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SECONDARY METABOLITE PRODUCTION FROM ROSEMARY(*ROSMARINUS OFFICINALIS* L.) USING PLANT TISSUE CULTURE TECHNIQUE

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ABSTRACT

The yield of *Rosmarinus officinalis* L., for some secondary metabolites, was investigated. Yield of tissue culture was compared with intact plant production. Many experiments were implemented. Callus was initiated on MS medium supplemented with a combination of 0.0, 0.1, 0.5, and 1.0mg/L of BA and 0.0, 0.5, 1.0, 1.5 and 2.0 mg/L of 2,4-D. Maximum callus fresh weight was obtained by the combination of 0.1 mg/L of BA and 0.5mg/L of 2,4-D, under 16hrs light and 8hrs dark. Highest callus fresh weight initiated in full dark occurred at 0.5mg/L BA and 0.5mg/L of 2,4-D. Many combinations were examined for callus maintenance. It was found that the best combination for callus maintenance under 16hrs light and 8hrs dark was 0.5mg/L of BA and 0.1mg/L of 2,4-D, where as maximum callus fresh weight was obtained in the absence of light when 0.5mg/L of both plant growth regulators were added to the culture medium. The plant content of essential oil was estimated monthly. Essential oil was at its highest levels during the winter season. The chemical analysis was carried out using gas chromatography-mass spectrometry (GC-MS). The essential oil of rosemary leaves was characterized by a higher content of ά-pinene (21.093%), camphor (12.889%) borneol (9.808%) eucalyptol (8.257%) and camphene (7.973%), while the main constituents of callus essential oil were camphor (19.961%), borneol (19.160%), thymol (8.497%) and eucalyptol (10.126%).

Key words: Rosemary plant, growth regulators(2,4-D;BA),secondary metabolites, gas chromatography.

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إنتاج مركبات الأيض الثانوية من نبات اكليل الجبل *Rosmarinus officinalis* L. باستخدام تقنية زراعة الأنسجة النباتية

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الخلاصة

درست إنتاجية إكليل الجبل *Rosmarinus officinalis* L. لبعض مركبات الأيض الثانوية بطرائق الزراعة النسيجية وقورنت مع تلك المنتجة من النبات الأم. نفذت العديد من التجارب، إذ حفز الكالس على النشوء في وسط MS المجهز بتوليفة من التراكيز 0.0، 0.5 أو 1.0 ملغم/لتر من السايتوكاينين BA و 0.0، 0.5، 1.5 أو 2.0 من الأوكسين 2,4-D. كان أفضلها من حيث وزن الكالس الطري التوليفة 0.1 ملغم/لتر من BA مع 0.5 ملغم/لتر من 2,4-D عند 16 ساعة ضوء/ 8 ساعة ظلام وان أعلى زيادة في وزن الكالس الطري كانت في ظرف الظلام كا عند المستويات 0.5 ملغم/لتر من BA و0.5 ملغم/لتر 2,4-D. أختيرت عدة توليفات من BA و2,4-D لإدامة الكالس ووجد إن أفضل توليفة لإدامة الكالس بوجود الضوء هي 0.5 ملغم/لتر BA و0.1 ملغم/لتر من 2,4-D وفي الظلام كانت 0.5 ملغم/لتر لكل من منظمي النمو. تم إختيار محتوى النبات الكامل من الزيوت الطيارة في أشهر السنة ووجد ان أعلاها يحصل في أشهر الشتاء. كما تم تحليل الزيوت الطيارة المستخلصة من الأوراق وأنسجة الكالس بوساطة جهاز Gas Chromotoography Mass (GC-MS) Spectrophotometry وتم تحديد معظم المركبات الموجودة فيها وتقدير نسبها المئوية. ووجد إختلاف في المركبات الموجودة في الزيوت الطيارة ونسبها المئوية بين أوراق النبات الكامل وأنسجة الكالس، إذ بلغ الفابينين 21.093%، كامفور 12.889%، بورنيل 9.808%، كاليبتول 8.257% وكامفين 7.973% هي المركبات الأكثر شيوعاً في مكونات النبات الكامل. أما أنسجة الكالس فقد تميزت بوجود كامفور 19.961%، بورنيل 19.16%، ثايمول 18.497%، نيوكاليبتول 10.126%.

