Establishment and characterization of a *Satureja khuzistanica* Jamzad (Lamiaceae) cell suspension culture: a new in vitro source of rosmarinic acid

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Abstract An in vitro approach to the production of rosmarinic acid (RA), a medicinally important caffeic acid ester, in a cell suspension culture (CSC) of *Satureja khuzistanica* Jamzad (Lamiaceae) has been investigated for the first time. The CSC was established from friable calli derived from shoot tip explants in Gamborg's B5 liquid medium 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). The effect of nitrogen source (KNO_3 and $(\text{NH}_4)_2\text{SO}_4$) and their different concentrations on the fresh and dry weight (g/L), as well as RA content (mg/g dry weight)

were measured. CSC growth measurements indicated a maximum specific cell growth rate of 1.5/day, a doubling time of 7.6 days and a high percentage of cell viability (96.4 %) throughout the growth cycle. Maximum cell fresh weight (353.5 g/L), dry weight (19.7 g/L) and RA production (180.0 mg/g) were attained at day 21 of culture. Cell growth and RA content were affected by nitrogen deficiency. Media containing 8.3 mM of total nitrogen ($\frac{1}{4}$ of B5 standard medium) led to a minimum cell fresh weight (243.0 g/L), dry weight (17.4 g/L) and RA content (38.0 mg/g) after 21 days. The established CSC provided useful material for further optimization experiments aimed at a large-scale production of RA.

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Abbreviations

RA	Rosmarinic acid
CSC	Cell suspension culture
BA	Benzyladenine
IBA	Indole-3-butyric acid
FW	Fresh weight
DW	Dry weight
SGR	Specific growth rate
PGRs	Plant growth regulators
HPLC	High performance liquid chromatography
PVP	Polyvinylpyrrolidone
CRD	Completely randomized design

MS	Mean square
DF	Degrees of freedom
CV	Coefficient of variation

Introduction

Secondary plant metabolites play important roles in plant–plant and/or plant–environment interactions. The production of these compounds is often low (less than 1 % dry weight) and depends greatly on the physiological and developmental stage of the plant (Dixon 2001; Oksman-Caldentey and Inzé 2004). Phenolic compounds, an important group of secondary metabolites, are reported to play several roles as medicinal agents, showing antioxidant and anti-inflammatory effects (Shetty 2001). Rosmarinic acid (RA) is a common water-soluble phenolic compound found mainly in Lamiaceae and Boraginaceae plant species (Khojasteh et al. 2014; Georgiev and Weber 2014). RA, derived from caffeic acid and (*R*)-(+)-3-(3, 4-dihydroxyphenyl) lactic acid (Fig. 1), represents one of the most common caffeic esters in plant material, being originally identified in *Rosmarinus officinalis* L. (Ellis and Towers 1970). RA possesses various biological activities, such as antimicrobial, anti-inflammatory, antimutagenic, antioxidant, and cancer chemoprevention, improvement of cognitive performance, prevention of the development of Alzheimer's disease, cardioprotective effects, and reduction of the severity of kidney diseases (Petersen and Simmonds 2003; Bulgakov et al. 2012). RA is also used commercially for food preservation (Park et al. 2008). Although many RA-containing products are available on the market, the pure compound has been used as a commercial drug only recently. These features highlight RA as an interesting product for both pharmaceutical and cosmetic industries.

Fig. 1 Chemical structure of rosmarinic acid (RA)

As the demand for bioactive products grows, plant cell cultures constitute an increasingly attractive alternative to the extraction of metabolites from limited natural resources. Plant cell cultures are also widely employed as a model system to investigate the production of specific secondary metabolites such as phenolic acid derivatives, since they offer experimental advantages both to basic and applied research and to the development of models with scale-up potential (Corchete et al. 1990; Pasqua et al. 2005; Georgiev et al. 2009; Bonfill et al. 2011; Cesarino et al. 2013). Additionally, the commercial cultivation of plant cells contributes to preserving the genetic diversity of wild populations of medicinal plants (Irvani et al. 2010).

