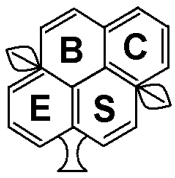

Journal

IN VITRO CALLUS FORMATION, REGENERATION AND THE ACTIVE CONSTITUENTS CONTENT IN *ROSMARINUS OFFICINALIS* L.

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ABSTRACT

The present work was carried out to investigate *in vitro* callus production and active constituents content in Rosemary (*Rosmarinus officicnalis* L.).The results showed that modified MS (1962) basal solid medium supplemented with 4.0 and 6.0 mg/l benzyl amino purine BAP recorded the best results of callus production from node stem and shoot tip explants, callus fresh weight, dry weight and size degree. While the modified MS medium without plant growth regulators failed to format callus production and callus regeneration. The maximum level of rosmarinic acid in the callus product from node stem and shoot tip was 4.049 and 5.105 mg/g dry weight on modified MS medium supplemented with 6.0 and 8.0 mg/l BAP, respectively. The shootlets product from node stem and shoot tip in medium supplemented with 6.0 and 8.0 mg/l BAP+5.0 mg/l GA_3. Were formed the best levels, 24.293 and 30.630 mg/g dry weight, respectively. The highest percentage of regeneration was for node stem and shoot tip cultures on modified MS medium supplemented with 6.0 mg/l BAP+5.0 mg/l GA_3 which gave 54.55 and 65.0 %, number of shootlets (5.0 and 13.6), length of shootlets (1.8 and 3.0 cm) respectively *in vitro* culture. Increase of BAP concentration from 1.0 to 4.0 mg/l combined with 5.0 mg/l GA_3 significant on essential oil % in shootlets produced from node stem as level α-pinene, camphene, limonene, 1,8-cineol and linalool respectively.

While the essential oil in shootlets formed from shoot tip which received BAP dose from 1.0 to 8.0 mg/l+5.0 mg/l GA_3 gradually

increased in limonene from 2.38 to 4.86 % and the treatment 8.0 mg/l BAP+5.0 mg/l GA_3 which recorded the highest percentage 25.87 and 21.11% of α-pinene and 1,8-cineol. Also, increasing BAP concentration from 1.0 to 4.0 mg/l+5.0 mg/l GA_3 gradually led to an increase in the level from 21.64 to 23.24 % of camphor.

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.), is one of the most important medicinal and aromatic species of lamiaceae family that is considered native to the Mediterranean region. Its growth habit is as a shrub of great longevity, well adapted to the Mediterranean warm and dry climates (subhumid and arid regions); and great capacity of adaptation to different soils (Serrano *et al*., 2002).

Rosemary has become one of the natural antioxidants used to replace synthetic antioxidants or allow the use of synthetic in lesser quantities to preserve food and cosmetic products. Many antioxidants trials in which rosemary and its extracts have been tested for their activities and have become to be regarded as potentially safer than chemical synthetic antioxidants (Etter, 2004). It contains volatile oils monoterpens such as alpha-pinene, camphene, beta-pinene, limonene, 1,8-cineol, linalool, camphor, endo borneol, terpineol-4-ol, alpha terpineol and verbenol (Isman *et al*., 2008). It also contains flavonoids, phenolic diterpenes and phenolic acids such as rosmarinic acid (Troncoso *et al*., 2005).

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. It has a number of interesting biological activities, e.g. antiviral, antibacterial, anti-inflammatory and antioxidant. The presence of rosmarinic acid in medicinal plants, herbs and spices has beneficial and health promoting effects. In plants, rosmarinic acid is supposed to act as a preformed constitutively accumulated defence compound (Petersen and Simmonds, 2003).

Murashige and Skoog (1962) medium is very popular, because most of plants react with it favorably. However, it should be appreciated that this nutrient solution is not necessarily always optimal for growth and development since the salt content is so high. Plant growth regulators are one of the most important factors affecting cell growth, differentiation and metabolite formation in plant cell and tissue cultures (Rokem and Goldberg, 1985; Liang *et al.,* 1991). Four basal classes of plant growth regulators are important in plant tissue

culture: the auxins, cytokinins, gibberellins and abscisic acid (Skoog and miller, 1957). The cytokinins are often used to stimulate the growth and development *in vitro* (Harding and Smigocki, 1994). For the development of lateral buds, the main shoot must lose apical dominance and the explants require exogenous cytokinin application (Ovesna *et al.,* 1993). GA_3 biosynthetic genes are expressed in specific cell and tissue types during development and their transcript levels are often elevated in rapidly growing regions, such as the rib meristem of shoot apex, elongating internodes and embryo axes (Olszewski *et al.,* 2002). In some plants GA_3 had been found conducive for organogenesis in callus tissue or for promotion of growth, biomass production and fiber length in transgenic trees according to (Pratibha, 2002).

