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Growth Optimization of *Zataria multiflora* Boiss. Tissue Cultures and Rosmarinic Acid Production Improvement

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Abstract: In order to improve the growth of *Zataria multiflora* tissues *in vitro* experiments in different hormonal treatments and culture media were carried out. Shoot cultures, established from plantlets shoot apex, were treated by different levels of 6-benzylaminopurine and 1-naphtalen acetic acid added to Murashige and Skoog medium with two conditions of active charcoal. The highest proliferation rate was obtained with 1 mg L⁻¹ 6-benzylaminopurine, without charcoal. Callus cultures of *Zataria multiflora* were established from plantlets shoot nodes on MS supplemented with 1 mg L⁻¹ kinetin and 0.5 mg L⁻¹ 2, 4-dichlorophenoxyacetic acid. Callus growth rate on three different media (MS salts and vitamins, MS salts with Gamborg vitamins and Gamborg salts and vitamins) was evaluated. The best growth was recorded on Gamborg medium (salts and vitamins) with 0.75 mg L⁻¹ 6-benzylaminopurine and this medium was retained for rosmarinic acid production optimization experimentation. Sucrose and glucose were tested at different doses to compare the effect of carbohydrates on the growing rate and the production of rosmarinic acid in shoot cultures and callus cultures. Experiments also were done at two light exposures (total obscurity or 16 h light/8 h dark). Very high level of rosmarinic acid was detected in callus tissues (158.26 mg g⁻¹ dry weight) as compared with propagated shoots (12.28 mg g⁻¹ dry weight). The dose of carbohydrate had a direct effect on the production of rosmarinic acid dependent of the type of tissues. The best ratio production/growth was obtained in callus cultures treated with glucose 75 g L⁻¹ under light exposure. These results indicate that, in *Zataria multiflora*, the undifferentiated state of tissues appears particularly more efficient for metabolic production *in vitro*, such as rosmarinic acid, in comparison to differentiated shoots.

Key words: Callus culture, micropropagated shoots, Labiatae, metabolite production

INTRODUCTION

Rosmarinic acid (α -o-Caffeoyl-3,4-dihydroxyphenyllactic acid) is an hydroxycinnamoyl ester most commonly found within the families of Boraginaceae and Labiatae (Mizukami *et al.*, 1992). Rosmarinic Acid (RA) has antimicrobial, antiviral and antiphlogistic effects, which make it a valuable product for the pharmaceutical and cosmetic industry (Petersen and Simmonds, 2003).

Zataria multiflora Boiss, a medicinal plant that belongs to the Labiatae, is distributed in Iran, Afghanistan and Pakistan. In Iran, *Zataria multiflora* is used in traditional folk remedies for its antiseptic, analgesic and carminative properties. Recently Zarei Mahmoudabadi *et al.* (2006) suggested a role for RA in the anti-Candida activity of *Zataria multiflora*.

RA has been reported to be accumulated in cultured cells of a number of species including *Coleus blumei* (Razzaque and Ellis, 1977), *Anchusa officinallis* (De-Eknamkul and Ellis, 1987), *Lithospermum erythrorhizon* (Fukui *et al.*, 1984),

Orthosiphon aristatus (Sumaryono *et al.*, 1991), *Salvia officinalis* (Hippolyte *et al.*, 1992), *Ocimum basilicum* (Bais *et al.*, 2002). RA has been also detected in tissue cultures of *Zataria multiflora* (Mohagheghzadeh *et al.*, 2004).

Various approaches have been applied to improve metabolic production in tissue or cell cultures as selection and screening, medium manipulation (nutrients, phytohormones and precursors), culture conditions change (inoculums size, pH, temperature, light) or the use of specialized techniques as elicitors, immobilization, permeabilization (Dornenburg and Knorr, 1995; Ramawat and Merillon, 1999; Namdeo, 2007). Biomass production is an interesting way for the production of secondary metabolites for tissues showing a positive relation between growth and metabolites synthesis.

In this investigation, after optimizing the growth of callus tissues and shoot cultures of *Zataria multiflora* Boiss., we compared the level of RA in these two types of culture and measured the variation in RA production of these tissues under different carbohydrate treatments.