Various studies have been carried out on RA production in callus and cell suspension cultures of Lamiaceae species, including *Solenostemon blumei* (Benth.) M. Gomez (syn.: *Coleus blumei*) (Bauer et al. 2004), *Salvia officinalis* (Hippolyte et al. 1992; Kintzios et al. 1999), *Lavandula vera* (Georgiev et al. 2004), *Agastache rugosa* (Kim et al. 2001), and *Ocimum sanctum* (Hakkim et al. 2011a, b). Callus culture for the production of RA has been previously generated from *Satureja hortensis* (Tepe and Sokmen 2007). In our ongoing efforts to investigate natural sources of phenolic acid derivatives, we found *S. khuzistanica*, endemic to the Southwest of Iran, to be a particularly potent species (Hadian et al. 2011). The extracts of the plant contain phenols, flavones, triterpenoids, steroids, and tannins (Moghaddam et al. 2007; Nejad Ebrahimi et al. 2008). The pharmacological and biological properties of *S. khuzistanica* make it potentially valuable for application in the pharmaceutical and food industries (Farsam et al. 2004; Hosainzadegan and Delfan 2009). Owing to over-exploitation of wild plants for commercial purposes and a low propagation rate in nature, *S. khuzistanica* is now almost extinct and is listed as an vulnerable species in Iran (Jalili and Jamzad 1999). Given the importance of the plant and its extracts for clinical medicine (Sangwan et al. 2001), in vitro culture techniques seemed a promising method to study sustainable RA production in *S. khuzistanica* under controlled conditions. In vitro callus induction and RA production in a *S. khuzistanica* callus culture has been recently reported by our laboratory (Sahraroo et al. 2014). In the present study, we extended our work with the aim of optimizing RA production in a *S. khuzistanica* cell suspension culture. This would not only

help to meet the industrial demand for RA, but also lead to the conservation of a valuable species in its natural habitat.

Materials and methods

Chemicals and reagents

Basal media, salts, vitamins, sucrose, plant growth regulators (PGRs) and a RA standard were purchased from Merck (Darmstadt, Germany) and Sigma (Sigma-Aldrich Corporation, St. Louis, MO, USA). Methanol at 99.5 % purity was obtained from Merck (Darmstadt, Germany). High performance liquid chromatography (HPLC) grade water was used throughout the analysis.

Establishment and maintenance of cell suspension culture

Stem cuttings of *S. khuzistanica* were collected from wild-growing plant populations in Khuzistan Province

(33° 00' 334" N, 47° 40' 999" E at an altitude of 490 m) in the Southwest of Iran (Fig. 2a) and were aseptically multiplied (Fig. 2b) as described before (Sahraroo et al. 2014). A voucher specimen of *S. khuzistanica* (MPH-1582) has been deposited at the Herbarium of Medicinal Plants and Drugs Research Institute (MPH), Shahid Beheshti University, Tehran, Iran. A *S. khuzistanica* callus culture was grown in B5 solidified culture media (Gamborg et al. 1968) containing 30 g/L sucrose, 2 g/L, polyvinylpyrrolidone (PVP), 20 mg/L L-glutamin, 200 mg/L casein hydrolysate, 5 mg/L benzyladenine (BA) and 1 mg/L indole-3-butyric acid (IBA) (Sahraroo et al. 2014; Fig. 2c). Subcultures were carried out at 28-days intervals and maintained under 23 ± 2 °C in darkness. Friable calli (Fig. 2d) were used to establish suspension cultures after 6 months of culture. Approximately 1.5 g fresh weight of callus as the initial inoculum was transferred to 100 mL Erlenmeyer flasks containing 20 mL of the abovementioned liquid medium without PVP. The pH of the medium was adjusted with NaOH or HCl to 5.8 before autoclaving for 20 min at 121 °C. The suspension cultures were kept in an incubator rotary shaker under continuous agitation

Fig. 2 Establishment of *S. khuzistanica* cell suspension culture. **a** Wild plant that served as explant source. **b** 3-month-old in vitro propagated plantlets. **c** 30-day-old grown calli from the basal end of the plantlets shoot tips. **d** Proliferated friable calli.

e Cell suspension culture of the plant established in flasks. **f** Photomicrograph of cell suspension culture showing viable cells (v) and non viable cells (nv) ($\times 10$). **g** Photomicrograph of cell suspension culture. Bars = 1 cm

Table 1 Nitrogen sources and concentrations used for nitrogen starvation stress

Treatment	Concentration (mg/L)		Concentration in B5 medium	Total nitrogen (mM)
	KNO ₃	(NH ₄) ₂ SO ₄		
N1	631.8	134.0	¼ NO ₃	8.3
N2	1263.7	134.0	½ NO ₃	14.5
N3	2527.5	134.0	Normal	28.8
N4	2527.5	33.5	¼ NH ₄	25.2
N5	2527.5	67.0	½ NH ₄	25.8

(75 rpm) at 23 ± 2 °C in darkness. Subculturing of suspension cultures to fresh media was done every 21 days. Analysis of growth kinetics and RA production was performed in individual flasks at days ¼, 1, 2, 3, 4, 7, 14, 21 and 28 (Fig. 2e). Cells were separated from the medium by filtration and weighed as fresh weight. The dry weight of the cells was recorded after drying them to a constant weight at 35–40 °C for 5 days. The specific growth rate (μ) was calculated as described by Sujanya et al. (2008).