The aim of this study was to look fore callus formation, regeneration from node stem and shoot tip explants *in vitro* culture and active constituents content in *Rosmarinus officinalis* L. var. Ternifolius plant.

MATERIALS AND METHODS

This work was conducted in the tissue culture lab and farm of Applied Research Center of Medicinal Plants at Abo Elhol region, National Organization for Drug Control and Research (NODCAR).

Tissue culture prepare

Cutting from mother stock plants (one year old) were taken from field of the Applied Research Center of Medicinal Plants (ARCMP) and planted in controlled greenhouse at 27±1ºC during 2-3 months. Two types 1.0-1.2 cm in length node stem as well as shoot tip segments of explants source were used, explants kept in antioxidant solution 100 mg/l ascorbic acid+100 mg/l citric acid + 100 mg/l poly vinyl pyrrolidone for three hours and washed several time by tap water, then rinsed with a small amount of liquid soap for 5 minutes to remove the assuring of most external contamination, and rinsed again under running tap water for 30 minutes to remove all the remaining detergent, after that the sterilization began under aseptic condition. This procedure and all the steps of the sterilization were done under complete aseptic condition in the laminar air flow. Explants were immersed in 95 % ethanol for 2 sec. surface sterilization was in 0.1 % mercuric chloride ($HgCl_2$) for 3-5 minutes. After surface sterilization,

approximately 2 mm was removed from cut ends of the explants and they were thrice washed with sterile distilled water for 5 min duration each. Explants were then kept for 30 min in poly vinyl pyrrolidone (PVP) solution 100 mg/l. The sterile explants were planted in sterile jars 360 ml containing 35 ml of tested medium. MS (1962) medium was subject to the following modification, medium in having (mg/l): 200 KNO_3, 1000 NH_4NO_3, 300 $CaCl_2.2H_2O$, 200 $MgSO_4.7H_2O$, 80 KH_2PO_4, 16.7 $FeSO_4.7H_2O$, 22.4 Na_2 EDTA and no change Myo-inositol and 0.1 Pyridoxine.HCl, respectively. The used MS modified medium was supplemented with 30 g/l sucrose and 10-50 mg/l adenine sulphate (AS) and 50 mg/l malt extract (ME) and solidified by 5.0 g/l agar. The pH value was adjusted to 5.7 –5.8 by adding suitable amount of 0.1 N HCl and 0.1 N KOH by using the pH meter prior to autoclaving at 1.3 kg/cm^2 for 20 minutes (Pratibha and Chaturvedi, 1991).

The combination treatments include two cases:

1- Effect of MS modified medium supplemented with benzyl amino purine (BAP) at 0.0, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/l individually on callus formation from node stem and shoot tip explants and active constituents content.

The following data were recorded after 6 weeks from planting explants:

1- Callus fresh weight (g / jar).
2- Callus dry weight (g / jar).
3- Callus percentage %.
4- Callus size (Degree).
5- Rosmarinic acid (RA) level (mg / g dry weight).

2- Effect of MS modified medium supplemented with benzyl amino purine (BAP) at 0.0, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/l combined with gibberellic acid (GA_3) at 5.0 mg/l on callus regeneration from node stem and shoot tip explants and active constituents content.

The following data were recorded after 6 weeks from planted callus:

1- Percentage of regeneration.
2- Number of shootlets.
3- Shootlet length (cm).
4- Chemical analysis of active constituents.

A. percentage of the total monoterpenes in the essential oil extracted.

B. Rosmarinic acid (RA) level (mg / g dry weight).

The vigor degree of the formed callus degree was recorded as follows according to Pattino (1981).

a) 1 = dead explants (no growth).

b) 2 = size degree below average.

c) 3 = medium sized of callus.

d) 4 = size above average growth of callus.

e) 5 = maximum callus growth.

Extraction and separation of rosemary oil

A- Investigation of volatile constituents using GC/MS

The method used was GC/MS-5989B, with the following conditions: Searched library: Wiley 275. LIB according to Gouda *et al.* (2009).