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) which belongs to the Labiateae family is described as a medicinal plant, is a small evergreen shrub, growing to over 1.5 meter(1). The production of secondary metabolites using plant tissue culture techniques is a strategy already adopted by many plant biotechnologists. There are many examples where yield in plant cell cultures is exceeded the parent plant. Callus cultures, cell suspensions and differentiated cultures are usually used as sources for production, when cultures are considered suitable for commercial production; cultures are grown in bioreactors(2).

As a medicinal plant, rosemary has been used as an external stimulant and as a relaxant for nervousness muscle spasms, and headaches. It was used in wines as a carminative, and it is through to act as a stimulant to the kidneys(3). Several studies have proved that rosemary essential oil possesses anti-oxidative and antimicrobial activities which are used for food preventative and human microbial diseases control(4). Plants are probably the best cell factories on this planet from which more than 100,000 low molecular secondary metabolites have been discovered, with the estimated total number in plants exceeding 500000. Plant metabolites not only are used for food purpose but also serve as an important historical source of medicines(5). Many of these secondary products are utilized as medicine, scent, dyes, pesticides and are of commercial importance. Nevertheless, their ecological roles have been extensively studied and have received more attention in the past few years. Environmental factors including biotic and a boitic stimuli, carbon nutrition balance, genotype and ontogenesis usually control and regulate the biosynthesis of secondary metabolites in plants(6).

Many publications have presented data on the essential oil composition of rosemary. Essential oil alone comprises more than sixty individual components. The quantitative composition of rosemary essential oils is well defined and fixed. However depending on the geographical origin and the specific ecological sites from which the rosemary is collected for distillation, its quantitative composition can vary greatly(7). Chemically, essential oil is derived from terpenes and their oxygenated compounds(8). Oxygenated compounds derived from these hydrocarbons include, aldehydes, esters, ethers, ketones, phenols and oxides. Their effects on blood circulation, nerve growth, nucleic acid, liver, protein, lipid, carbohydrate, cholesterol metabolisms are also monitored as well as any effect on the activity of the adrenal gland and the body's immune system(9).

There are a series of distinct advantages to produce a valuable secondary product in plant cell culture, rather than *in vivo* (whole) crop plant. These include production of more reliable, simpler, and more predictable isolation of the photochemical in rapid and efficient, as compared to extraction from complex whole plants. Compounds produced *in vitro* can directly parallel compounds as in the whole plant. Interfering compounds that occur in the field grown plants can be avoided in the cell culture. Cell cultures are a super model to test elicitation(10). Research in the area of plant tissue culture technology has resulted in the production of many pharmaceutical for new therapeutics. Advances in this area have made possible the production of a wide variety of pharmaceuticals like alkaloids, terpenoids,

steroids, saponins, phenols, flavonids and amino acid. Successful attempts to produce some of these valuable substances in relatively large quantities by cell cultures(11).

The main aim of this project was to enhance secondary metabolites production using plant tissue culture techniques, then quantify and identify essential oils in plant leaves and callus cultures and the comparison between the yield of essential oil in leaves and callus cultures.

MATERIALS AND METHODS

Rosemary plant, *Rosmarinus officinalis* was collected from a local public garden in Sulaimani city. Plant leaves were used as a source of essential oils and ethanol extract. Mature leaves were excised, rinsed with tap water for 10 minutes then submerged in 1% of sodium hypochlorite for 15 min., rinsed with sterilized distilled water, then submerged in 70% ethanol for 30 seconds. Explants were then rinsed with sterilized water for three times inside a laminar air flow cabinet. The ends of each leaf were cut to remove tissues affected by sterilization solution. Finally, leaf disks 1cm in diameter was transferred to the culture medium. Murashige and Skoog basal salts (MS) medium was prepared as stock solutions (12), then 100mg/L myoinositol, 30000mg/L sucrose were added. The pH was adjusted to 5.7. A quantity of 8g/L agar-agar was added to the medium.