Cell viability

The Cells viability (Fig. 2f, g) was determined by Evan's blue staining test (Rodríguez-Monroy and Galindo 1999). 2 mL samples from each flask were incubated in 0.25 % Evan's blue stain for 5 min, after which at least 500 cells were counted; this was repeated twice ($n = 6$).

Nitrogen treatment

Experiments were performed in liquid B5 medium with different concentrations of KNO₃ and (NH₄)₂SO₄, as shown in Table 1. Cultures were kept in the dark at 23 ± 2 °C and shaken at 75 rpm. Data were collected every 7 days for 21 days.

Extraction and RA analysis

Ground dried cells of *S. khuzistanica* (20 mg) were suspended in 9 mL methanol and incubated for 10 min in an ultrasonic bath with two vigorous mixings. After filtering through Whatman filter papers, methanolic extracts were evaporated under reduced pressure (vacuum evaporator) at 40–45 °C. The residues were dissolved in 1 mL methanol and filtered through a syringe filter (0.2 μ m, Millipore, Bedford, MA, USA). An aliquot of 20 μ L of the filtrate was injected into the

HPLC for RA analysis. The HPLC column was a Spherisorb ODS-2 (5 μ m) reversed phase 4.6 mm $\times$ 250 mm. Elution was carried out at a flow rate of 1.0 mL/min at 25 °C and detection at 330 nm. Two mobile phases, A and B, were used: A was 0.1 % (v/v) formic acid solution in water, while B was acetonitrile (MeCN). A ratio of 88 % A and 12 % B was applied in the first 30 min, followed by 80 % A and 20 % B for the next 15 min, and finally, 70 % A and 30 % B for an additional 15 min. The chromatographic peak of RA was confirmed by comparing its retention time with that of the reference standard (Fig. 3). Working standard solutions were prepared as recently described by Sahraroo et al. (2014) and then were injected into the HPLC and peak area responses obtained. Standard graphs were prepared by plotting concentration versus area. The equation ($Y = 1636.10x + 328.30$) with correlation coefficients (R^2) of 0.998, generated from the curve by the external standard method was used to calculate the amount of RA present in the cell samples.

Statistical analysis

Experiments were performed in triplicate. Data were analyzed in a factorial completely randomized design (CRD). Data were analyzed using statistical programs SAS (Version 9.1.3). Statistically significant averages were compared using Duncan's multiple range tests. Differences were regarded as significant at $P \leq 0.05$.

Results

Growth kinetics and RA content in suspension cultures

Growth was estimated by measuring fresh weight (FW), dry weight (DW) and specific growth rate

Fig. 3 HPLC-UV chromatograms (330 nm) corresponding to the MeOH extract of in vitro cell culture of *S. khuzistanica* (a) and the authentic standard of rosmarinic acid (b). Separation conditions involved: Spherisorb ODS-2-C18 column

(250 × 4.6 mm, 5 μm); mobile phase H₂O:MeCN, with 0.1 % formic acid, flow rate: 1.0 mL/min with an injection volume of 20 μL

(SGR). Figure 4a show the specific growth rate of the culture that had been grown for 28 days. Suspensions were white-brown in color until day 14, thereafter turning dark brown. Cultures initially grew slowly and showed a lag phase until day 7, followed by a linear growth phase of 14 days. Based on calculations, the specific growth rate of the cells was 1.5 per day and doubling time was 7.6 day. Maximum growth was reached at 21 days of cultivation, after which the growth declined (Fig. 4a). From an initial inoculum of 75.0, 353.5 g/L FW (19.7 g/L DW) was obtained at the end of the growth phase (Fig. 4b). After staining with Evan's blue, viable and dead cells (Fig. 2f, g) were light microscope (Olympus Optical Co., Tokyo, Japan). The percentages of cell viability varied between 94.6 (39.0 × 10⁵ cells/mL) and 87.0 % (34.4 × 10⁵ cells/mL) at the end of the first and third week, respectively. These results confirm that the *S.*

khuzistanica cell suspension culture has been successfully established. The concentration of RA in the cell suspension decreased for 3 days upon subculture and then increased, representing 110.2 and 140.6 mg/g DW at days 3 and 7 after subculture, respectively (lag phase). The RA content also increased during the linear growth phase and the highest accumulation was achieved at day 21 (180.0 mg/g DW). RA production in this culture system was 2.4- and 150-fold higher than in callus cultures (75.0 mg/g DW) and intact plants (1.2 mg/g DW), respectively (Fig. 4c).