- Column: DBI, 30 m, 0.53 mm ID, 1.5 µm film.
- Carrier gas: Helium (flow rate 1ml/min.).
- Ionization mode: EL (70 eV).
- Temperature program: 40º C (static for 2 min.), then gradually increasing (160º C at a rate of 2º C/ min.) up to 250º C (static for 7.5 min.).
- Detector temperature and injector temperatures are 250º C.

Qualitative identification of the essential oils was achieved by library searched data base Willey 275. LIB and by comparing their retention index and mass fragmentation patterns with those of the available references and with published data, the percentage composition of volatile oil components was determined by computerized peak area measurements (Qualitative estimation).

B- Gas Chromatography for compared different treatments

This assay was performed according to **Tawfik *et al.* (1998).** To determinate the levels of monorterpenes in proliferated explant and regenerated plantlets, extraction using hexane as an organic solvent was considered to be the preferable method to use. The plant tissues were placed in glass vials and thoroughly soaked in a sufficient amount of hexane 2.0 ml / 0.5 g fresh weight. The vials were then capped and sealed, and extraction continued for 12-15h at 4º C. Water was

removed from the extract using sodium sulfate anhydrous (Na_2 SO_4) then decanted into clean vials, and the final volume for all treatments was adjusted to 2.0 ml using hexane. A 1.0 µl aliquot of the oil extract injected into a Hewlett Packard.

Gas Chromatographic condition

GC Model: 6890

- Columns: HP-S (phenyl methyl silica) capillary column 25m*0.25mm, 0.33µm film thickness.
- Detector: (FID) temperature of 275˚ C.
- Injector: temperature of 225º C.
- Mode: A split less injection,
- Oven programmed: from 60º C to 300º C.
- Carrier gas: nitrogen at a flow rate of 1.0 ml/min.

After system stabilization inject 1.0 micro liter of each test to compared different treatments according to (Serrano *et al.,* 2002).

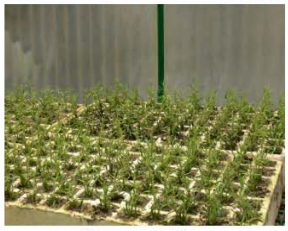
Determination of rosmarinic acid (RA)

Determination of rosmarinic acid (RA) was done according to Lopez-Arnoldos *et al,* (1995). Thus approximately 50 mg of fresh weight (FW) of tissue samples (explants) and 300 mg for callus cultures were placed in 3 ml of 50% methanol (diluted with distilled deionizer water to 50% v/v). Samples were incubated at 55°C for 2 h walk-in growth incubator. After cooling the sample at room temperature, exactly 1 ml of the methanol extract was taken and diluted with 5 ml of 50% methanol to make a total dilution of 1:18 (for callus cultures, no dilution was used, since they contained very low concentration of rosmarinic acid). The diluted extracts were mixed with a vortex mixer and absorbance was measured at 333 nm by spectrophotometer (UV Spectrophotometer hp 8453 + Quartz cell path length 1mm type Q-104) concentration of RA was calculated using the equation: $A = \varepsilon bc$, where the extinction coefficient $\varepsilon = 19000$ $Lmol^{-1}$ cm^{-1} and width of the disposable cuvettes b =1 cm. the RA is expressed per unit dry weight (DW) of the biomass.

The obtained data of experiments were subjected to the statistically analysis of variance procedure and the mean values were compared using the LSD method at 5% level of significance according to Gomez and Gomez (1984).

Mother plants in the field (one year old)

Cutting from mother plants in greenhouse



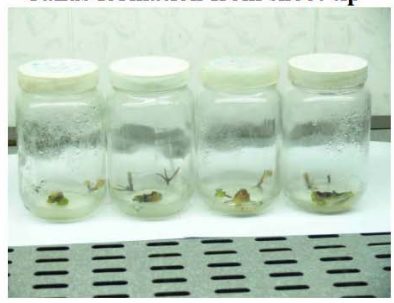
One month

Tow months

Three months

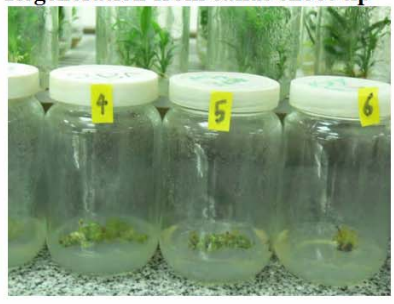
Callus formation from node stem

Callus formation from shoot tip



Regeneration from callus node stem

Regeneration from callus shoot tip



RESULTS AND DISCUSSION

1- Effect of MS modified medium supplemented with benzyl amino purine (BAP).

