Effect of Some Biotic and Abiotic Elicitors on Phenolic Acids and Diterpenes Production from Rosemary (*Rosmarinus officinalis* L.) Leaf and Callus Analyzed by High Performance Liquid Chromatography (Hplc)

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Abstract

The yield of phenolic acids; rosmarinic acid (RA), caffeic acid (CAF) and phenolic diterpenes; carnosic acid (CA), carnosol (CAR), rosmanol (ROL) from rosemary (*Rosmarinus officinalis* L.) was investigated. Aqueous extraction was conducted for leaf and callus tissues, the chemical analysis for extracts was carried out using high performance liquid chromatography (HPLC). Data showed that RA and CAF production was 4.5, 2.7 µg/ml respectively. CA and CAR production reached 3.3, 2.8 µg/ml when the fungal elicitor; *Fusarium oxysporum* was added at the concentration 2.0 mg/l (2×10^4CFU/ml), except ROL which was found at high levels (4.3, 4.2 and 4.6 µg/ml) only from leaf extracts, untreated callus and when callus treated with 0.4 mg/l of $CaCl_2$ respectively.

Keywords: Biotic and Abiotic elicitors, HPLC, Rosemary.

Introduction

Rosemary (*Rosmarinus officinalis* L.) which had long been known as a spice and medicinal herb belongs to the Lamiaceae family and receiving increasing attention due to its antimicrobial, anti-inflammatory, and antioxidative constituents [1]. Plant materials from rosemary are of commertial interest for its essential oil content and its antioxidant compounds. Carnosic acid and carnosol were shown to be the major phenolic diterpenes in leaves of rosemary [2]. The Lamiaceae family seems to be a rich source of plant species containing large amounts of phenolic acids, have already been analyzed using HPLC. The later was used for the determination of rosmarinic acid and other phenolic acids [3]. Phenolic compounds are characterized by having an aromatic ring bearing one or more hydroxyl substituents, including functional derivatives [4]. Rosemary leaves contain (2-3)% phenolic acids such as caffeic acid, chlogenic acid, labiatic acid, neoclorogenic acid and rosmarinic acid [5]. The principle compounds carnosic acid and carnosol were shown to be the major phenolic diterpenes in leaves of *R. officinalis* and *Salvia officinalis* [5]. It has been demonstrated that carnosic acid is a precursor of phenolic diterpenes featuring γ and δ-lactone structure *in vitro*. In the presence of oxygen, carnosic acid is degraded within several days to carnosol and rosmanol [6]. Elicitations are considered to be an important strategy towards improved *in vitro* production of secondary metabolites. In cell cultures, biotic and abiotic elicitors have effectively stimulated the production of plant secondary metabolite [7]. Biotic and abiotic elicitors are often applied for the examination of secondary metabolism and the responses of cultured plant cells to UV, Methyl jasmonate, and Yeast extract have been characterized at various levels of detail in several species [8]. The aims of this study are to investigate the effect of biotic and abiotic elicitors in increasing the amount of rosmarinic acid in rosemary tissues and to determine the phenolic acids and phenolic diterpenes as well using HPLC techniques.

Materials and Methods

This study was carried out in the plant tissue culture lab., Molecular and Biotechnology Department (Atomic Energy Commissions, Syria), during the period 16/11/2008-16/5/2009. Rosemary plant was collected from the garden of Atomic Energy Commissions, Syria), in winter (Mid-January to Mid-February 2008).

Preparation of abiotic elicitors

Calcium chloride ($CaCl_2$) was added as abiotic elicitor at different concentrations (0.0, 0.4 or 0.6 mg/l) to Murashige and Skoog, 1962 medium (MS). The pH was adjusted to 5.8. before autoclaving at 121˚C and a pressure of 1.04 kg/cm^2 for 20 min.

Preparation of biotic elicitors

The fungus *Fusarium oxysporum* and the bacteria *Pseudomonas aeruginosa* were used as biotic elicitors. The strains were kindly supplemented by department of microbiology (AEC-Syria). The fungal cultures (*F. oxysporum*) were maintained as slants on potato dextrose agar (PDA), while the bacterial cultures (*P. aeruginosa*) were maintained on Mueller-Hinton Agar (MH) [9]. Liquid medium was prepared similarly without agar which used for growing fungi or bacteria for elicitor preparation. Cultures that maintained on agar slants, were transferred to 100 ml liquid medium in 250-ml flasks and incubated at room temperature. The bacterial cultures were kept on a rotary shaker (90 rpm), while the fungal cultures were allowed to stand without shaking. The cultures were collected after reaching stationary phase (5-7 days for fungal cultures and 48 hrs for bacterial cultures). Flasks which contain bacterial or fungal cultures were then autoclaved and the solution obtained was stored at 4˚C for future use. The pH was adjusted to 5.8 before autoclaving at 121˚C and a pressure of 1.04 kg/cm^2 for 20 min.