Nitrogen source treatment

In a series of experiments, both growth and RA production were monitored in cells cultured with a reduced ratio of nitrogen (Table 1). Nitrogen source treatments had significant effects on fresh and dry

Fig. 4 **a** Specific growth rate of *S. khuzistanica* cell suspension culture. **b** Cell fresh and dry weight of *S. khuzistanica* cell suspension culture during a 28 day period. **c** Rosmarinic acid content in the cell biomass. Bars represent standard deviation ($n = 5$)

weight as well as RA content (Table 2). The effect of nitrogen deficiency on cell FW and DW is shown in Figs. 5a, b. Reduction of nitrate (treatment N1 and N2, Table 1) to $\frac{1}{2}$ or $\frac{1}{4}$ with respect to normal level in B5 medium resulted in a browning of cells and culture media and decreasing FW. In contrast, reduction of nitrate (N1 and N2 media) or ammonium (N4 and N5 media) did not substantially affect the DW of cultures over a 28 day period. The pattern of RA accumulation throughout the experiment differed between the control (N3) and the different treatments (Fig. 5c). Decline in RA production was also seen when the nitrate was reduced to $\frac{1}{2}$ or in the presence of low

amounts of ammonia; however, the decrease occurred almost 2 weeks later than in media with a very low nitrate concentration.

Discussion

As far as our literature survey could ascertain, growth and RA production in *S. khuzistanica* cell cultures have not previously been reported. We have recently demonstrated that *S. khuzistanica* calli retain the capacity to synthesize RA (75.0 mg/g DW) (Sahraroo et al. 2014), which prompted us to test RA production in suspended cultures. Production and optimization of RA by *S. hortensis* L. callus cultures has been previously reported by Tepe and Sokmen (2007). They mentioned that RA is readily accumulated in undifferentiated plant cell cultures, in some cases in concentrations much higher than in the plant itself. Among all treatments they tested, highest fresh weight and RA content were 10.0 g/L and 82.0 mg/g DW after 56 days, respectively. These values were attained in cultures in which the sucrose concentration had been manipulated to alter production (Tepe and Sokmen 2007). We achieved similar RA values to those reported in the aforementioned species using the basal B5 medium containing 3 % sucrose. Highest fresh biomass and RA content obtained from *S. khuzistanica* suspension culture were 353.0 g/L and 180.0 mg/g DW after 21 days, respectively. So far, the highest content of RA in suspension cultures was reported in *Salvia officinalis* (360.0 mg/g DW) by Hippolyte et al. (1992) and *Coleus blumei* (210.0 mg/g DW) by Ulbrich et al. (1985). Literature survey revealed that the growth rate of *Ocimum basilicum* L. and *Lavandula vera* cell cultures was higher than that of *S. khuzistanica*, and in contrast with our results, they reached the highest biomass production on days 14 and 8, respectively (Kintzios et al. 2003; Georgiev et al. 2009). CSC growth measurements indicated a maximum specific cell growth rate of 1.5/day, and a doubling time of 7.6 days. A similar behavior was previously reported in the establishment of other suspension cultures of *Cleome rosea* (Simões et al. 2011) and *Stevia rebaudiana* (Mathur and Shekhawat 2013). When cell viability remained around 50 %, it is considered that the suspension culture establishment has failed (Qui et al. 2009; Mathur and Shekhawat 2013). Culture browning might have been caused by

Table 2 ANOVA test for experimental nitrogen source influencing cell fresh weight, dry weight and RA content

Source of variation	DF	MS		
		FW	DW	RA content
N treatment	4	133.5**	0.0215*	5489.33**
Time	2	3634.5**	6.75**	11078.29**
Time × N treatment	8	57.65**	0.014*	2803.44**
Error	28	8.95	0.006	162.86
CV		9.39	3.77	9.88

MS mean square, DF degrees of freedom, FW fresh weight, DW dry weight, CV coefficient of variation