Callus formation from node stem and shoot tip explants

Data in Table (1) showed that, the MS modified medium free hormones had no effect on callus initiation from different explants, but MS modified medium supplemented with low concentration from (BAP) gave a low effect on callus. Furthermore, the optimum value of callus formation was obtained from node stem and shoot tip treated with 4.0 and 8.0 mg/l BAP respectively. Node stem and shoot tip treated with 4.0 and 6.0 mg/l BAP respectively recorded the highest significant value of callus fresh weight 3.011 and 3.739 g/jar respectively and dry weight 0.241 and 0.253 g/jar respectively. The callus size (Degree) was 4.55 and 4.85 respectively as maximum callus growth. While node stem and shoot tip treated with 1.0 mg/l BAP formed the lowest significant value of callus production fresh weights 0.338 and 0.951 g/jar respectively and dry weights 0.019 and 0.058 g/jar respectively and callus size (Degree) was 1.65 and 1.95 respectively as below average. These results were in agreement with the findings of Tawfik *et al.* (1998) who reported that the induction of callus depends on growth regulators and the type of organ used, stem segments, shoot tips. Yang *et al.* (1997) reported that shoot and callus cultures of rosemary were grown on ½ MS/BAP medium, containing half strength Murashige and Skoog (MS) salts (1962) and Benzyl amino purine (BAP) level 1.0 mg/l for shoot multiplication, maintenance and plant regeneration.

Table (1). Effect of MS modified medium supplemented with benzyl amino purine (BAP) at 0.0, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/l individually on callus formation from node stem and shoot tip explants of *R. officinalis* L.

Treatment mg / l	Callus fresh weight gm /jar		Callus dry weight gm /jar		Callus %		Callus size (Degree)	
	Node stem	Shoot tip	Node stem	Shoot tip	Node stem	Shoot tip	Node stem	Shoot tip
0.0	-	-	-	-	-	-	1	1
1.0	0.338	0.951	0.019	0.058	8.42	13.33	1.65	1.95
2.0	1.481	2.564	0.112	0.230	36.89	26.66	2.92	3.32
4.0	3.011	1.502	0.241	0.144	75.00	40.00	4.55	2.55
6.0	2.558	3.739	0.178	0.253	63.72	60.00	4.04	4.85
8.0	0.788	2.600	0.052	0.218	19.63	80.00	2.34	3.37
L.S.D at 5 %	**0.3248**	**0.4872**	**0.0146**	**0.0221**	-	-	**0.5084**	**0.5673**

Rosmarinic acid (RA) levels in callus formation from node stem and shoot tip

Data in Table (2) showed that, MS modified medium free hormones had no effect on RA level in the callus formation from node stem and shoot tip, but increasing BAP concentration from 1.0 to 6.0 mg/l caused an increased rosmarinic acid (RA) level in the callus formation from node stem. The maximum significant level of RA was recorded 4.049 mg/g dry weight for 6.0 mg/l BAP and the low concentration of BAP 1.0 mg/l was formed the minimum significant level of RA 1.039 mg/g dry weight in the callus formation from node stem. While increasing BAP concentration from 1.0 to 8.0 mg/l caused an increased rosmarinic acid (RA) level in the callus formation from shoot tip, the maximum significant level of RA was recorded 5.105 mg/g dry weight for 8.0 mg/l BAP and lower level of BAP 1.0 mg/l formed the minimum significant value of RA 2.0 mg/g dry weight in the callus formation from shoot tip.

Table (2). Effect of MS modified medium supplemented with benzyl amino purine (BAP) at 0.0, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/l individually on rosmarinic acid (RA) level in callus formation from node stem and shoot tip explants of *R. officinalis* L.

Treatment (mg / l)	Rosmarinic acid (mg / g dry weight)	
	Node stem	Shoot tip
0.0	0.0	0.0
1.0	1.039	2.0
2.0	1.762	2.447
4.0	2.739	2.647
6.0	4.049	4.846
8.0	3.489	5.105
L.S.D at 5 %	**0.5187**	**0.2409**

2- Effect of MS modified medium supplemented with benzyl amino purine (BAP) at 0.0, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/l combined with gibberellic acid (GA_3) at 5.0mg/l.