The growth regulators 2,4-Diclorophynoxy acetic acid (2,4-D) and benzyl adenine (BA) were added at 0.0, 0.5, 1.0, 1.5 or 2.0mg/L and 0.0, 0.1, 0.5 or 1.0mg/L, respectively. Medium components were placed on a hotplate magnetic stirrer until boiling. Aliquots of 10ml were dispensed into 50ml culture tube then left at room temperature until use. Tissue culture medium was autoclaved at 12°C and a pressure of 1.04kg/cm^2 for 15min., while instruments were wrapped with aluminum foil, placed in a stainless steel tray and autoclaved as mentioned above or placed in the oven at 140-160°C for 4hrs(13). Leaf explants were placed on MS medium containing 2, 4-D and BA.

The response of these explants to callus initiation was evaluated after 45 days in culture. After surface sterilization, explants were placed into the culture vessels under aseptic conditions. All replicates were placed under two different conditions. First group was placed in the growth-chamber (incubator) at 25°C for 16/8hrs light/dark photoperiod using day light inflorescent with light intensity of 1500lux and the other half was incubated under the same temperature but in full dark. A quantity of 50mg of initiated callus was removed from explants using forceps and scalpel, then sub cultured on fresh medium supplemented with the same concentrations of 2,4–D and BA and placed in the growth chamber. Leaves were collected from field grown plants.

Callus cultures were harvested from samples subjected to both light and dark conditions. Essential oil was extracted by hydro-distillation from dried plant leaves for 3 hrs using Clevenger-type apparatus (14). A quantity of 25g of dried leaves was placed in 125ml distilled water then placed on a heater adjusted at 60°C for 3hrs, then the essential oil was separated and stored at 10°C (14). A quantity of 25g of dried homogenized leaves were mixed with 125ml of ethanol (100%) and left at room temperature for 24-28 hrs, filtered through a filter paper (Whatman No.1), concentrated for a proper volume using rotary

evaporator at 40°C (15). Gas chromatography mass spectrometry (GC-MS) analysis was performed using a Varian chromatograph.

The analysis of volatile oils were run on a (GC-MS:1200, ion trap MS) using a Varian MS capillary column factor four VF-5 ms low bleed column ($30mm \times 0.25mm$ D, film thickness; 0.25μm # CP8944; carrier gas helium, adjusted to a linear velocity of 34 cm/s). The oven temperature program was 230°C, quadruple temperature 150°C, ionization energy, 70eV; scan range, 50-505 amu; 2.2 scans/s. injector port: 250°C and detector: 280°C. The volume of the injected samples was 0.1μL originally taken from a concentration of 1% solution which diluted with hexane in the split ratio of 1:50. Essential oil analysis was conducted by hydro-distillation of dried leaves and callus cultures in Clevenger-type apparatus. GC-MS analyses were performed(16).

Trepans and steroids were detected by taking 1g of dried ethanol extract suspended with a few drops of chloroform, and then a drop of acetic anhydrate and a drop of concentrated sulfuric acid were added. Appearance of brown color indicated the presence of terpenes. The appearance of dark blue color after few minutes indicated the presence of steroids in the extracts(17). Saponins were detected in aliquots of 5ml plant extract added to 1-3ml of ferric chloride solution. The development of white precipitates indicated the presence of saponins(18). Flavonoids were detected after ethanol extraction of the plant material which partitioned with petroleum ether. The aqueous layer was mixed with the ammonia solution. The appearance of dark color was an indication for the presence of flavonoids(19). Data were analyzed using two ways ANOVA test. Least significant differences (LSD) were computed. Means were compared at probability of ($p \leq 0.05$). Statistical analysis was performed using SPSS version 11.5 (SPSS 12 Inc. Chiago 111).

