Original Article

ESSENTIAL OIL AND TRICHOME DENSITY FROM *Origanum majorana* L. SHOOTS AFFECTED BY LEAF AGE AND SALINITY

ÓLEO ESSENCIAL E DENSIDADE DO TRICOMA DOS REBENTOS DA Origanum majorana *L. AFETADOS PELA IDADE DA FOLHAGEM E PELA SALINIDADE*

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ABSTRACT: Essential oil (EO) from *Origanum majorana* L. (Lamiaceae) shoots, extracted by hydrodistillation from plant cultivated under control and salt conditions. Essential oil composition was determined by GC-MS. Plant material was harvest at three vegetative stages; early (EVS), late (LVS) and early flowering (P.F.S). Essential oil yield were 0.11% and 0.071 for E.V.S, 0.19% to 0.37% for L.V.S 0.23% and 0.47% for P.F.S, at the control and in the presence of 75 mM NaCl, respectively. Salt stress and development of vegetative stage affected the formation of the major compounds: *cis-sabinene hydrate* and *terpinene-4-ol*. Leaves were observed with scanning electron microscope (SEM), to determined trichomes number, size and distribution. Results showed that globular trichome density decrease with leaf maturity but increased with salinity.

KEYWORDS: Essential oil. *Origanum majorana* L. Salinity. Trichomes.

INTRODUCTION

Glandular and non glandular trichomes are known to be present on the surfaces of leaves (BAATOUR et al. 2012). Previous reports have shown that there are glandular and non glandular trichomes that function in plants to protect it. It seemed that under salinity, leaves are more involved in essential oil production (BAATOUR et al. 2012). Number of environmental conditions factors have high impact on the performance of plant including EO content and composition (FAROOQI et al. 1999).

Salinity is one of the major factors that affect essential oil biosynthesis and secretion (HEUER et al. 2002). In Tunisia, salt-affected soils cover about 10% of the total area of the country (HACHICHA, 2007). Thus it is important to consider this factor in every study. Lamiaceae is the important family of aromatic and medicinal plant, it is known for its popular species such as, basil, lavender, thyme or clary sage (*Salvia sclarea)* and oregano. Among this later genus *Origanum majorana* Syn. *Majorana hortensis* (M.), is one of the most common medicinal and aromatic species in Tunisia. Its essential oil was known for its antimicrobial (MOHAMMED et al. 2011), antimutagenic (AL-HARBI, 2011) antihyperglycemic, antilipidemic (AL-HARBI, 2011) and antiulcer (PIMPLE et al. 2012) and antioxidant activity (BAATOUR et al. 2012 c). It is used in perfumery for its spicy fragrance and herbaceous notes (FILIPPO et al.2000). *Origanum majorana* L., as described by Wagner et al. (2004) in most Lamiaceae species, we have observed epidermal trichomes in leaves and stems. Trichomes were divided into two subcategories, glandular and non-glandular (BAATOUR et al. 2012). Three type of EO secretion referred to their contrast dark red droplets, clear lipids droplets, secretion of lucid appearance were seen under cuticule of head cells of peltate trichomes (BAATOUR et al. 2012). Many phytochemical studies have been conducted to investigate the chemical composition of *Origanum majorana* L. In our knowledgment, there is no study in salt effect on: i) glandular distribution, ii) EO content and composition at development staged. Except study of Karray et al. (2009) in *Mentha Pulegium*, who reported that under saline condition, glandular trichomes exhibited no visible reduction in their sizes, maintained the same distribution between leaf sides in young as well as in mature leaves. But, there is an increased in the densities of the two types of glandular trichomes (peltate and capitate). That's why in the present work we investigate, the effect of salinity on Tunisian *O. majorana* shoot essential oil content at three development stage, and we explained to understand the phenomenon through a study concerning trichomes distribution under saline and non-saline conditions.