MATERIALS AND METHODS

Source of culture: Seeds of *Zataria multiflora* were collected in Kashan province (Iran). Seeds washed with water supplemented with 0.1% (v/v) Tween 20 were surface sterilized in 70% (v/v) ethanol for 2 min, sodium hypochlorite (1% (v/v)) for 5 min and then were rinsed with sterile distilled water three times. After that, seeds were placed on MS solid medium (Murashige and Skoog 1962) and were maintained at 25°C under 16/8 h light/dark photoperiod.

Growth improvement: Plantlet shoot apex from germinated seeds were cut and grown on MS medium with 30 g L⁻¹ sucrose and 6-benzylaminopurine, BAP (0, 0.5, 1, 1.5 mg L⁻¹) and/or 1-naphtalen acetic acid, NAA (0, 0.2, 0.5, 1.5 mg L⁻¹) with and without 0.1% (w/v) charcoal. The cultures were maintained under 16 h light/8 h dark photoperiod regime and proliferation of shoots was evaluated by the number, the length of shoots and the number of internodes after 4 weeks.

Callus induction from the shoot node culture of *Zataria multiflora* plantlets was realized on MS medium with an hormonal composition of 2,4-D (0.5 mg L⁻¹) and Kinetin (1 mg L⁻¹), casein hydrolysate (200 mg L⁻¹) and sucrose (30 g L⁻¹). Then, the same quantity of callus explants was cultured in three kinds of media (MS+MS vitamins, MS+B5 (Gamborg, 1968) vitamins, B5+B5 vitamins) with BAP (0.75 mg L⁻¹) under 16 h light/8 h dark photoperiod. After 6 weeks of culture, fresh weight was measured and dry weight was obtained by the mean of electronic moisture analyzer (Sartorius MA).

Rosmarinic acid production in tissues: The more efficient culture medium for the proliferation of shoot and callus cultures was selected for the next experimental purpose. Proliferated shoots and callus tissues were treated with sucrose (30 and 60 g L⁻¹) or glucose (60, 75 and 90 g L⁻¹) added to the medium. For each treatment the same quantity of callus tissues was distributed onto the experimental medium. Cultures were maintained under obscurity (callus cultures only) or photoperiod regime as described before and growth measurement and RA analysis was done after 6 weeks of culture for the two experimental cases.

For RA determination, stock standard solutions were prepared by accurately weighing 20 mg of rosmarinic acid (Fluka) and dissolving in methanol (10 mL). The primary solution (500 µL) was dissolved into methanol (25 mL) and was prepared as standard solution (40 ppm). RA was extracted from the tissues according to Hippolyte *et al.* (1992) and RA content was determined according to the method described by Ilieva and Pavlov (1997) with some

modifications. Briefly lyophilized samples (50 mg) were extracted with methanol (5 mL) at 40°C for 90 min with vigorous shaking. The slurry was centrifuged at 10000 rpm for 15 min and the supernatant was evaporated. The residue was dissolved in methanol (2 mL) and was subjected to HPLC analysis using a column, ODS-3, Inertsil 250×4.6 mm. The mobile phase consisted of solvent A (2% (v/v) acetic acid) and solvent B (2% (v/v) acetic acid/acetonitrile (7:3, v/v)) with a linear gradient from 50-30%. The flow rate was set at 1.5 mL min⁻¹ and the RA fraction was determined at 333 nm. The chromatographic peak of RA was confirmed by comparing their retention times.

Statistical analysis: The experiments were done according to a completely randomized experimental design. All data on shoot growth are the means of two independent experiments with 35 shoots/treatment; 25 samples of callus per treatment were analyzed for growth determination. For RA determination, the data represented the mean of three independent samples (callus tissues). Data were statistically analyzed in a randomized complete block design and t-test using SPSS 9.

RESULTS

Shoot culture improvement: Apical shoot explants were cultured in MS medium containing different concentrations of NAA and BAP. Figure 1 indicates that shoot proliferation was more efficient at 1 mg L⁻¹ BAP added alone or in a less extent added with 0.2 mg L⁻¹ NAA to the medium. By these treatments, the growth of these shoots was noticeable for higher length and more elevated internodes number. The presence of activated charcoal did not modify this result. Higher concentrations of BAP resulted in decrease of shoot induction and collagenoses was stimulated.