RESULTS AND DISCUSSION

The effect of different concentrations of 2,4-D and BA on callus fresh weight initiated on leaf explants is shown in Table(1). The highest mean callus fresh weight initiated achieved on explants cultured on Ms medium supplemented with 1.0mg/L of BA reached 1772.14mg, and at the concentrations 0.5mg/L and 0.1mg/L led to a significant increase in callus fresh weight reached (1564.320 and 1619.440mg) subsequently compared with 0.0mg/L of BA (1172.52mg).

Maximum callus fresh weight (2644.58mg) occurred on explants cultured on a medium containing 0.5mg/L, of 2,4-D, while the concentrations of 1.0mg/L, 1.5mg/L and 2.0mg/L produced callus fresh weight of (2127.270mg, 1683.687 and 989.752mg) respectively. All values were significantly different at ($p \leq 0.05$) from each other as well as with the control treatment (215.246mg).

The interaction between the two growth regulators achieved maximum callus fresh weight in the combination of 0.1mg/L BA and 0.5mg/L 2,4-D reached 3018.32mg, and the lowest was 142.866 mg in the combination of 0.1mg/L BA and 0.0 mg/L 2,4- D. All treatments were significantly different compared with the control one (11.7mg).

Table(1) : Effect of different concentrations of 2,4- D and BA and their interaction on callus fresh weight (mg) grown at 16hrs light and 8hrs dark, (n= 12).

BA (mg/l) / 2,4-D (mg/l)	0.0	0.1	0.5	1.0	Mean
0.0	11.700	142.866	259.841	446.575	215.246
0. 5	2426.190	3018.320	2331.650	2802.150	2644.580
1.0	1961.020	2091.800	2035.490	2420.760	2127.270
1.5	989.483	1592.260	1853.900	2299.110	1683.687
2.0	474.208	1251.970	1340.710	892.125	989.752
Mean	1172.520	1619.440	1564.320	1772.140	
LSD 0.05	2,4-D = 208.5759 BA= 186.5559 2,4-D× BA = 418.6539				

The effect of different concentrations of BA and 2,4-D on mean callus fresh weight is shown in table 2. Mean callus fresh weight reached maximum value (994.22mg) at a BA concentration 0.5mg/L, while the concentrations 0.1mg/L, 0.5mg/L and 1.0mg/L led to a callus fresh weight of (897.00, 994.22 and 892.85mg) subsequently. This was significantly higher than callus fresh weight formed on explants not treated with BA.

Mean callus fresh weight initiated on explants treated with different concentrations of 2,4-D was fluctuated. It reached maximum fresh weight (1238.83mg) in the culture supplemented with 0.5mg/L. Callus fresh weight decreased to (829.625, 605.452, 720.318 mg) at 2,4-D levels 1.0, 1.5 and 2.0mg/L consequently. These were significantly higher than callus initiated in the absence of 2,4-D (484.69mg). Maximum callus fresh weight was achieved in a balanced ratio (0.5mg/L) of BA and 2,4-D reaching 1649.4mg, and all the interaction treatments were significantly different in comparison with the control one (98.067mg) as shown in Table(2). Callus induction requires a balanced ratio of auxin(s) and cytokinin(s) as stated by Skoog and Miller(20). In a number of plant species, callus induction favors higher auxins than cytokinins. Rosemary leaf explants may contain some levels of endogenous auxins that made a balanced ratio with the exogenous auxin and may be even cytokinin. Establishment of a callus on the explants was illustrated by Dodds and Roberts (21) who divided the process into three developmental stages: induction, cell division and differentiation. The period of these phases depends mainly on the physiological status of the explants cells as well as the culture conditions including the appropriate combination of plant growth regulators(20). Production of large quantity of callus fresh weight as has been recorded in Table(1) is advantageous, since the yield of secondary metabolites is a proportional to biomass yield. Quick callus induction and fast growth rate is favorable in tissue culture experiments and production of secondary metabolites alike.

Table(2): Effect of different concentrations of 2,4-D and BA and their interaction on callus fresh weight (mg) grown in a full dark (n= 12).

