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Effect of medium type, cytokinin kind and sodium chloride stress on *in vitro* callus, shoot formation and composition of volatile oil of three cultivars of rosemary plant

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ABSTRACT

Three rosemary cultivars which grown under Egyptian conditions, *Rosmarinus officinalis* L. (CV.1), *R. officinalis* L. *Pyramidalis* "Upright Rosemary" (CV.2) and *R. officinalis* L. *Angustifolius* "Pine scented" (CV.3) were evaluated morphologically by measuring different attributes. It was found that the longest leaf and the highest no of axilary shoots were recorded for CV.2 then CV.1 followed by CV.3 while CV.3 had the highest number of leaves followed by CV.2 then CV.1.

To study the effect of medium type and cytokine, sterilized leaf explants of each cultivar were planted on two types of medium Murashige and Skoog, 1962 (MS) including vitamins and McCown Woody plant medium 1980 (WPM), both media were supplemented with one kind of cytokinen (CPPU or TDZ) at concentration (1mg/l) to examine their effect on callus and shoot formation for each cultivar. It was found that, the highest mean of callus weight was 9.02g/explant followed by 8.02 g/explant for CV.2 and CV.1, respectively recoded with MS medium supplemented with 1mg/l CPPU while, The highest mean No. of shoot was 2.40 shoots/explant then 1.60 shoots/explant produced from WPM+1mg/l CPPU for CV.1 then CV2. Concerning CV.3 the highest mean of callus weight was 2.52g/explant resulted from WPM+1 mg/l CPPU while the highest mean No. of formed shoots was 11.00 shoots/explant resulted from the same combination of medium and PGR.

To determine the effect sodium chloride NaCl stress on callus induction, shoot formation and volatile oil composition of callus for each rosemary cultivar sterilized leaves explants of each cultivar were planted on MS medium supplemented with 1.0 mg/l CPPU for all treatments and 200, 400, 600, 800, 1000,1200, 1400 µM/l of NaCl. The results indicated that, the highest mean of callus weight was detected at 1000 µM/l for both CV.1 and CV.2 as they gave 8.46 g/explant and 8.55g/explants, respectively, while CV.3 showed highest weight 1.04 g/explant at 200 µM/l. The highest mean No. of shoots formed under stress was obtained from CV.1 was (8.33 shoots/explant) at concentration of 400 µM/l, while CV.2 had 4.00 shoots/explant were obtained from addition of 200 and 800 µM/l of NaCl to the medium. However,CV.3 recorded the highest mean No. of formed shoots 21.76 shoots/explant at 600 µM/l of NaCl. The major component of volatile oil formed in callus was Bornyl acetate as 33.38%, 28.31% and 15.11% for CV.1, CV.3 and CV.2, respectively followed by .(-)-α-Pinene by 17.47% and16.55% for CV.1 and CV.3, respectively while CV.2 didn't contained(-)-α-Pinene . Cineole also was formed in callus of each three cultivars as CV.1 had 13.72% then CV.3 had 14.16% followed by CV.2 which had it at 10.92%.

Keywords: *:* Rosemary, *Rosmarinus officinalis L., R. officinalis L., Pyramidalis* "Upright Rosemary", *R. officinalis L. Angustifolius "Pine scented",* MS,WPM, CPPU, TDZ, Salt Stress, NaCl

Introduction

Rosemary, *Rosmarinus officinalis* L. (Family Lamiaceae), is a perennial evergreen shrub. It is a Mediterranean region plant used for flavoring food, in cosmetic and in traditional medicine for

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choretics, hepatoprotective and antimorigenic activity. Rosemary extracts have great interest for the food industry as a source of active compound and medicine as a great part of drugs. Rosemary volatile oil is used as a seasoning for food stuffs, such as meat salami and sauces (Lo Presti *et al*., 2005), but due to its chemical active constituents properties, it is used as an antioxidant (for food preserving), antibacterial and antifungal agents against some spoilage organisms (Rezzoug *et al*., 2005). The oil is also used in traditional medicine as tonic pulmonary antiseptic, choleretic and colagoguic agents (Pintore *et al*., 2006). it has been reported to possess a number of therapeutic applications in folk medicines in curing or managing of a wide range of diseases such a diabetic mellitus (DM), respiratory disorders, stomach problems and inflammatory diseases (AL-Serreiti *et al*.,1999 and Kültür,2007).