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MATERIAL AND METHODS

Plant material

Marjoram seeds (collected in 2010) were taken from a local plant nursery at Nabeul in Northeast of Tunisia. Plants of *O. majorana* were raised from seed for 8 days in a culture chamber with a 16 h photoperiod (150 µmol m^{-2} s^{-1}). Temperature and average relative moisture were 22 °C and 40%, respectively, during the day and 18°C and 86% at night. Plants were transferred to eight-strength Hoagland and Arnon (1950) nutrient solution (1.25 mmol L^{-1} KNO_3, 1.25 mmol $L^{-1}Ca(NO_3)_2.4H_2O$, 0.50 mmol L^{-1} $MgSO_4.7H_2O$, 0.25 mmol L^{-1} KH_2PO_4, 0.01 mmol L^{-1} H_3BO_3, 0.001 mmol L^{-1} $MnSO_4.4H_2O$, 0.0005 mmol L^{-1} $CuSO_4.5H_2O$, 0.0005 mmol L^{-1} $ZnSO_4.6H2O$ and 0.00005 mmol L^{-1} $(NH_4)_6Mo_7O_{24}.4H_2O$) in a culture chamber with a 16 h photoperiod (150 µmol m^{-2} s^{-1}). After 20 days of acclimation, NaCl (75 mmol L^{-1}) was added to the nutritive solution. The plant aerial parts were harvested 17, 24 and 31 respectively at early (EVS), late (LVS) and preflowering vegetative stage (PFS).

Essential oil isolation, identification and quantification

Isolation

A 50 g portion of air-dried material was subjected to hydrodistillation for 90 min in a simple laboratory according to Msaada et al. (2007). Quik-fit apparatus consisting of a 1 L steam generator flask, a distillation flask, a condenser and a receiving vessel. The obtained distillate was extracted using diethyl ether as solvent and dried over anhydrous sodium sulfate. The organic layer was then concentrated at 35°C in a Vigreux column and the essential oil was stored at −20°C until analysis. In order to quantify the essential oil constituents, 6- methyl-5-hepten-2-one was used as an internal standard. Essential oil isolation was done in triplicate.

Identification and quantification

GC-FID: Gas chromatography analysis was carried out on a Hewlett–Packard 6890 gas chromatograph equipped with a flame ionization detector (FID) and an electronic pressure control (EPC) injector. A polar HP Innowax (PEG) column and an apolar HP-5 column (30 m × 0.25 mm, 0.25 µm film thickness) were used. The carrier gas (N_2, U) flow was 1.6 ml min^{-1} and the split ratio 60:1. EO analysis was performed using the following temperature program: oven temps isotherm at 35°C for 10 min, from 35 to 205°C at the rate of 3°C min^{-1} and isotherm at 225°C during 10 min. Injector and detector temperatures, were held, respectively at 250 and 300°C .

GC–MS: GC–MS analysis was performed on a gas chromatograph HP 5890 (II) interfaced with a HP 5972 mass spectrometer with electron impact ionization (70 eV). A HP-5MS capillary column (30m ×0.25 mm, 0.25 µm film thickness) was used. The column temperature was programmed to rise from 50 to 240°C at a rate of 5°C min^{-1}. The carrier gas was helium with a flow rate of 1.2 ml min^{-1}; split ratio was 60:1. Scan time and mass range were 1 s and 40– 300 m/z, respectively **(Baatour et al. 2010)**.

Scanning electron microscopy (SEM)

Leaves from the top (third node from the apex) and the base (tenth node from the apex) of control and treated plants were harvest for each treatment, without causing any damage to the surfaces, 10 fresh leaves were observed on the two faces by a FEIQUANTA 200 environmental scanning microscope.

Statistical analysis

All data were subjected to analyses of variance and means were separated using Duncan's multiple range test at a 5%.

RESULT AND DISCUSSION

Variation of essential oil yield and composition with leaf age and salinity

Essential oil (EO) yields from *O. majorana* shoots, varied with stages (Table.1). In the control, EO yield increased by about 1.61 times and 2.32 times respectively at L.V.S and P.F.S. as compared to EVS (Table. 1). At 75 mM NaCl, this yield decreased significantly by about 0.6 times at EVS, but increased by about 1.94 and 2 times at L.V.S. and P.F.S., respectively. The increase at LVS was in agreement of most previous works such as those of Verdian-Rizi (2008) in *Origanum onites* and *Laurus nobilis*; Karray et al. (2009) in *Mentha pulegium* and Hamrouni et al. (2009) in *Origanum majorana*. This decrease in EO yield may be due to the low rate of biosynthesis of volatile compounds during vegetative stage and suggest biosynthesis of volatile compounds that to reach a maximum during the pre flowering stage. At our knowledgment this stage could be favoured to ensure the maximum of essential oil yield. EO composition is affected by many factors, among others, the development stage (KIM; LEE, 2004).

Table 1. Quantitative (µg/ g DW) changes of essential oil compounds from *Origanum majorana* shoots under NaCl 75 mM at the early vegetative stage (E.V.S), late vegetative stage (L.V.S) and preflowering stage (P.F.S.)