Callus cultures with elicitors

A quantity of 500 mg of callus pieces was placed on the surface of MS medium supplemented with biotic or abiotic elicitors as mentioned previously. Cultured tubes were incubated in growth chamber at 25±2˚C for 16/8 hrs (light/dark) photoperiod.

Preparation of leaf extracts

Samples weighting 50 g of powdered rosemary leaves were soaked into 250 ml of double distilled water (DDH_2O) in glass flask and left for 24 hrs on a rotary shaker, then filtered using sterile filter paper (Wattman no.1) into a clean conical flask and subjected to evaporation in water bath, where the aqueous solvent was evaporated at 100˚C. The extracts were then stored in a refrigerator at 4˚C for future use.

Preparation of callus extracts

Callus was dried at 40˚C for 24 hrs, ground into powder using grinding machine, then subjected to extraction. A quantity of 10 g of callus powder was extracted with 50 ml of DDH_2O in a glass flask and left for 24 hrs on a rotary shaker. The solution then evaporated to dryness using a rotary evaporator at 40ºC in order to remove the water, then the extract was stored at 4ºC in a refrigerator for future use.

Preparation of standard solutions

Stock solutions of standards were prepared by dissolving 0.01g of each standard in 50 ml of methanol (70%) to obtain a final concentration of 200 mg/ml. Stock solutions were then stored in a deep freezer at -20˚C. Sample volume was 15 µl for every injection into HPLC [10].

HPLC Analysis

Symmetry C18 (150 x 3.9 m, 5 µm) water column was used. A mobile phase consisting of Methanol and water (70:30), pH was adjusted to 2.5 using formic acid. The flow rate was 0.5 ml/min and the injection volume 15 µl. All analyses were carried out at room temperature. MWD detector was working in the range of 210-254 nm, and chromatograms were acquired at different wave lengths according to absorption maxima of analyzed compounds.

Identification of the compounds

Phenolic and terpenic compounds were identified by comparison of recorded mass spectra with those of a computer library (Wiley 275 library) or with those of authentic compounds. The identification was confirmed by comparison of their retention indices with those of authentic standard compounds.

Results and Discussion

HPLC analysis of rosemary extracts

The average contents of phenolic and terpenic compounds in rosemary leaves and callus treated with different concentrations of elicitors were presented in Table (1). RA concentration in leaves and untreated callus was 3.3, 3.7 µg/ml respectively. Callus treated

with 0.4 mg/l of $CaCl_2$ produced 4.3 µg/ml of RA, while RA concentration was 3.2 µg/ml when callus treated with 0.6 mg/l of $CaCl_2$. RA concentration reached 3.9 µg/ml at the treatment of 1 ml/l (1.5×10^5) of *P. aeruginosa* elicitor, while it reached 3.1 µg/ml when callus treated with 2 ml/l (3×10^5).

Treatment with 1 ml/l (1×10^4 CFU/ml) of *F. oxysporum* elicitor produced 4.1 µg/ml of RA, while the higher concentration 2 ml/l (2×10^4 CFU/ml) gave 4.5 µg/ml.

Also *P. aeruginosa* at 1.5×10^5 CFU/ml was more effective than 3×10^5 CFU/ml. *F oxysporum* was more effective at 2×10^4 CFU/ml than 1×10^4 CFU/ml in production of RA. The amounts of RA compound were found to be more in callus tiossues than those found in leaves. It was observed that treatment with biotic elicitors (*P. aeruginosa*, *F. oxysporum*), and abiotic elicitors ($CaCl_2$), increased also the productivity of callus tissue from phenolic diterpenes. Callus was accumulated more RA when treated with these elicitors.

Table (1).
Effect of some biotic and abiotic elicitors on phenolic and terpenic compounds quantity of rosemary callus as compared to leaf extracts*.