Some cytokinin-like compounds of nonpurine structure have been used in tissue-culture research. These compounds, which include forchlorfenuron (CPPU) and thidiazuron (TDZ), have exhibited stronger cytokinin-like effects than conventional cytokinins on a wide range of species Mohamed *et al*.,(1992). N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU, Forchlorfenuron) it is a strong cytokinin bioactively and has been shown stimulate fruit growth of kiwi fruits, apple and grapes (Retamales *et al*., 1995). Castro *et al*., (1995) mentioned that, optimal condition for *in vitro* shoot proliferation in Avocado cultivars 'Lula' and 'Velvick' were 0.5 and 0.1 mg/1 CPPU obtaining up to 92% of establishment rate and 1.4 shoots per explant. Lower levels of browning were detected when using CPPU, in comparison to TDZ and BA. The use of biotic or abiotic elicitors to stimulate product formation has become an important progress strategy and can be very useful in reducing the process time to attain high product concentrations and increased volumetric productivity (Dornenburg and Knorr, 1996).

In this study, the effect of medium type, cytokinine kind and sodium chloride stress on callus and shoot formation and volatiles composition of callus of three rosemary cultivars was examined.

Materials and Methods

This study had been carried out at the tissue culture laboratory, Horticulture Research Institute, Agriculture Research Center, Giza, Egypt, to study the effect of medium type, cytokinin kind and sodium chloride stress on *in vitro* callus, shoot formation and composition of volatile oil of three cultivars of rosemary plant callus during the period from 2016 to 2017. Leaf explants were taken from terminal cuttings of mother plants. The three cultivars *Rosmarinus officinalis* L. CV.1 (Fig1), *Rosmarinus officinalis* L. *Pyramidalis*" Upright rosemary" CV.2 (Fig.2), and *Rosmarinus officinalis* L. *Angustifolius* "Pine scented" CV.3 (Fig.3) which growing in the garden of the Gene Bank, Agriculture Research Center, Ministry of Agriculture, Giza, Egypt. The following experiments had been carried out:

Fig.1 *Rosmarinus officinalis* L. CV.1

Fig.2 *R. officinalis* L. *Pyramidalis* "Upright Rosemary " **CV.2**

Fig.3 *R. officinalis* L. *Angustifolius* "Pine scented" **CV.3**

1. Morphological attributes of the three rosemary cultivars

Morphological and quantitative attributes of rosemary (*Rosmarinus officinalis* L.) cultivars under study were detected as follow:

Growth habit , plant color, leaflet length , leaflet width , stem color, mean No of leaves/middle branch of the plant, leaf area (cm2), No. of axillary shoot/middle branch of the plant. All measurements had 3 replicates.

2. Effect of medium type and cytokinin kind on callus and shoot formation of rosemary cultivars.

Rosemary leaves of each three cultivar were sterilized according to (Sakr *et al*., 2018).Two types of medium were examined Murashige and Skoog, 1962 (MS) including vitamins and McCown Woody plant media 1980 (WPM), both media were supplemented with one kind of cytokinen (CPPU or TDZ) at concentration (1mg/l). Leaves explants were planted on small jars poured with 30 ml/each , every treatment had 3 replicates and every replicate had 3 explants. After 60 day data of fresh callus weight and developed shoots were recorded then transplanted on rooting medium.