			L.V.S	L.V.S	P.F.S	P.F.S
Essential oil yield (%)			**0.19±0.002[b]**	**0.37±0.005[a]**	**0.232±0.024[b]**	**0.479±0.005[a]**
Compounds	**RI[a]**	**RI[b]**	**Content of total volatiles (µg/ g DW)**			
			0 (control)	**75**	**0 (control)**	**75**
Toluene			-	0.006 [a]	0.023±0.01[b]	0.031±0.01[b]
Tricyclene	927	1014	-	-	0.006±0.00[b]	0.035±0.02[a]
α- pinene	931	1035	0.09±0.03[a]	0.001 ±0.01[b]	0.017±0.00[b]	0.719±0.24[a]
β- pinene	980	1095	-	0.012±0.03 [a]	0.26±0.14[b]	0.404±0.04[a]
sabinene	976	1132	9.75± 0.87 [a]	0.034±0.23 [b]	1.41±0.03[b]	0.374±0.04[a]
Δ-3-Carene	1014	1134	-	0.010±0.08[a]	0.01±0.00[b]	4.04±0.23[a]
myrcene	991	1174	0.06±0.10[b]	0.015±0.21 [a]	0.19±0.24[b]	0.041±0.24[b]
myrtenal	1192	1684	-	0.077±0.10 [a]	1.1±0.24[b]	0.039±0.24[b]
α phellandrene	1006	1176	-	-	-	-
limonene	1030	1203	-	-	3.74±0.22[a]	-
1.8 cineole	1033	1213	-	0.002±0.02[a]	0.83±0.12[b]	9.95±1.12[a]
γ-terpinene	1062	1266	0.63±0.10 [a]	0.003±0.04 [b]	0.05±0.01 [b]	0.07±0.01 [a]
p-cymene	1026	1280	-	0.04±0.23[a]	-	-
terpinolene	1088	1290	0.14±0.10 [b]	0.03±0.10 [a]	1.46±0.1[b]	7.45±0.21 [a]
cis p-menth-en-1-ol	1129	1562	0.11±0.01 [a]	0.001±0.00 [b]	0.081±0.01[b]	0.09±0.001 [a]
B-tujone	1115	1336	-	0.002±0.02 [a]	0.068±0.01[b]	12.96±0.45[a]
trans p-menth-en-1-ol	1130	1638	3.63±0.87 [a]	0.019±0.14[b]	5.97±0.24[a]	-
Linalool	1098	1553	0.11±0.10 [a]	0.003±0.01 [a]	0.107±0.01[b]	25.19±0.31 [a]
camphor	1143	1532	-	0.004±0.07 [a]	0.016±0.01[b]	0.289±0.04 [a]
β murolene	1469	1672	-	0.001±0.00 [a]	0.130	0.06
cis sabinene hydrate	1082	1556	16.33±0.10 [a]	0.90±0.08 [b]	20.32±0.19 [a]	68.85±0.27 [a]
trans sabinene hydrate	1053	1474	1.07±0.05 [b]	0.18±1.98 [a]	2.09±0.11[b]	6.13±0.31[a]
linalyl acetate	1257	1556	0.18	0.11±0.25[a]	0.53±0.11[b]	1.39±0.02[a]
bornyl acetate	1295	1597	0.71±0.01 [b]	0.03±0.13 [a]	1.32±0.02 [a]	4.02±0.31 [a]
Carvone	1245	1598	-	-	-	-
β-elemene	1391	1601	0.37±0.12[a]	0.006±0.02[b]	0.617±0.31[a]	-
terpinene 4-ol	1176	1611	10.75±0.10[a]	0.48 ±0.20[b]	18.85±1.01[b]	52.74±3.41 [a]
β caryophyllene	1419	1612	0.21±0.02[a]	0.002±0.00 [b]	0.43±0.11 [a]	0.04±0.01[b]
σ terpineol	NI	NI	-	0.005±0.01[b]	0.02±0.00[b]	9.53±3.01 [a]
α humulene	1454	1687	-	0.003±0.09[b]	0.184±0.01 [a]	-
α terpineol	1189	1713	0.10±0.03[b]	0.15±0.11 [a]	2.74±1.04 [a]	-
isobornylacetate	-	-	-	0.027±0.01 [a]	0.374±0.21[b]	0.56±0.22[a]
germacrene-D	1480	1726	-	0.003±0.02 [a]	0.06±0.01[b]	0.12±0.01[a]
β-bisabolene	1503	1741	-	0.21±0.00 [a]	0.18±0.01[b]	0.46±0.11[a]
bicyclogermacrene	1344	1705	-	0.003±0.02 [a]	0.07±0.00[b]	0.12±0.07[a]
neryl acetate	1385	1733	0.36±0.04 [a]	0.003±0.01[b]	0.011±0.01[b]	0.04±0.01 [a]
geranyl acetate	1383	1765	0.10±0.02[b]	0.009±0.01 [a]	0.018±0.01[b]	0.07±0.01[a]
Nerol	1228	1797	-	0.001±0.14 [a]	-	-
cis-piperitone	1265	-	-	0.01±0.22 [a]	0.158±0.02 [b]	0.42±0.11[a]
spathulenol	1572	2144	-	0.02±0.24 [a]	0.05±0.01[b]	1.42±0.02[a]
Eugenol	1401	2030	-	0.23±0.03[a]	0.09±0.01[b]	0.28±0.11 [a]
Penol			-	-	0.861±0.21[a]	0.21±0.01[b]

