g/l (the concentration that is used commonly in different tissue culture media) resulted in growth diminution (Hakkim *et al*., 2011; Kikowska *et al*., 2012; Santos-Gomes *et al*., 2003), while others reported enhancement of cell growth by increasing sucrose concentration (Françoise *et al*., 2007; Ghorbanli, 2002). Concerning RA accumulation, maximum content (187 ± 7 mg RA per g DW) was measured in cultures supplemented with 45 g/l sucrose after 14 days (Figure 3d), which represents a 1.3-fold increase compared with the control cells at the same time point. Optimum sucrose

levels for RA production vary depending on the plant species. Similar to our results, RA up to 19% of the cell DW was obtained in suspension cultures of *C. blumei* after only 10 days of cultivation in a medium with a high sucrose concentration (Petersen *et al*., 1994). In cell suspension cultures of *Anthoceros agrestis* Paton, RA was achieved up to 5.1% DW in the medium supplemented with 2% (w/v) sucrose at day 8 (Vogelsang *et al*., 2006). In contrast, 7% (w/v) sucrose was the optimal concentration for increasing RA production in cell cultures of *L. Vera* (Ilieva and Pavlov 1997). A remarkable RA yield (about 4350 mg RA per liter) was observed in the medium supplemented with 45 and 60 g/l sucrose at the end of cultivation (day 21), (Figure 3e), although overall RA production throughout the culture period was higher in sucrose at 45 g/l than 60 g/l. It seems that although sucrose concentration affects the RA production in different plants, but the best concentration of this carbohydrate source vary among different species and should be optimize by testing different concentrations.

CONCLUSION

Considering the high RA yield obtained in *S. khuzistanica* undifferentiated cells grown in a low cost production media, cell cultures of this plant species could represent a promising biotechnological platform for commercial RA production. Our results indicated that it is possible to improve RA production in *S. khuzistanica* cell suspension by supplementing Phe and altering sucrose concentrations in the media. Phe at 3 mM concentration not only increased RA contents of cell dry weight, but also advanced the time require for achieving the highest concentration of this medicinal important substance. In addition, increase in sucrose concentration of culture media can increase RA production in this plant. These findings have great practical importance for establishing and exploitation of *S. khuzistanica* cell suspension. Finally, it can be expected that both substances (3 mM Phe plus 45 g/l sucrose) can be used simultaneously for more RA biosynthesis in cell suspension culture of *S. khuzistanica*.

ACKNOWLEDGEMENTS

We appreciate the Iranian Ministry of Science, Research and Technology, University of Tehran and University of Salamanca for financial supporting this work.

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