3. Effect of salt stress of NaCl on callus induction , shoot formation and volatile oil composition

a. On callus induction and shoot formation

Sterilized rosemary leaves explants were planted into small jars (125ml) poured with 30ml/jar of MS medium supplemented with 1.0 mg/l CPPU for all treatments and 200, 400, 600, 800, 1000,1200, 1400 µM/l of sodium chloride NaCl to determine the effect of it as biotic stress on callus weight and shoot formation for each cultivar. Every treatment had 3 replicate and every replicate had 3 explants. After 30 day of incubation produced callus was subcultered on medium with same components then after 30 day callus weight were recorded and isolated to determine the chemical volatile oil composition by using Headspace gas chromatography (via solid-phase micro extraction (SPME). Also formed embryos were separated and subcultred on MS free medium with same salt stress supplemented with enhance shoots proliferation and after 3 weeks number of elongated shoots were registered.

b. On volatile oil composition of produced callus which analyzed via Head space chromatography with solid-phase microextraction (SPME)

b.1. Chemicals and Materials

SPME fibers of stableflex coated with divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS, 50/30 µm) or PDMS (polydimethylsiloxane) were purchased by Supelco (Oakville, ON, Canada).

b.2. Volatile oil analysis

The HS-SPME volatile oil analysis was carried out according to Farag *et al*., (2015) with slight modifications. Callus (2g) were placed in SPME screw cap vials (20 ml) and spiked with 2 µg (Z)-3-hexenyl acetate as an internal standard per vial dissolved in water. The SPME fiber was inserted manually into vial containing samples placed in an oven kept at 55°C for 30 min. The fiber was subsequently withdrawn into the needle and then injected into the injection port of the gas chromatography-mass spectrometer (GC-MS). GC-Ms analysis was performed on a Schimadzu GC-17A gas chromatogram equipped with DB-5 column (30 m x 0.25 mm i.d. x 0.25 µm film thickness; Supelco) and coupled to Schimadzu QP5050A mass spectrometer. The interface and the injector tempreatures were both set at 220°C. The following gradient temperature program was used for volatiles analysis. The oven temperature was kept first at 40°C for 3 min, then increased to 180°C at a rate of 12°C min-1, kept at 180°C for 5 min, and finally ramped at a rate of 40°C min-1 to 240°C and kept at this temperature for 5 min. The carrier gas Helium was used at a total flow rate of 0.9 ml/min. Splitless injection mode was used for analysis considering the lower levels of volatiles in samples. SPME fiber was prepared to the next analysis by placing it in the injection port for 2 min at 220 °C to ensure

complete elution of volatiles. Blank runs were made during samples analyses. The HP quadruple mass spectrometer was operated in EI mode at 70 eV. A scan range was set at m/z 40-500.

b.3. GC-MS data processing and multivariate analysis:

Volatile oil components were identified by comparing their retention indices (RI) relative to n-alkanes (C6-C20), mass matching to NIST, WILEY library database and with standards whenever available. Peaks were first de-convoluted using AMDIS software (www.amdis.net) prior to mass spectral matching.

4. Experiments layout

The layout of the experiments was randomized complete block design (two factors split design using rosemary cultivars as main plot) and data were statistically analyzed according to Gomez and Gomez (1984) by (M-STATC) software computer program, version 2.10. analysis of variance was used to compare statistical difference between means using L.S.D at probability 0.05%.

Results and Discussion

1. Morphological attributes of rosemary cultivars

Within the tested biotypes, some variability in growth habit, plant color, leaflet length, leaflet width, stem color, mean No. of leave/middle plant branch, leaf area, mean No. of axillary shoots /middle plant branch between *Rosmarinus officinalis* L. cultivars had been registered.

Data at Table (1) showed that, the longest leaf was for CV.2 then CV.1 then CV.3 with value 3.2mm, 2,3mm and 1.98 mm. No notable differences were observed in leaf width as all in rang 1.6-1.8 mm. However, CV.3 had the highest number of leaves recorded by (465) followed by CV.2 (412) then CV.1 (312) while the highest no of axilary shoots registered with CV.2 with (12.4) then CV.1 (11.2) then CV.3 (10.9). De-Mastro *et al*., (2004) on rosemary clones obtained from different environments of southern Italy they mentioned that, the leaf type and the No. of axillary shoots are correlated with each other and this finding was in agreement with results of this study. On the other hand, this results were not agreed with the leaves number, as the leaf No. of CV.2 is higher than CV.1, although CV.2 had longer and wider leaves. This could be explained by the effect of genotype on morphological attributes .