BA (mg/l) / 2,4-D (mg/l)	0.0	0.1	0.5	1.0	Mean
0.0	98.067	321.250	785.53	729.90	484.695
0. 5	468.74	1212.250	1649.40	1624.90	1238.830
1.0	462.33	905.208	1159.60	791.29	829.625
1.5	340.90	628.416	783.64	668.84	605.452
2.0	225.28	1417.860	588.80	649.32	720.318
Mean	319.06	897.00	994.22	892.85	
LSD 0.05	2,4-D = 132.9696		BA= 118.9316	2,4-D× BA = 285.4736	

Calli that induced on leaf explants were dissected from explants grown on the best combination of BA and 2,4-D, then inoculated into the fresh medium containing the same combinations of plant growth regulators used for callus induction in order to determine the appropriate concentration for callus maintenance (Table 3).

Inclusion of BA at the concentration of 0.5mg/L significantly increased callus fresh weight (2499.728mg), when the medium supplemented with 0.1mg/L and 1.0mg/L, it gave (2153.625 and 2265.862mg) respectively. These values were significantly higher than the treatment where no BA was supplemented (1581.964mg).

The highest callus fresh weight obtained in 2,4-D treated callus cultures were at the concentration 1.0mg/L recording 2572.508 and the callus fresh weight 2572.508, 2413.300 and 2416.330mg was recorded at 2,4-D levels 1.0, 0.5 and 1.5mg/L, respectively which were all significantly higher than 2,4-D free treatment. The later exhibited the lowest fresh weight (1099.041mg). The interaction between the two growth regulators at the levels of 0.5mg/L BA and 1.0mg/L of 2,4-D resulted in a maximum callus fresh weight reached 3054.135mg, and all the treatments were significantly higher than the control one (455.978mg). Callus tissues showed a reduced fresh weight when inoculated into a medium lacking both growth regulators.

The best combinations of 2,4-D and BA used for callus induction at darkness was examined to determine the appropriate combinations for callus maintenance (Table 4). Inclusion of 2,4-D at the concentration of 0.5mg/L gave the highest callus fresh weight recorded (2284.287mg). Higher levels (1.0 and 1.5mg/L) showed a reduced callus fresh weight (1971.104 and 1743.233mg) respectively. All 2,4-D treatments were significantly higher than the lowest fresh weight (941.327mg) obtained in 2,4-D free medium. Treatment with BA at the concentrations 0.1, 0.5 or 1.0mg/L led to a significant increase in callus fresh weight reached (1660.206, 2007.045, and 2000.412mg) subsequently. The last two values were not significantly different.

Table(3): Effect of different concentrations of 2,4-D and BA and their interaction on callus fresh weight (mg) initiated on leaf explants grown for 16hrs in light and 8hrs in dark and grown on a maintenance medium. Initial weight was 100mg (n= 12).

BA (mg/l) / 2,4-D (mg/l)	0.0	0.1	0.5	1.0	Mean
0.0	455.978	1042.200	1375.185	1522.800	1099.041
0. 5	1956.971	2347.192	2717.228	2631.807	2413.300
1.0	1736.785	2895.778	3054.135	2603.335	2572.508
1.5	2178.121	2329.328	2852.364	2305.507	2416.330
Mean	1581.964	2[illegible]3.625	2499.728	2265.862	
LSD 0.05:	2,4-D = 186.3632	BA= 186.3632	2,4-D× BA = 372.7263		

Table (4): Effect of different concentrations of 2,4-D and BA and their interaction on callus fresh weight (mg) initiated on leaf explants in the absence of light grown on a maintenance medium. Initial weight was 100 mg (n= 12).