Values (means of three replicates± SD) with different superscripts (a–b) are significantly different at $P < 0.05$ between salt levels within each vegetative stage; RI retention indices relative to n-alkanes on aHP-5 and bHP-Innowax columns; not detected: Nd;not identified: Ni

At L.V.S., the major constituents were cis - sabinene hydrate (16.33 µg/g DW) and terpinene-4-ol (10.75 µg/g DW) at control (Table 1). These compounds decreased significantly about 18.14 and 22.39 times with salt treatment. In addition, in the presence of NaCl, new compounds appeared, such as camphor, β-murolene, α-humulene, 1.8-cineole, *p*-cymene, isobornylacetate, germacrene-D, β-bisabolene, bicyclogermacrene, nerol, *cis*-piperitone, spathulenol, eugenol, σ-terpineol. Besides, other compounds disappeared such as

sabinene, *trans p*-menth-en-1-o and *cis* -sabinene hydrate.

At P.F.S., the chemotype of marjoram was *cis*-sabinene-hydrate and terpinene-4-ol in absence and presence of salt. These major compounds increased significantly about 3.38 and 2.79 times. In addition, salinity had a significant increase ($p<0.05$) on the content of 1.8-cineole, terpinolene, β-tujone, linalool and σ-terpineol by about 12, 5.1, 190, 235,42 and 476.5 respectively.

According to our result it seems that chemical composition of *O.majorana* EO varied with physiological stage and salt treatment. Thus, according to amounts of major constituents *O.majorana* EOs could be divided into two chemotypes

1. At early vegetative stage, the chemotype changed from **trans sabinene hydate/terpinene 4-ol** in the control to became **sabinene/cis-sabinene hydrate** at salt treatment.
2. ***Cis* -sabinene hydrate/terpinene-4-ol** characterizing the late vegetative stage and early flowering stage.

In P.F.S., the high content of one or both of these two compounds in EO followed by linalool and β-tujone, could be explained by trichomes distribution.

Some study such as Rodrigues's (2002), have reported that terpinene-4-ol was the major component flowed by ɤ-terpinene and *cis*-sabinene hydrate. In Argentinean *O.majorana* EOs, terpinene-4-ol and *trans*-sabinene hydrate seemed to be the main components (Banchio et al. 2008).

In our species and at the control, essential oil extract at the three stages delaminated contained respectively 3.963; 11.323, 6.60 µg/ DW monoterpene hydrocarbons and 22.750; 42.93, 52.04 µg/ DW terpenes alcools. At 75 mM NaCl, it contained respectively 9.168; 0.01; 27.33 µg/ DW monoterpene hydrocarbons and 9.163; 0.22; 172.67 µg/ DW terpene alcools (Table 2).

When comparing our result to some data, it seems as mentioned by Hamrouni et al. (2009) and Davaranauskaite et al. (2009) that EO yield, composition and classes could be affected by physiological stage.

The accumulation of EO during the P.F.S. could be related to ecological roles such as intensifying antifungal defences and attracting pollinators. To obtain favoured EO content, we suggest to harvest *O. majorana* at P.F.S.