Table 1: Morphological attributes of some rosemary (*Rosemarinus officinalis* L. cultivars.

Attribute	***Rosmarinus officinalis* L. CV.1**	***R. officinalis* L.** *Pyramidalis* "Upright Rosemary " **CV.2**	***R. officinalis* L.** *Angustifolius* "Pine scented" **CV.3**
Growth habit	Upright	Upright	Upright
Plant color	Grayish green	Light green	Dark green
Leaflet length (cm)	2.3	3.2	1.98
Leaflet width (mm)	1.7	1.8	1.6
Stem color	Hardy Gray	Light brown	White
Mean No of leaves/middle branch of the plant	362	412	465
Leaf area (cm2)	0.61	0.78	0.56
No. of Axillary shoot/ middle branch of the plant	11.2	12.4	10.9

2. Effect of medium type and cytokinin kind on callus and shoot formation of rosemary cultivars

Data in Table (2 and 3) showed that, the highest mean value of callus weight 8.02 g/explant for CV.1 recoded with MS medium supplemented with 1mg/l CPPU followed with 6.9 g/explant WPM supplemented with mg/l TDZ. On the other hand, the highest mean No. of shoot formed 2.040 shoots/explant produced from WPM+1mg/l CPPU followed with 2.00 shoots/explant produced from

WPM+ 1mg/l TDZ. For CV.2 the highest significant weight of formed callus resulted from MS+1 mg/l CPPU by 9.02g/explant followed by 8.38g/explant produced from MS+WPM+1mg/l TDZ. Although the highest means of callus weight resulted from MS medium, data at Table (3) showed that the highest mean No. of formed shoots 1.60 shoots/explant produced from WPM+1 mg/l CPPU followed by 1.20 shoots/explant from WPM+1mg/l TDZ. Concerning CV.3 the highest mean weight of callus was 2.52g/explant produced from WPM+1 mg/l CPPU followed by 1.80g/explant from WPM+1mg/l TDZ while the highest mean No. of formed shoot resulted from the same combination of medium and PGRs which gave 11.00 and 6.20 shoots/explant respectively.

From previous results it can be concluded that MS medium support callus initiation especially for CV.1 and CV.2, while WPM improve embryos and shoot formation for all cultivars. These results agreed with Misra and Chaturvedi (1984) who mentioned that lower salt concentration of Whit's medium is better for seed germination, exceed root growth and initial culture of shoot explants rather than MS medium which have high salt concentration with presence of NH4NO3 which more suitable for shoot proliferation. Aman and Afrasiab (2014) concluded that, subculture of primary somatic embryos on modified WPM without hormones gave rise to clusters with secondary somatic embryos and embryogenic calli. These clusters were sub-cultured every 4 weeks, and an average of 10% of the secondary somatic embryos developed into plantlets in each subculture. El-Zefzafy *et al*. (2016) on *Rosmarinus officinalis* L. used BAP or TDZ at 0.125, 0.250, 0.500 and 1.000 mg/l on MS –modified , and found that callus induction was completely inhibited in the absence of both auxin and cytokinin and the different ratios of auxin to cytokinin significantly affected the physiological callus responses.

Table 2: Effect of medium type supplemented with (1 mg/l) CPPU or TDZ on fresh callus weight g/explant of rosemary cultivars.

Cultivar type	MS-CPPU	MS-TDZ	WPM-CPPU	WPM-TDZ	Mean (A)
Rosmarinus officinalis L. CV.1	8.02	3.67	3.95	6.90	5.63
R. officinalis L. Pyramidalis CV.2	9.02	8.38	5.08	5.27	6.94
R. officinalis L. Angustifolius CV.3	0.85	1.02	2.52	1.80	1.55
Mean (B)	5.96	4.36	3.85	4.66	
LSD 0.05%	A= 0.903		B=1.044	AxB=1.808	

* A= CV. B=Media

Table 3: Effect of MS-medium or WPM medium supplemented with (1 mg/l) CPPU or TDZ on No. of formed shoots/explant for each rosemary cultivar.