BA (mg/l) / 2,4-D (mg/l)	0.0	0.1	0.5	1.0	Mean
0.0	321.433	912.758	1237.691	1293.425	941.327
0. 5	1813.266	2454.808	2534.875	2334.200	2284.287
1.0	1659.866	1717.383	2150.175	2356.991	1971.104
1.5	1294.583	1555.875	2105.441	2017.033	1743.233
Mean	1272.287	1660.206	2007.045	2000.412	
LSD 0.05:	2,4-D = 163.2781	BA= 163.2781	2,4-D× BA = 326.5561		

The interaction between the two growth regulators at the levels of 0.5 and 0.5mg/L BA and 2,4-D resulted in a maximum callus yield reached (2534.875mg), as shown in Table (4). All the interaction values were significantly higher than the control treatment.

According to the results stated above, callus was induced and maintained on MS medium containing different concentrations of 2,4-D and BA under two regimes (16hrs light, 8hrs dark or full dark). Cultures grown on MS medium under a photoperiod of 16hrs light and 8 hrs dark were mostly green, grew fast, large in size, and friable. While callus induced and maintained in dark was mostly pale white, grew slowly. Light has been shown to be an important regulator for betacyanin formation in betalain- producing cell cultures and whole

plants in a number of species. Intensively pigmented cell cultures became colorless when maintained in the dark (22). Induction and maintenance of callus cultures of rosemary seem
to favor moderate levels of 2,4-D and BA rather higher levels. Increasing the levels of the two plant growth regulators suppressed callus growth. Results revealed that the concentration 0.5mg/L of 2,4-D and 0.5mg/L BA was the best combination in both regimes for callus induction and maintenance. On contrary to most plant species that requires higher auxin levels than cytokinin(23). Rosemary required higher cytokinin/auxin ration for callus induction in light, while a balanced ration for callus induction in dark and during maintenance.

Data presented in Table(3) showed that generally callus fresh weight increased with increasing BA levels in the medium without 2,4-D, and decreased by increasing 2,4-D levels in the medium lacking BA. This may be due to the high endogenous auxin levels in rosemary leaves. Callus cultures may be affected by light duration(23). Light has significantly enhanced callus initiation, the highest callus fresh weight was found in cultures initiated at 16hrs light, 8hrs dark. However, the highest callus fresh weight (3054.135mg) was found in cultures grown on a maintenance medium at 16hrs light, 8hrs dark and the highest callus fresh weight in the absence of light grown on a maintenance medium was (2534.875mg) as shown in Table(4).

This large difference in the callus fresh weight between the two light regimes confirms the critical role of light in callus yield and maintenance. The daylight fluorescent tubes give important emission bands at red and blue spectra wavelength. These conditions have been found to give good culture growth rates(23).

Although various aspects of light-mediated regulation of enzymes related to photosynthesis and chloroplast development have been reported(24). The increase of callus mass is important for the production of secondary metabolites since they are proportionally related(10). It would be convenient from the practical point of view to induce and maintain callus on the same growth nutrients and plant growth regulators. This was not applied for rosemary tissue cultures.

Results showed that the quantity of the essential oil in the rosemary leaves is not the same through the year. It reached its maximum values in February (2.64ml/100g) and the minimum level in September and December Figure(1). This is confirmed by Laitinen(7) who stated that the yield of essential oil is largely affected by climatic conditions. Also results showed that productivity of rosemary essential oil recorded maximum levels at winter time when temperature is low. Quantities of essential oil accumulated in leaves, stems, callus grown in dark and callus grown in light were not the same. Rosemary leaves contained (2.64ml/100g), while stems (0.25ml/100g).

Callus grown in dark recorded (1.442ml/100g), while callus grown in 16hrs light/8hrs dark gave higher quantity of essential oil (3.605ml/100g) than the rest using the same extraction procedure. The tissue cultures of some medicinal plants produce secondary metabolites more than intact plant(25). This was confirmed in Figure(2).