Compound (µg/ml)	***Concentration (µg/ml)***							
	Leaf	***Untreated Callus***	***$CaCl_2$***		***P. aeruginosa***		***F. oxysporum***	
			0.4 mg/l	***0.6 mg/l***	***1 ml/l***	***2 ml/l***	***1 ml/l***	***2 ml/l***
RA	3.3	3.7	4.3	3.2	3.9	3.1	4.1	4.5
CAF	0.4	0.5	0.3	0.2	2.3	2.2	2.5	2.7
CA	2.8	2.6	2.3	1.9	3.1	2.3	2.4	3.3
CAR	1.7	1.9	0.6	0.5	2.4	1.7	2.1	2.8
ROL	4.3	4.2	4.6	3.2	0.8	0.5	0.6	0.9

**** RA= Rosmarinic Acid, CAF=Caffeic Acid, CA=Carnosic Acid, CAR=Carnosol, ROL= Rosmanol***

The retention times and peak areas (%) for RA production following treatment with biotic and abiotic elicitors are shown in Table (2) as compared with standard RA. From these results, $CaCl_2$ significantly increased RA level in rosemary callus compared with untreated callus (control) and with leaves. The most effective stimulator was *F. oxysporum* at a concentration of 2×10^4 CFU/ml, which gave 4.5 µg/ml of RA. It was found that fungal elicitor at 2.0 ml/l was more effective among others, which produced 15.5891% of RA, while bacterial elicitor gave 13.3219%. $CaCl_2$ at the concentration 0.4 mg/l produced 14.8751% of RA compared to control treatment which gave 13.0921%.

Table (2).
Retention time and peak areas (%) for RA obtained from rosemary callus treated with some biotic or abiotic elicitors analyzed by HPLC.

RT (min)	***RA Peak area%***	***Treatment***
15.112	13.0921	Untreated callus
15.092	14.8751	0.4 mg/l $CaCl_2$
15.112	13.3219	1.5×10^5 CFU/ml *P. aeruginosa*
15.092	15.5891	2×10^4 CFU/ml *F. oxysporum*

It was noted that tannin-like phenolics are considered to be plant defense metabolites and their production in the plant increases under stress conditions. This fact is used to stimulate the production of RA in cell culture with considerable results [11]. It was found that RA is the main phenolic acid detected in all

investigated samples, the other phenolics were presented at lower concentrations, which is in accordance with [12].

Contents of phenolic and terpenic compounds in rosemary samples are relatively comparable to those reported in literature, but it has to be emphasized that results may differ because of several factors may influence significantly on the phenolic and terpenic compounds in this plant, time of extraction, temperature, pH and the type of solvent used were considered to avoid chemical modification, degradation and other biochemical changes of the components in the sample [10]. CAF was presented in all rosemary samples but in lower concentrations. Analysis of leaf extract produced 0.4 µg/ml of CAF, while callus extract produced 0.5 µg/ml. Callus treated with $CaCl_2$ produced 0.3 µg/ml at the concentration of 0.4 mg/l of $CaCl_2$, while it gave 0.4 µg/ml at 0.6 mg/l of $CaCl_2$. Treatment with *P. aeruginosa* as elicitor at 1.0 ml/l produced 2.3 µg/ml of CAF, while treatment with *P. aeruginosa* at 2.0 ml/l produced 2.2 µg/ml. CAF productivity increased following treatment with *F. oxysporum as* eliciter, it produced 2.5 µg/ml when treated with 1.0 ml/l, while it reached 2.7 µg/ml after the addition of 2.0 ml/l of *F. oxyspoum* to the callus culture medium.

Levels of CA reached 2.8, 2.6 µg/ml in leaves and callus extracts respectively. Callus treated with 0.4 mg/l of $CaCl_2$ gave 2.3 µg/ml of CA, while 1.9 µg/ml was obtained when $CaCl_2$ added at 0.6 mg/l to the callus culture medium. *P. aeruginosa* gave 3.1 µg/ml of CA in callus cultures treated with 1 ml/l, while it recorded 2.3 µg/ml of CA when treated with 2 ml/l. Callus treated with *F. oxysporum as elicitor* at 1 ml/l gave 2.4 µg/ml of CA, while it recorded 3.3 µg/ml after treatment with 2 ml/l.