Cultivar type	MS Medium		MC-Woody Plant Medium		
	MS-CPPU	MS-TDZ	WPM-CPPU	WPM-TDZ	Mean (A)
Rosemarinus officinalis L. CV.1	1.40	1.00	2.40	2.00	1.700
R. officinalis L. Pyramidalis CV.2	1.00	0.60	1.60	1.20	1.100
R. officinalis L. Angustifolius CV.3	3.80	2.80	11.00	6.20	5.950
Mean (B)	2.067	1.467	5.00	3.133	
LSD 0.05%	A= 2.15		B= 2.90	AxB=5.022	

* A= CV. B=Media

3. Effect of salt stress on callus induction and shoot formation

Data in Table (4) showed that, with increasing salt stress callus weight increased at CV.1 and CV.2 it's recorded highest mean of weight at 1000 µM/l which resulted 8.46 g/explant for C1 and 8.55g/explant for CV.2 while CV.3 showed highest weight 1.04 g/explant at 200 µM/l followed by 0.85g/explant with 1200 µM/l. most initiated callus developed embryos and shoots.

Tabulated data at Table (5) showed the highest mean No. of shoots which formed on stress conditions obtained from CV.1 was (8.33 shoots/explant) at concentration 400 µM/l, with increasing concentration of salt stress mean No. of shoots started to decrease till it gave 6.00 shoots/explant at 1200 µM/l of NaCl but it sharply decreased to be not significant result by 2.33 shoots/explant at 1400 µM/l. Concerning CV.2 the highest mean No. of shoots was 4.00 shoots/explant was obtained from addition of 200 and 800 µM/l of NaCl to the medium, while 1200 µM/l produced 3.33 shoots/explant then number of shoots decreased to be not significant at 1400 µM/l which gave only 2.00 shoots/explants. On the other hand, CV.3 recorded the highest mean No. of formed shoots 21.76

shoots/explant at 600 µM/l of NaCl then number of formed shoots start to decrease to be 5.33 shoots/ explant at 1000 µM/l of NaCl then its increased again to be 14.00 shoots/explant at 1400 µM/l of NaCl.

Table 4: The effect of different concentrations of NaCl salt stress on callus fresh weight (g) of leaf explants cultured on MS media supplemented with 1 mg/l CPPU for each rosemary cultivar

NaCl con. µM/l	***Rosmarinus officinalis* L. CV.1**	***R. officinalis* L.** *Pyramidalis* **CV.2**	***R. officinalis* L.** *Angustifolius* **CV.3**	**Mean (B)**
200	5.410	6.130	1.047	**4.196**
400	6.550	7.077	0.826	**4.818**
600	7.040	6.283	0.426	**4.583**
800	7.953	5.517	0.330	**4.600**
1000	8.467	8.557	0.770	**5.931**
1200	6.507	6.813	0.850	**4.723**
1400	3.260	3.577	0.700	**2.512**
Mean (A)	**6.455**	**6.279**	**0.707**	
LSD 0.05%	**A=0.765**	**B=1.169**	**AxB=2.025**	

*A= CV. B=salt stress

Table 5: Effect of abiotic salt stress on mean number of formed shoots of each rosemary cultivar leaf explants cultured on MS media supplemented with 1 mg/l CPPU.