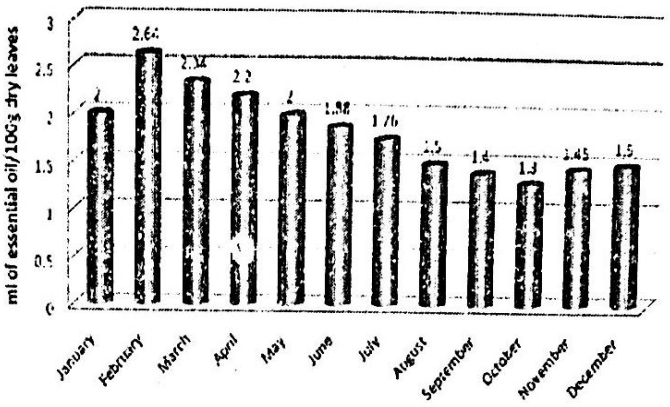

Fig.(1): Fluctuation of essential oil content in *R. officinalis* leaves through out the year round in samples collected from Sulaimani city

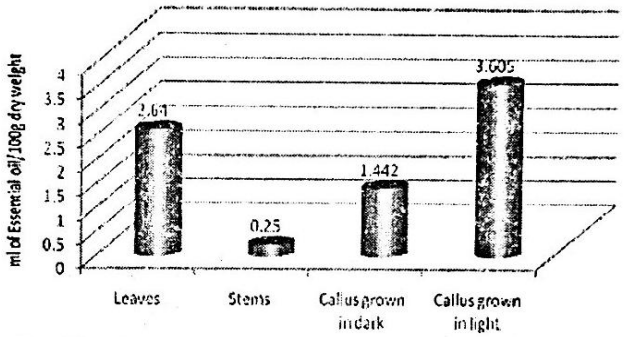

Fig.(2): Essential oil content in leaves and stems of Rosemary collected in February compared with callus cultures subjected to light regimes.

Light may affect several metabolic processes during secondary metabolite production.Light affects several metabolic processes during chloroplast development in plant. Blue light induced responses induced lipid biosynthesis, increased carbon: nitrogen assimilation and oxidative phosphorylation. Significant increases in total lipid, carotenoids or RNA occur only after adequate photo- phosphorylation(26).

GC- MS methods were used for analysis of rosemary essential oil in plant leaves and callus extracts. The essential oil composition found in samples is reported in Table(5). Samples were tested one month after hydro- distillation. Twenty four compounds were identified in rosemary leaves and twenty compounds were identified in callus grown at 16 hrs light, 8hrs dark.

Chemical analysis showed that essential oil in rosemary leaves is characterized by a high content of α-pinene (21.093%), camphor (12.889%), borneol (9.808%), eucalyptol (8.257%), camphene (7.973%), thymol (6.365%) and D-Limonene (4.010%). The most abundant compounds in callus cultures are camphor (19.961%), borneol (19.16%), thymol (19.661%), eucalyptol (10.126 %), α-terpinyl isovalerate (5.340%) and 5-Caranol (3.936%). α-pinene showed the highest percentage (21.093%) in plant leaves, while α-pinene was not detected in callus cultures that initiated from leaf explants grown at 16 hrs light and 8hrs dark. High percentage of camphor (19.961%) was found in callus cultures

Table (5): Components of essential oil found in samples from rosemary plant.

No.	Compounds	In mature leaves collected from Sulaimani city		In callus cultures	
		Retention indicate	%	Retention indicate	%
1	3-Carene	7.1	0.371	7.362	0.353
2	α-Pinene	7.384	21.093	-------	-------
3	Camphene	7.837	7.973	7.826	0.136
4	β-Pinene	8.59	1.148	-------	-------
5	Limonene	10.017	4.010	10.003	0.072
6	Eucalyptol	10.134	8.257	10.12	10.126
7	Linaly isobutyrate	12.009	2.208	11.994	2.920
8	3,5-Heptadien-2-ol	12.754	1.065	12.104	0.644
9	*Trans*-Pinocarveol	13.461	0.336	13.443	1.023
10	Camphor	13.556	12.889	13.536	19.961
11	*Trans*-Pinane	13.914	0.704	13.901	0.873
12	Camphenol	14.102	0.332	14.089	0.730
13	Borneol	14.254	9.808	14.237	19.160
14	Terpineol, cis-beta	14.453	1.143	14.437	2.433
15	α-terpinyl isovalerate	14.861	2.157	14.848	5.340
16	Bornyl chloride	15.072	1.524	15.062	2.773
17	Thymol	15.274	6.365	15.265	19.661
18	*Trans*-Shisool	16.188	1.214	16.171	2.559
19	5-Caranol	16.388	1.802	16.371	3.936
20	Bornyl acetate	17.33	3.726	17.312	0.867
21	Ocimene	19.615	0.230	-------	-------
22	α -Caryophyllene	20.968	0.548	-------	-------
23	Caryophyllene oxide	24.985	0.247	24.964	0.093
24	*Cis*-Lanceol	26.641	0.345	26.623	0.388