Callus regeneration from explants

Data in Table (3) showed that, the highest percentage of regeneration was recorded for node stem and shoot tip cell cultures as compared with MS modified medium supplemented with 6.0 mg/l BAP+5.0 mg/l GA_3 which gave 54.55 and 65.0 % respectively. While

the lowest percentage of callus regeneration from node stem and shoot tip explants respectively was 8.54 and 16.50 % for MS modified medium supplemented with 8.0 and 1.0 mg/l BAP+5.0 mg/l GA_3.

Furthermore, the best results in the term of number and length of shootlets cm were recorded for that derived from node stem and shoot tip cultures respectively grown on MS modified medium supplemented with 6.0 mg/l BAP+5.0 mg/l GA_3 compared with other BAP concentrations. The recorded data were 5.0 and 13.6 shootlets/jar and 1.8 and 3.0 cm number and shootlets length. The treatments 8.0 and 1.0 mg/l BAP+5.0 mg/l GA_3 gave the lowest significant values in number of shoolets 1.0 and 1.8 shootlets/jar from node stem and shoot tip. The treatment 1.0 mg/l BAP+5.0mg/l GA_3 recorded the lowest values of shootlet length 0.50 and 0.75 cm from node stem and shoot tip explants.

Regeneration of *Salvia nemorosa* L from node segment of year-old tree *in vitro* cellular and development (Skaia *et al*., 2004), BA has been commonly used for the induction of organogenesis in plants (Mneny and Mantell, 2002). It was reported that BA is most effective for meristem, shoot tip, and bud cultures. BA was reported to be most suitable for the production of a larger number of shoot, all explants tested formed shoots with either BA or Kin, over all the number of shoots per explant high level of BA interaction and GA_3 were recorded decreasing in plant (Verron *et al.,* 1995).

Table (3). Effect of MS modified medium supplemented with benzyl amino purine (BAP) at 0.0, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/l combined with gibberellic acid (GA_3) at 5.0 mg/l on percentage of regeneration, number and length of shootlets (cm) from node stem and shoot tip cells cultures of *R. officinalis* L.

Treatment (mg/l)		Percentage of callus regeneration		Number of shootlets		Shootlets length (cm)	
BAP	GA_3	Node stem	Shoot tip	Node stem	Shoot tip	Node stem	Shoot tip
0.0	0.0	-	-	-	-	-	-
1.0	5.0	15.00	16.50	1.2	1.8	0.50	0.75
2.0		24.20	20.30	2.6	4.6	0.70	1.5
4.0		36.51	32.50	3.6	5.6	1.2	2.1
6.0		54.55	65.00	5.0	13.6	1.8	3.0
8.0		8.54	52.40	1.0	9.8	1.0	2.5
L.S.D at 5%		-	-	0.9745	1.6241	0.1624	0.4763

The percentage changes of essential oil in shootlets produced from explants

The data presented in Table (4) showed that, increasing BAP concentration from 1.0 to 4.0 mg/l+5.0 mg/l GA_3 led to increase the level of α-pinene, camphene, limonene, 1,8-cineol and linalool gradually. The minimum concentration of BAP 1.0 mg/l+5.0 mg/l GA_3 recorded the highest percentage 21.20, 25.58 and 0.88% for camphor, verbenol and bornyl acetate respectively and recorded the lowest percentage 3.72% for camphene. The treatment 4.0 mg/l BAP+5.0 mg/l GA_3 was more suitable and recorded the highest percentage 25.15, 6.86, 5.65, 19.50 and 2.61% for α-pinene, camphene, limonene, 1,8-cineol and linalool respectively, also this treatment recorded the lowest percentage 15.45, 7.66, 0.82 and 13.26 % for camphor, endo borneol, terpineol-4–ol and verbenol. Also 6.0 mg/l BAP+5.0 mg/l GA_3 recorded the highest percentage 12.74 and 3.28% for terpineol-4–ol and α-terpineol respectively and recorded the lowest percentage 11.77, 11.55 and 2.04 % for α-pinene, 1,8-cineol and linalool respectively. While the maximum concentration of BAP 8.0 mg/l+5.0 mg/l GA_3 recorded the highest percentage 13.44 % for endo borneol and recorded the lowest percentage 2.17, 1.83 and 0.67 % for limonene, α-terpineol and bornyl acetate respectively.