NaCl con. µM/l	***Rosmarinus officinalis* L. CV.1**	***R. officinalis* L.** *Pyramidalis* **CV.2**	***R. officinalis* L.** *Angustifolius* **CV.3**	**Mean (B)**
200	7.333	4.000	10.000	**7.111**
400	8.333	3.000	6.000	**5.778**
600	3.667	3.333	21.67	**9.556**
800	6.000	4.000	7.667	**5.889**
1000	3.000	2.667	5.333	**3.667**
1200	6.000	3.333	7.000	**5.444**
1400	2.333	2.000	14.000	**6.111**
Mean (A)	**5.238**	**3.190**	**10.24**	
LSD 0.05%	**A=2.958**	**B=4.518**	**AxB=7.827**	

*A= CV. B=salt stress

Although the concentrations of salt stress considered very weak but the previous results showed that the ability of each cultivar to grow under salt stress conditions differ from each other as it found CV.3 was the most tolerant then CV.1, while CV.2 showed the least tolerance, it's also noticed that some concentrations of salt stress at medium can enhance the role of cytokinin to produce more number of shoots and embryos in comparison of using cytokinin alone especially with CV.1 and CV.2. Youssef and Rady (2000) mentioned that callus growth was depressed as salt concentration increased in the culture medium, the presence of 1.5 % NaCl in the culture medium leads to reduction in callus growth by 19.34 % of control on fresh weight. The effect of NaCl salinity on growth of cell and callus cultures caused reduction of growth due to salt damage as a result of toxic effects caused by specific ions and negative effects caused by lowering the external water potential.

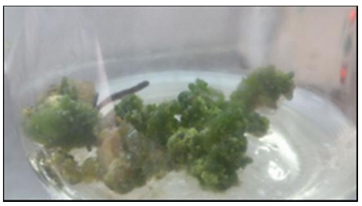

Fig. 4: Callus formed of CV.1 on MS medium +1 mg/l CPPU +1000 µM/l NaCl

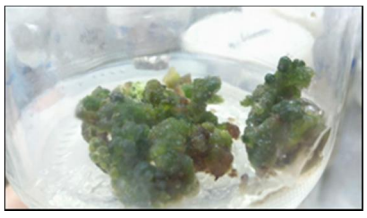

Fig. 5: Callus formed of CV.2 on MS medium +1 mg/l CPPU +1000 µM/l NaCl.

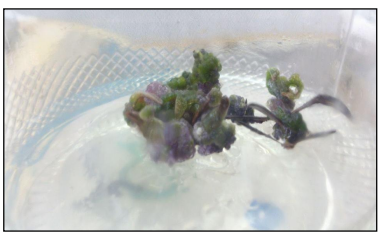

Fig.6: Callus formed of CV.3on MS medium +1 mg/l CPPU +200 µM/l NaCl.

4. Volatile oil profiling in rosemary cultivars callus analyzed *via* Head space chromatography with solid-phase microextraction (SPME)

Volatile oil compositions were determined on callus of CV.1 and CV.2 obtained from the highest weight of callus grown under salt stress at 1000 µM/l NaCl while for CV.3 determined callus was obtained from that resulted at 1200 µM/l. Table (6) showed that the highest component of volatile oils formed in callus was Bornyl acetate at 33.38%, 28.31% and 15.11% for CV.1, CV.3, and CV.2 respectively followed by (-)-α-Pinene by 17.47% and16.55% for C1 and C3 respectively while CV.2 didn't show (-)-α-Pinene content. Cineole also was formed at callus of each three cultivars as CV.1 had 13.72% then CV.3 had 14.16% followed by CV.2 which had it at 10.92%. Limonene also was appeared at results, the highest concentration was 9.57% obtained from CV.2, followed by 6.46% for CV.1 then 5.12% for CV.3. Camphor which considered the main component of essential oil for CV.2 was only appeared at concentration of 9.69 % for CV.2 and 4.12 % for CV.1, Although the essential oil of CV.3 didn't had Camphor but it papered of formed callus under salt stress with concentration of 3.47%. Verbenone which was the main component of essential oil for both CV.3 and C1 it's appeared at very weak percent at callus with concentration of 1.28% For CV.3 and 4.89% for CV.1 while CV.2 show also it at results at1.83%.