Callus culture: Callus culture was initiated from the shoot node culture on MS medium with 2,4-D (0.5 mg L⁻¹), Kinetin (1 mg L⁻¹), casein hydrolysate (200 mg L⁻¹) and sucrose (30 g L⁻¹). When subcultured in this initiation medium, the callus tissues turned brown. For this reason, three kinds of media were tested in order to promote the growth of callus tissues. Results indicated that the highest level of the growth was obtained with Gamborg (B5+vit B5) medium with 0.75 mg L⁻¹ BAP (Table 1). In this medium, the growth index was multiplied by 3.6 against MS medium and the tissues grew as a homogenous friable callus with yellow color. This culture medium that gave the best growth of callus tissues was retained for the study of carbohydrate effect on RA production.

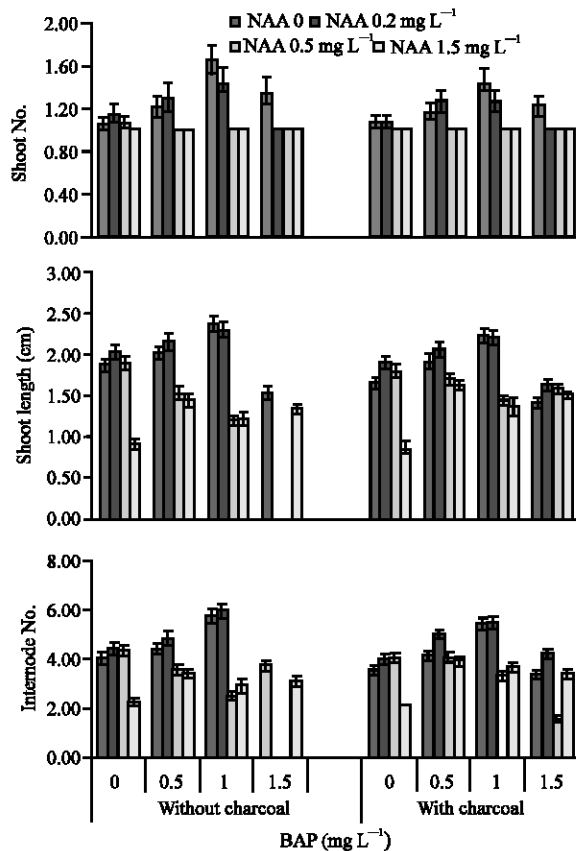


Fig. 1: BAP and NAA effects on the shoot length, shoot and internode number in *Zataria multiflora* shoots culture on MS Medium with or without active charcoal

Table 1: Effect of three different media supplemented with 0.75 mg L⁻¹ BAP on the growth of *Zataria multiflora* callus tissues maintained under 16 h light/8 h obscurity photoperiod, after 6 weeks

Medium	Fresh weight (g) ±SE	Dry weight (g) ±SE	Growth index
MS salts and vitamins	1.281±0.330	0.124±0.034	1.738±0.941
MS salts and vitamins B5	1.867±0.463	0.179±0.053	3.608±1.165
B5 salts and vitamins	2.77±0.537	0.258±0.059	6.243±1.133

Table 2: Effect of sucrose and glucose treatments on callus tissues growth in B5 media supplemented with BAP (0.75 mg L⁻¹) under 16 h light/8 h obscurity photoperiod or total obscurity after 6 week

Carbohydrate (g L ⁻¹)	Fresh weight (g)±SE		Dry weight (g)±SE	
	Obscurity	Photoperiod illumination	Obscurity	Photoperiod illumination
Sucrose				
30	2.07±0.331	1.88±0.136	0.160±0.012	0.116±0.023
60	2.21±0.271	1.99±0.158	0.192±0.026	0.159±0.018
Glucose				
60	1.03±0.270	1.30±0.183	0.101±0.011	0.098±0.007
75	1.76±0.310	1.51±0.299	0.117±0.020	0.113±0.008
90	0.37±0.082	0.33±0.078	0.042±0.007	0.040±0.007

Table 3: Carbohydrate and illumination effects on Rosmarinic Acid (RA) content in tissues cultures of *Zataria multiflora*. after 6 weeks of treatment

Carbohydrate (g L ⁻¹)	Illumination regime	Callus tissues RA (mg g ⁻¹ DW) ±SE	Propagated shoots RA (mg g ⁻¹ DW)
Sucrose			
30	16 h light/8 h obscurity	83.330±0.688	12.28
30	Obscurity	96.342±1.074	-
60	16 h light/8 h obscurity	111.370±0.576	5.68
60	Obscurity	120.220±0.563	-
Glucose			
60	16 h light/8 h obscurity	131.350±0.568	10.12
60	Obscurity	93.760±0.818	-
75	16 h light/8 h obscurity	152.760±0.862	4.03
75	Obscurity	144.618±0.869	-
90	16 h light/8 h obscurity	158.260±1.086	4.52
90	Obscurity	113.578±0.809	-

Growth and rosmarinic acid content: The growth levels under obscurity condition were higher than light condition. The sucrose 60 (g L⁻¹) treatment produced the maximum callus growth and the highest growth reduction was registered for tissues treated by glucose (90 g L⁻¹) under light condition (Table 2).