To better explained the improvement in EO yield with salt and development of vegetative stage in shoots of *O. majorana* under environmental stress conditions, we accomplish a study on trichomes distribution responsible for the production of these secondary metabolites

Table 2. Chemical classes (µg/ g DW) of essential oil from *Origanum majorana* shoots under NaCl 75 mM at the early vegetative stage (E.V.S), late vegetative stage (L.V.S) and flowering stage (P.F.S)

		E.V.S	**E.V.S**	**L.V.S**	**L.V.S**	**P.F.S**	**P.F.S**
		0 (control)	**75**	**0 (control)**	**75**	**0 (control)**	**75**
Classes	Monoterpene hydrocarbons	3.963	9.168	11.323 [b]	0.01 [a]	6.60[b]	27.33 [a]
	terpene alcools	22.750	9.163	42.93	0,22[b]	52.04 [b]	172.67 [a]
	Sesquiterpene hydrocarbons	1.473	0.628	0.213 [a]	nd	0.99 [b]	1.377 [a]
	Aldehydes	nd	Nd	Nd	nd	1.10 [a]	0.039 [b]
	ketones	0.106	0.226		0.345	0.17	0.718 [b]
	Esters	0.507	0.189	1.377	0.02 [b]	0.40 [a]	0.689 [a]

Variation of trichome density with leaf age and salinity

Origanum majorana leaf has a typical dorsi-ventral structure. It is composed of an heterogenous mesophyll, typical of dicotyledones; its palisade parenchyma is made of three to four layers of elongated cells. Spongy parenchyma, consists of two to three layers of cells. The leaf is caracterised by a reticulate venation. The midrib vein is surrounded by a sheath of parenchymatous cells and protected on the dorsal and ventral sides by three to four assizes of collenchymateuses cells. The leaf is enveopped by an unistratified epidermis, covered with thick cuticule. Epidermis cells were remarkably thicker on the ventral side.

As previously study at EVS by Baatour et al. (2012) has been confirmed by in this paper. In fact, all leaves of *Origanum majorana* L. had non-glandular and glandular trichomes on both surfaces (Figure 1: A, A'). The number of non-glandular trichomes increased at 75 mM NaCl at each vegetative stage (Figure 2).