initiated at 16hrs light and 8hrs dark while the less percentage was found in plant leaves (12.889%). Callus cultures accumulated (19.160%) of borneol while leaves accumulated (12.889%). Thymol was found at high levels (19.661%) in callus cultures initiated from leaf explants and grown at 16hrs light and 8hrs dark. Meantime, its percentage in plant leaves was found to be lower than callus cultures (6.365%).

Eucalyptol was found at high percentage in callus cultures reached (10.126%) while plant leaves recorded lower eucalyptol content (8.257%). α-terpinyl isovalerate in callus cultures grown in the presence of light recorded (5.340%) which is more than twice the amount found in plant leaves (2.157%). Callus cultures were also containing 5-Caranol (3.936%), while in leaves (12.889%).

Linalyl isobutyrate, Bornyl chloride, trans-Shisool and Terpineol, cis-beta showed higher percentages in callus cultures exposed to light (2.920, 2.773, 2.559 and 2.433%) respectively, while lower values were recorded in leaves (2.208, 1.524, 1.214 and 1.143%) respectively. Higher percentages of trans-Pinocarveol, trans-Pinane, Camphenol and appeared in callus cultures initiated from leaves exposed to light (1.023, 0.873, cis-Lanceol 0.730 and 0.388%) compared to those found in leaves which recorded (0.336,

0.704, 0.332 and 0.345%) respectively. Camphene, Bornyl acetate, Limonene, 3-Carene and Caryophyllene oxide were higher in plant leaves (7.973, 3.726, 4.010, 0.094, 0.371 and 0.247%) respectively, compared with the content of callus cultures grown in light which recorded (0.136, 2.773, 0.072, 0.353 and 0.093%) respectively. β -Pinene, 3,5-Heptadien- 2-ol, α-Caryophyllene and Ocimene were found in leaves at levels (1.148, 1.065, 0.548 and 0.230%) respectively, while they were not detected in callus cultures initiated from rosemary leaves grown at 16hrs light and 8hrs dark.

The report that rosemary oils are mostly represented by the biochemical specificity of 1,8- cineole(11). The geographical distribution of varieties influences significantly the chemical composition of their essential oils. Such compounds are generally bio-efficacious, economically and environmentally safe and can be an ideal candidate for the use as antimicrobials, antioxidants and agrochemicals, for example; thymol is a constituent of oil of thyme, a naturally occurring compound in the plant *Thymus vulgaris* L. Thymol is an active ingredient in pesticide products registered for the use as animal repellents, fungicides/fungistats, medical disinfectants, tuberculocides, and virucides(7). Eucalyptol comprises up to 90 percent of the essential oil of some species of the *Eucalyptus* sp. essential oil.

Various stress factors affect the qualitative and quantitative accumulation of valuable secondary products in nature. Environmental stresses, primarily a response to varying soil nutrients, light, water, and other biotic and a biotic factors are known to cause many physiological, biochemical and molecular changes in plant metabolism and possibly alter the yield of secondary metabolites and their composition in plants(6).

The major advantages of a cell culture system over the conventional cultivation of whole plants which are: first, useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions. Second, cultured cells would be free of microbes and insects. Third, cells of, tropical or alpine, plants could easily be multiplied to yield specific metabolites. Forth, automated control of cell growth and rational regulation
of metabolite processes would reduce labor cost and improve productivity. Fifth, organic substances are extractable from callus cultures easily(7).

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