It is clearly that the production of monoterpene constituents from proliferated explants cultured *in vitro* and from regenerated plants was influenced by the components of the culture media, such as plant growth regulators. These results partially agree with the results reported for *Mentha spicta* (Hirata *et al.,* 1990) and *Zingiber officinale* (Sakamura *et al.,* 1986). The effects of plant growth regulators on the yield of some secondary products in plant cell cultures have been investigated by Bajaj (1996). This effect varied greatly, depending on the kinds of metabolites being produced and on the type of auxin and cytokinin added to the cultured medium (Tawfik, 1992).

Table (4). Effect of MS modified medium supplemented with benzyl amino purine (BAP) at 0.0, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/l combined with gibberellic acid (GA_3) at 5.0 mg/l on essential oil (%) in shootlets produced from node stem after 6 weeks from culturing *in vitro* of *R. officinalis* L.

Compounds	Treatments (mg/l)					
	0.0 BAP + 0.0 GA_3	1.0 BAP + 5.0 GA_3	2.0 BAP + 5.0 GA_3	4.0 BAP + 5.0 GA_3	6.0 BAP + 5.0 GA_3	8.0 BAP + 5.0 GA_3
α - pinene	-	13.27	18.17	25.15	11.77	14.60
Camphene	-	3.72	6.18	6.86	4.12	5.36
Limonene	-	3.47	4.15	5.65	2.28	2.17
1,8 - Cineol	-	13.29	13.55	19.50	11.55	12.95
Linalool	-	2.42	2.54	2.61	2.04	2.16
Camphor	-	21.20	17.77	15.45	18.88	19.69
Endo borneol	-	12.89	11.70	7.66	12.93	13.44
Terpineol -4- ol	-	0.91	1.17	0.82	12.74	2.76
α - terpineol	-	2.37	2.15	2.22	3.28	1.83
Verbenol	-	25.58	21.75	13.26	19.56	24.37
Bornyl acetate	-	0.88	0.87	0.82	0.85	0.67

Data in Table (5) showed that, increasing BAP concentration from 1.0 to 4.0 mg/l+5.0 mg/l GA_3 gradually led to an increase in α-pinene, limonene, camphor and bornyl acetate. Also, increasing BAP concentration from 1.0 to 8.0 mg/l+5.0 mg/l GA_3 increased the level for limonene from 2.38 to 4.86 %. The minimum concentration of BAP 1.0 mg/l BAP+5.0 mg/l GA_3 recorded the highest percentage 17.70, 1.46, 3.03 and 30.09 % for endo borneol, terpineol-4-ol, α-terpineol and verbenol respectively, while this treatment recorded the lowest percentage 5.36, 1.93, 2.38 and 12.24 % for α-pinene, camphene, limonene, 1,8-cineol respectively. But 4.0 mg/l BAP+5.0 mg/l GA_3 recorded the highest percentage 23.24 % for camphor and recorded the lowest percentage 0.0, 0.77 and 4.97 % for terpineol-4-ol, α-terpineol and verbenol, respectively. In this concentration 6.0 mg/l BAP + 5.0 mg/l GA_3 formed the highest percentage 8.56, 3.54 and 4.27 % for camphene, linalool and bornyl acetate, respectively and recorded the lowest percentage of camphor 15.02 %. Moreover, the maximum concentration of BAP 8.0 mg/l BAP+5.0 mg/l GA_3 was more suitable for α-pinene, limonene and 1,8-cineol and recorded the highest percentage 25.87, 4.86 and 21.11 % respectively, also that treatment recorded the lowest percentage for linalool, endo borneol and bornyl acetate 2.77, 8.28 and 0.68 % respectively.

The changes in the proportion of the monoterpenes caused by thaidiazuron TDZ and benzyl amino purine (BAP) suggested that the cytokinin may have an effect on the biosynthesis of camphor. Since

camphor can be derived from borneol (Croteau and Karp, 1976), the cytokinin may affect the enzymes responsible for the conversion of borneol or borneol acetate to camphor. Tawfik *et al.* (1992) reported that increasing BAP in the shoot tip culture medium of *Salvia officinalis* L. increased camphor and decreased borneol. On the other hand, Drawert (1988) reported the biotransformation of terpenes such as: from borneol to camphor, from citronellal to citronellol in cell suspension culture of *Salvia officinalis* and *Melissa* sp., respectively. The rate and conversion time of one terpene to another depended on the kind of monoterpene.

Table (5). Effect of MS modified medium supplemented with benzyl amino purine (BAP) at 0.0, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/l combined with gibberellic acid (GA_3) at 5.0 mg/l on essential oil (%) in shootlets produced from shoot tip after 6 weeks from culturing *in vitro* of *R. officinalis* L.