RA production was improved by the increased of carbohydrate dose added to the medium and the best performance was recorded with glucose in a significant manner (Table 2). There was a positive relation between the synthesis of RA and growth level in sucrose condition but this relation was contrary with glucose. The highest level of RA (158.26±1.086 mg g⁻¹ DW) was obtained in tissues treated by 90 g L⁻¹ glucose. Treatment with 75 g L⁻¹ glucose under light conditions has also increased the RA production by callus tissues (152.76±0.086 mg g⁻¹ DW) without growth reduction in a significant manner (Table 2 and 3).

High doses of carbohydrate in the culture medium of shoot apex reduced considerably the elongation of shoots and the better growth was obtained with 30 g L⁻¹ sucrose (not shown). In this carbohydrate condition the level of RA was higher as regards the other treatments. Nevertheless the production rate of RA in shoot apex culture remained very low (12.28 mg g⁻¹ DW) in comparison with callus tissues (Table 3).

DISCUSSION

The best way to improve the production of metabolites *in vitro* is to combine optimization of growth and metabolites production. Producer tissues have to be selected and optimization of growth and production may be attained by changing culture conditions.

Lack of differentiation in cultures has been reported to be one cause of poor product expression. For a reported number of metabolites a state of differentiation in cultures is required to accumulate product. We previously measured an accumulation of RA in *Z. multiflora* callus tissues (Bernard *et al.*, 2006) but no comparison with differentiated tissues was done. So, by producing high proliferated shoot and callus cultures as described in results, we have been able to compare the capacity of production of these two types of culture. The results presented in the present study show significantly that callus tissues of *Z. multiflora* may produce considerable amounts of RA contrary to propagated shoots. Such an accumulation of RA in undifferentiated cells has been measured in other cultures of Labiatae species (Razzaque and Ellis, 1977; Sumaryono *et al.*, 1991; Hippolyte *et al.*, 1992). These results confirm that accumulation of RA do not required a specialization of cells.

The highest amount obtained under light exposition may result from the activation of phenylalanine ammonolyase (PAL), key enzyme of phenyl propanoid pathway (Mori *et al.*, 2000).

Sucrose and glucose are the preferred carbon source for plant tissue cultures and the concentration of the carbon source may affect cell growth and yield of secondary metabolites. High carbohydrate content in the culture medium generates stress on the tissue thus favoring secondary metabolites pathways activity with the detriment of the growth. But sucrose concentrations up to 6% showed positive effect on cell growth and rosmarinic acid synthesis in *O. aristatus* (Sumaryono *et al.*, 1991) and *S. officinalis* (Hippolyte *et al.*, 1992) cell cultures. Zenk *et al.* (1977) reported that increasing the sucrose concentration in B5 medium up to 7.5% greatly stimulated both cell growth and rosmarinic acid formation in *C. blumei* cultures. But Kim *et al.* (2001) observed a reverse relation between the growth and rosmarinic acid content in the suspension cultures of *Agastache rugosa*. In the callus culture of *Zataria multiflora* treated by glucose, a reverse relation was also noted between the growth and RA accumulation. Nevertheless the best treatment for RA production was 75 g L⁻¹ glucose under light conditions because accumulation of RA was elevated (15.2% of dry weight) and the growth was not affected obviously in this condition. This production was about 40 fold higher than the level of RA detected in culture of *Z. multiflora* by Mohagheghzadeh *et al.* (2004). These differences with the present work may be explained by different conditions of culture (medium, light, phytohormones) and perhaps

also explant origin. The use of 2,4-D to induce callus cultures may be a factor of stress and/or somaclonal variations (Dolezel *et al.*, 1987) and it may be one reason for higher RA accumulation in callus tissues as compared with shoot cultures.

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