CAR levels reached 1.7 µg/ml in leaf extract, while it produced 1.9 µg/ml in callus extracts. Treatment with $CaCl_2$ at a concentration of 0.4 mg/l produced 0.6 µg/ml, whereas it gave 0.5 µg/ml when callus treated with 0.6 mg/l of $CaCl_2$. Addition of 1 ml/l *P. aeruginosa* as elicitor recorded 1.9 µg/ml, while it recorded 1.7 µg/ml when callus treated with 2 ml/l. *F. oxysporum* addition to the callus cultures resulted in production of 1.2 µg/ml of CAR, while it reached 2.8 µg/ml after the addition of 2 ml/l.

ROL production recorded 4.2 µg/ml in rosemary leaf extracts, while it gave 4.3 µg/ml in untreated callus. When callus tissues treated with 0.4 mg/l of $CaCl_2$ they gave 4.6 µg/ml while they yielded 3.2 µg/ml of ROL when callus treated with 0.6 mg/l. Callus treated with 1 ml of *P. aeruginosa* as elicitor produced 0.8 µg/ml of ROL, while it gave 0.5 µg/ml when 2 ml/l were added. *F. oxysporum* added at 1 ml/l produced 0.6 µg/ml of ROL, while it reached 0.9 µg/ml when the concentration of elicitor increased to 2 ml/l. Although numerous phenolics, flavonoids and diterpenes have been reported in rosemary extract, only RA, CAF, CA, CAR and ROL were presented in sufficient amount to be identified and quantified in this study. The determined compounds were similar in their content and concentration to the data reported in a previous study showing RA and CA as the most abundant compounds in rosemary extracts [13].

It was concluded that phenolic compounds are synthesized in plant cells via the phenylpropanoid pathway localized in the cytosol, with the final stages of biosynthesis and accumulation in the vacuole [14]. They are present in leaves, stems, petals, callus which are consistent with the results obtained for RA in this study. On the other hand, diterpenes such as CA are synthesized in plants via the non-mevalonate isopentenyl diphosphate pathway [15]. Results in this research show that CA in leaves (which are photosynthetic tissue) compared with those non-photosynthetic tissues, such as callus. The concentration of growth regulators often affected [16, 17]. In the present study unorganized callus of rosemary could synthesize CAR, but at low concentrations. This finding raised the following question, whether this compound (CAR) is a true compound of callus cultures or a product of degradation of CA. This study provided the evidence that this compound is present in undifferentiated cultures. So far neither CAR nor CA have been found in undifferentiated rosemary callus cultures [18]. Biotic and abiotic factors lead to increase the production of secondary metabolites in rosemary callus.

F. oxysporum enhanced accumulation of RA and the phenolic diterpenes in callus tissue. These results are in agreement with [19], who reported similar results for enhancing RA accumulation in suspension cultures of *Coleus blumei* treated with fungal elicitors.

It was reported by [10] that there is strong seasonal variation in concentrations of phenolic acids and phenolic diterpenes in rosemary. Usually solar radiation during the summer, resulting in water and light stress, decreases concentrations of some phenolics, while they are increased during winter. Carnosic acid may give rise to carnosol after enzymetic dehydrogenation or to highly oxidized diterpenes such as rosmanol, isorosmanol. Oxidative stress *in vivo* induced by drought or high light stress enhances the formation of highly oxidized diterpenes due to the antioxidant activity of CA [6].

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الخلاصة

درست انتاجية نبات اكليل الجبل (*Rosmarinus officinalis* L.) من الحوامض الفينولية: حامض الروزمارينك (RA)، حامض الكافيك (CAF) والتربينات الثنائية الفينولية: حامض الكارنوسك (CA)، الكارنوسول (CAR)، الروزمانول (ROL). تم اجراء الاستخلاص المائي لأوراق وكالس نبات اكليل الجبل، اجري التحليل الكيميائي للمستخلصات باستعمال تقنية HPLC. اظهرت البيانات بان انتاج RA و CAF كان 4.5 و 2.7 مايكروغرام /مل على التوالي. وصل انتاج CA و CAR الى 3.3 و 2.8 مايكروغرام /مل على التوالي عند اضافة المؤثر الفطري *Fusarium oxysporum* بتركيز 2 ملغم/ لتر(2×10^4 CFU/ml)، عدا الروزمانول ROL والذي وجد بمستويالت عالية بلغت 4.3، 4.2 و 4.6 مايكروغرام / مل لكل من مستخلص الاوراق، الكالس غير المعامل والكالس المعامل بتركيز 0.4 ملغم /لتر من كلوريد الكالسيوم $CaCl_2$ على التوالي.