Compounds	Treatments (mg/l)					
	0.0 BAP + 0.0 GA_3	1.0 BAP + 5.0 GA_3	2.0 BAP + 5.0 GA_3	4.0 BAP + 5.0 GA_3	6.0 BAP + 5.0 GA_3	8.0 BAP + 5.0 GA_3
α - pinene	-	5.36	24.08	24.12	23.02	25.87
Camphene	-	1.93	7.60	6.58	8.56	7.90
Limonene	-	2.38	4.20	4.55	4.60	4.86
1,8 - Cineol	-	12.24	21.07	20.44	14.03	21.11
Linalool	-	3.41	2.94	2.88	3.54	2.77
Camphor	-	21.64	22.50	23.24	15.02	18.10
Endo borneol	-	17.70	9.30	10.94	9.83	8.28
Terpineol -4- ol	-	1.46	0.54	-	1.19	0.43
α - terpineol	-	3.03	1.55	0.77	1.98	2.21
Verbenol	-	30.09	5.32	4.97	13.96	7.79
Bornyl acetate	-	0.76	0.90	1.51	4.27	0.68

Rosmarinc acid (RA) formation in shootlets derived from explants

Data presented in Table (6) clearly showed that, MS modified medium free hormones had no effect on (RA) level in shootlets produced from node stem and shoot tip, but increasing BAP concentration from 1.0 to 6.0 mg/l + 5.0 mg/l GA_3 caused an increased rosmarinic acid (RA) level in shootlets produced from node stem, the maximum significant level of RA was recorded 24.293 mg/g dry weight for 6.0 mg/l BAP+5.0 mg/l GA_3 and the low concentrations of BAP 1.0 mg/l +5.0 mg/l GA_3 was formed the minimum significant level of (RA) 6.235 mg/g dry weight in shootlets produced from node stem. While increasing BAP concentration from 1.0 to 8.0 mg/l + 5.0 mg/l GA_3 was more effected on RA level and recorded the best results, the maximum significant level of RA 30.630

mg/g dry weight was recorded with 8.0 mg/l BAP+5.0 mg/l GA_3 and lower level of BAP 1.0 mg/l + 5.0 mg/l GA_3 formed the minimum significant value of RA 12.010 mg/g dry weight in shootlets produced from shoot tip.

Table (6): Effect of MS modified medium supplemented with benzyl amino purine (BAP) at 0.0, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/l combined with gibberellic acid (GA_3) at 5.0 mg/l on rosmarinic acid (RA) formation in shootlets derived from explants after 6 weeks from culturing *in vitro* of *R. officinalis* L.

Treatment mg / l		Rosmarinic acid (mg / g dry weight)	
BAP	**GA_3**	**Node stem**	**Shoot tip**
0.0	**0.0**	0.0	0.0
1.0	**5.0**	6.235	12.010
2.0		10.571	14.680
4.0		16.432	15.880
6.0		24.293	29.076
8.0		20.935	30.630
L.S.D at 5 %		**3.2483**	**1.4974**

Conclusion

The results clearly showed that modified MS basal solid medium supplemented with 4.0 and 6.0 recorded the best results in callus production from node stem and shoot tip explants 3.011 and 3.739 g /jar fresh weight respectively and dry weight 0.241 and 0.253 g/jar respectively. The callus size (Degree) was 4.55 and 4.85 respectively. Furthermore, the optimum value of callus formation was obtained from node stem and shoot tip treated with MS modified medium supplemented with 4.0 and 8.0 mg/l BAP and recorded 75 and 80 % respectively. While the maximum level of rosmarinic acid (RA) in the callus product from node stem and shoot tip was 4.049 and 5.105 mg/g dry weight on modified MS medium supplemented with 6.0 and 8.0 mg/l BAP, respectively.

The highest percentage of regeneration was for node stem and shoot tip cultures on modified MS medium supplemented with 6.0 mg/l BAP+5.0 mg/l GA_3 which gave 54.55 and 65.0 %, number of shootlets (5.0 and 13.6), length of shootlets (1.8 and 3.0 cm) respectively *in vitro* culture.