* $P < 0.05$; ** $P < 0.01$

the release of phenolic compounds to the media. Nutrient deficiency, an increase of lethal products and other restricting factors could have led to cell death and decline in growth after 21 days (Chattopadhyay et al. 2002; Dordas and Brown 2005; Abbasi Kajani et al. 2012). RA can accumulate in cell cultures to amounts much higher than those in intact plants. Its biosynthesis under in vitro conditions was confirmed for several species and diverse in vitro culture systems like: callus cultures, suspension cultures, shoot cultures and hairy roots cultures (Shetty 2007; Matkowski 2008). Our results showed that RA production in *S. khuzistanica* cell culture was 2.4- and 150-fold higher than in callus cultures and intact plants, respectively (Shekarchi et al. 2012; Sahrarou et al. 2014). Cell cultures of some Lamiaceae plants also produced higher amounts of RA than intact plants (Zhong 2001; Georgiev et al. 2004). This enhancement in production could be explained by the controlled environment of in vitro cultures. Appropriate nutrients, their concentrations and environmental factors are known to enhance the yield and productivity of metabolites in plant cell suspension cultures. However, it is essential to study and quantify the effect of selected key medium components on growth as well as product accumulation and strike a balance between the two to enhance the yield and productivity. The most important factors are the chemical constitution of the media used with respect to growth regulators (Nawa et al. 1993; Tepe and Sokmen 2007), nitrogen source and concentration (Do and Cormier 1991; Sato et al. 1996), and carbon source and concentration (Decendit and Merillon 1996; Tepe and Sokmen 2007). The

Fig. 5 a and b Cell fresh and dry weight of *S. khuzistanica* during nitrogen source treatment. c Rosmarinic acid content affected by nitrogen source treatment. Bars represent standard deviation (n = 5)

effect of $\text{NH}_4^+:\text{NO}_3^-$ ratio on the growth kinetics of cells and their secondary metabolism with phenolic structure for some cell cultures has been reported (Do and Cormier 1991; Hirasuna et al. 1991; Srinivasan and Ryn 1993) including the production of RA by *Anchusa officinalis* (De-Eknamkul and Ellis 1985) and *Lavandula vera* (Ilieva and Pavlov 1999). Usually the start of the biosynthesis of secondary metabolites is related to comparatively low levels of nitrogen in the medium. Our results showed that RA content dropped dramatically when nitrate in the culture medium was reduced to $\frac{1}{4}$. Decline in RA production was also seen

when the nitrate was reduced to $\frac{1}{2}$ or in the presence of low amounts of ammonia; however, the decrease occurred almost 2 weeks later than in media with a very low nitrate concentration. This result reflects the importance of nitrate for the biosynthesis of RA, as described by Ilieva and Pavlov (1999), who reported a 50 % decrease in RA production when nitrate was reduced in *Lavandula vera* cells. This finding is also in agreement with the results of Afzal et al. (2006) who obtained faster uptaking of NH_4^+ versus NO_3 in cell suspension culture of *Vigna radiata*. Most plant cell suspensions need both nitrogen forms, but ammonium is required at low concentration (Dougall 1977). It is also well known that biosynthesis of RA starts with the amino acids L-phenylalanine and L-tyrosine. Also, eight enzymes are involved in the RA biosynthesis (Petersen and Simmonds 2003). Since nitrogen is present in the structure of amino acids and enzymes, therefore increase in RA content by nitrogen fertilization could be explained by the increase in biosynthesis of RA precursors (L-phenylalanine and L-tyrosine) and catalyzer enzymes. Two inorganic nitrogen forms are necessary for RA production in cell suspension culture of *S. khuzistanica* but they have not the same importance. Alteration of secondary metabolite production by nitrogen manipulation in the culture media is well described in the literature, with nitrogen deficiency leading to the accumulation of polyphenol compounds (Oksman-Caldentey and Barz 2002).

Conclusion

The present study showed that a suspension culture of *S. khuzistanica* is a viable choice for the production of RA. It can also be seen that *S. khuzistanica* cells grew slowly in comparison with other cell cultures of Lamiaceae species. We also demonstrated that nitrogen sources (especially nitrate) can play an important role in the biosynthesis of RA in a *S. khuzistanica* cell culture. The lowest biomass yield (dry and fresh) and RA production was obtained in a medium containing 8.33 mM initial nitrogen sources, this being the critical concentration for the maintenance of *S. khuzistanica* cell cultures.

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