Table 6: Volatile oils profiling in each rosemary cultivar fresh callus as analyzed *via* Head space chromatography with solid-phase microextraction (SPME)

RT	NAME	Class	CV.1	CV.2	CV.3
7.008	.α-Thujene	Monoterpene hydrocarbon	0.00	5.54	0.00
7.143	**.(-)-α-Pinene**	**Monoterpene hydrocarbon**	**17.47**	**0.00**	**16.55**
7.45	camphene	Monoterpene hydrocarbon	2.10	5.78	2.18
7.948	β-Pinene	Monoterpene hydrocarbon	1.86	0.00	1.75
8.108	β-Myrcene	Monoterpene hydrocarbon	3.20	0.00	3.30
8.74	p-Cymene	Monoterpene hydrocarbon	5.28	5.25	5.76
8.807	**Limonene**	**Monoterpene hydrocarbon**	**6.46**	**9.57**	**5.12**
8.9	**Cineole**	**Oxygenated monoterpene**	**13.72**	**10.92**	**14.16**
9.283	γ-Terpinene	Monoterpene hydrocarbon	1.82	0.00	1.22
9.725	.Isoterpinolene	Monoterpene hydrocarbon	1.91	0.00	0.00
10.758	**Camphor**	***Oxygenated monoterpene***	**4.12**	**9.69**	**3.47**
11.1	unknown	*unknown monoterpene*	0.00	5.46	1.15
11.14	unknown	*unknown monoterpene*	0.00	5.17	1.06
11.426	α-Terpineol	*Oxygenated monoterpene*	0.00	0.00	0.97
11.643	**Verbenone**	***Oxygenated monoterpene***	**1.83**	**4.89**	**1.28**
12.523	**Bornyl acetate**	***Oxygenated monoterpene***	**33.38**	**15.11**	**28.31**
13.5	unknown	unknown monoterpene	1.57	0.00	0.00
13.586	(-)-cis-Myrtanyl acetate	*Oxygenated monoterpene*	1.76	0.00	1.37
14.252	Caryophyllene	Sesquiterpene hydrocarbon	1.89	0.00	1.27
14.433	(E)-β-Famesene	Sesquiterpene hydrocarbon	0.00	4.93	0.00
14.662	Humulene	Sesquiterpene hydrocarbon	1.63	5.58	0.85
15	Germacrene D	Sesquiterpene hydrocarbon	0.00	0.00	9.27
15.192	unknown	unknown sesquiterpene	0.00	4.73	0.00
15.317	cis-muurola-3,5-diene	Sesquiterpene hydrocarbon	0.00	0.00	0.96
19.83	Capric acid, ethyl ester	Fatty acid	0.00	7.37	0.00
			100.00	100.00	100.00

From previous results its noticeable that the main components of essential oil of each cultivar which obtained under normal conditions was decreased on percent comparing of it at callus volatile oils which grown under salt stress, most formed components were belong to oxigenated terpines and

mono terpines (Bornyl acetate, (-)-α-Pinene, Cineole, Limonene and Camphor) which are valuable for marketing. Also, it was found that the more sensitivity of cultivar to grow under stress conditions the less production of volatile oils for it at callus grown under salt stress, and this was clear with CV.2 comparing with CV.1 which showed the highest results at most components followed by CV.3, this may back up for their sensitivity and tolerance for salt stress. These results agreed with Youssef and Rady (2000) Who found that, the major constituents of essential oils of rosemary plant were found to be camphor (39.5 %), bornyl acetate (19.08 %) 1,8-cineole (17.97%) and linalool (15.5 %). While bornyl acetate was found to be the major constituents for callus tissues grown under different salt levels and reached the higher relative percent (74.1 %) at 1.5 % NaCl. Tawfik *et al*.,(1992) reported that maximum beta-pinene levels were obtained from *R. officinalis* callus cultures when grown on MS medium with 19 g/ l sucrose, while borneol levels decreased at the highest sucrose concentration. Ca2+ concentration in the culture media affected the yield of camphene, 1,8-cineole, linalool and bornyl acetate. The highest oil yield was obtained from fresh callus cultured on medium containing 0.99 mmol Ca2+ / l. Also, Rashid *et al.*, (2011) found that rosmanol (ROL) 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 $CaCl_2$, they also reported 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.

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