Concerning, the percentage changes of essential oil in shootlets produced from node stem the minimum concentration of BAP 1.0

mg/l+5.0 mg/l GA_3 recorded the highest percentage 21.20 and 25.58 for camphor and verbenol and the treatment 4.0 mg/l BAP+5.0 mg/l GA_3 was recorded the highest percentage 25.15, 6.86 and 19.50 % for α-pinene, camphene and 1,8-cineol respectively. Also 6.0 mg/l BAP+5.0 mg/l GA_3 recorded the highest percentage 12.74 % for terpineol-4–ol. While the maximum concentration of BAP 8.0 mg/l+5.0 mg/l GA_3 recorded the highest percentage 13.44 % for endo borneol. While the percentage changes of essential oil in shootlets produced from shoot tip the minimum concentration of BAP 1.0 mg/l BAP+5.0 mg/l GA_3 recorded the highest percentage 17.70 and 30.09 % for endo borneol and verbenol, but 4.0 mg/l BAP+5.0 mg/l GA_3 recorded the highest percentage 23.24 % for camphor. In this concentration 6.0 mg/l BAP + 5.0 mg/l GA_3 formed the highest percentage 8.56 % for camphene. Moreover, the maximum concentration of BAP 8.0 mg/l BAP+5.0 mg/l GA_3 was more suitable for α-pinene and 1,8-cineol and recorded the highest percentage 25.87 and 21.11 %.

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تكوين وتكشف الكالس معمليا ومحتوى المواد الفعالة فى نبات حصالبان

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أجريت هذه الدراسه بهدف إنتاج الكالس معملياً ومحتوى المواد الفعالة فى نبات حصالبان . أوضحت النتائج أن بيئة MS المعدله مضافاً إليها البنزيل أمينو بيورين بتركيز 4.0 و 6.0 ميلليجرام/لتر على التوالى سجلت أفضل النتائج لإنتاج الكالس من العقدة الساقية والقمة النامية من حيث الوزن الطازج والوزن الجاف وقوة نمو الكالس . مستوى حامض الروزمارينيك العالى فى الكالس المنتج من العقده الساقية كان 4.049 ميللجرام/جرام وزن جاف والقمه النامية 5.105 ميللجرام/جرام وزن جاف على بيئة MS المعدله مضافاً إليها البنزيل أمينو بيورين بتركيز 6.0 و8.0 ميلليجرام/لتر على التوالى . أما بالنسبة للمستوى العالى لحامض الروزمارينيك في الأفرع المُنتَجة مِنْ العقدة الساقية والقمة النامية فى البيئة مضافاً إليها 6.0 و 8.0 ميلليجرام/لتر بنزيل أمينو بيورين + 5.0 ميلليجرام/لتر جبرليك أسيد شكلوا أفضل المستويات 24.293 و 30.630 ميللجرام/جرام وزن جاف على التوالى . النسبه المئويه العاليه لتكشف الكالس من العقده الساقيه والقمه الناميه عند زراعة الكالس على بيئة MS المعدلة مضافاً إليها 6.0 ميلليجرام/لتر بنزيل أمينو بييورين + 5.0 ميلليجرام/لتر جبرليك أسيد اعطت 54.55 و 65.0 % وعدد الأفرع 5.0 و13.6 و طول الأفرع 1.3 و 3.0 سم على التوالى . زيادة تركيز البنزيل أمينو بيورين من 1.0 إلى 4.0 ميلليجرام/لتر + 5.0 ميلليجرام/لتر جبرليك أسيد كانت إيجابيه على النسبه المئويه للزيت الطيار فى الأفرع المنتجه من العقدة الساقية مثل مستوى ألفا بينين – الكامفين - الليمونين- 8,1 سينيول – اللينالول على التوالى. بينما للزيت الطيار فى الأفرع المتكونه من القمه الناميه التى تعرضت لتركيزات البنزيل أمينو بيورين من 1.0 إلى 8.0 ميلليجرام/لتر + 5.0 ميلليجرام/لتر جبرليك أسيد أدى إلى زيادة الليمونين تدريجياً من 2.38% إلى 4.86% والمعامله 8.0 ميلليجرام/لتر بنزيل أمينو بيورين+5.0 ميلليجرام/لتر جبرليك أسيد التى سجلت النسبه المئويه العاليه 25.87 و21.11% للألفا بينين و 8,1 سينيول. أيضاً زيادة تركيز البنزيل أمينو بيورين من 1.0 إلى 4.0 ميلليجرام/لتر + 5.0 ميلليجرام/لتر جبرليك أسيد أدى إلى زيادة مستوى الكامفور من 21.64 إلى 23.24% تدريجياً.