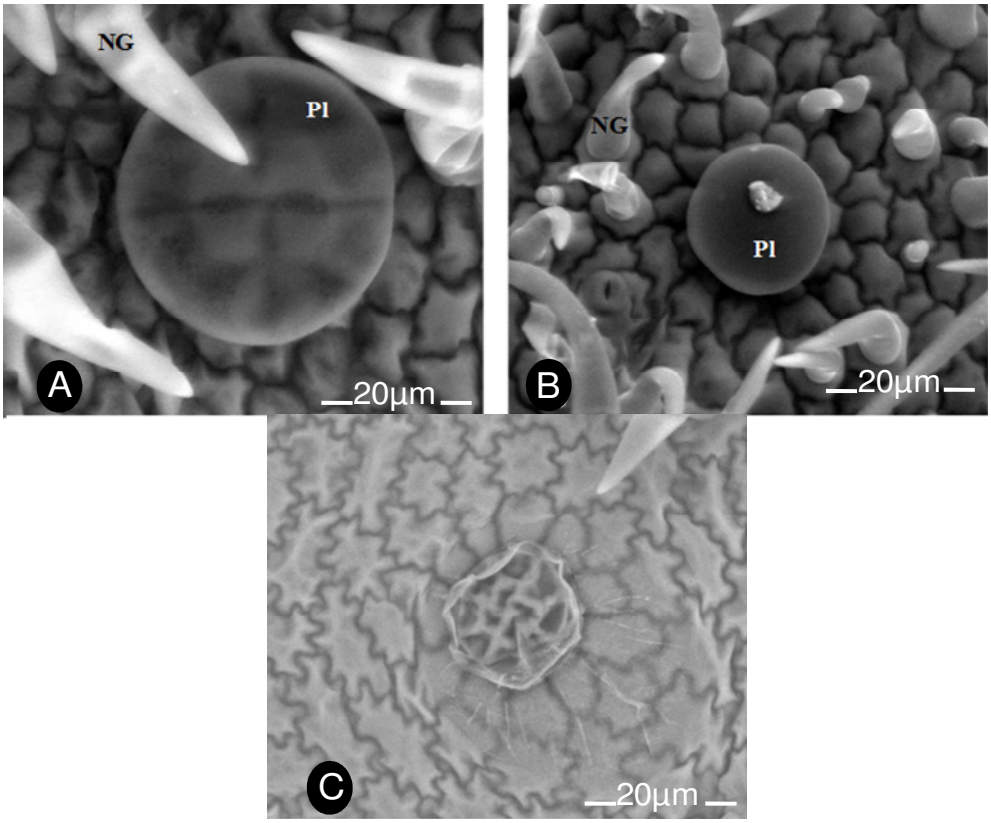

Figure 1. Scanning electron micrographs (A-C) of leaf portions from *Origanum majorana L.* **A**, trichomes on the adaxial side of a leaf; Peltate heads at eight cell stage (Pl). B, Note the higher density of non-glandular trichomes (NG) as compared with A at 75 mM NaCl. C: Mature peltate trichome with torn cuticular sheath, disclosing the head cells. Bar: 1. µm

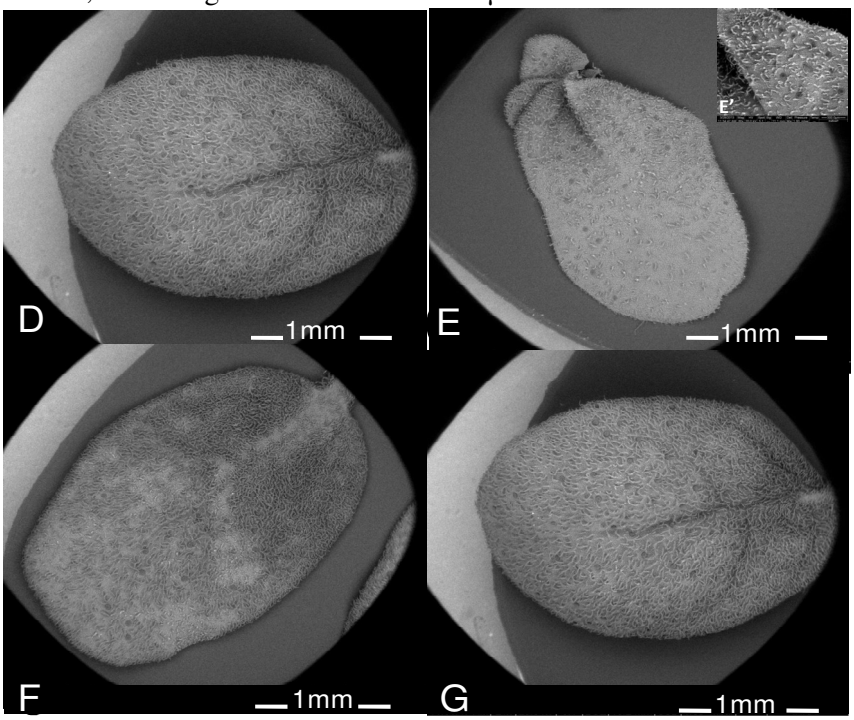

Figure 2. Scanning electron micrographs of the adaxial surfaces of *Origanum majorana* leaves, showing trichome density (mm-^{2}). **D-F**, views of early vegetative stage (E.V.S.) at 0 mM NaCl; **E-G,** views of late vegetative stage (L.V.S.) at 75 mM. Bar = 1µm

The density of trichomes, including non-glandular and glandular, gradually decreases with leaf maturity. The surface of young leaves is covered by important number of non glandular trichomes (23 ± 0.01^{c}) and (36 ± 0.24^{a}) respectively at 0 mM and 75 mM NaCl. As the leaf expands, the number and density of these trichomes decrease (Table 3).

Salinities increase the densities of the glandular trichomes at EV, LV and PF stages of development to reach (36 ± 0.24^{a}), (34 ± 0.14^{b}) and (25 ± 2.01^{c}) respectively as compared to control (23 ± 0.01^{c}), (19 ± 0.34^{d}) and (9 ± 0.14^{e}).

Our result was in agreement of several studies. In fact, Gruenwald et al. (1998), affirmed that stress may increase the absolute number of glands produced before leaf emergence. In this investigation, Farooqi et al. (1999) explained the increase of their density on leaves under environmental stress conditions by the fact that salt-treated plants exhibit less leaf area. The improvement of their density in *M. pulegium* under salt stress could explain the enhancement of EO yield.

Table. 3. Trichome density of (per cm^{-2}) of leaves in *Origanum majorana* under 75 mM NaCl at the early vegetative stage (E.V.S.), late vegetative stage (L.V.S) and preflowering stage (P.F.S.)

developement stage	salt treatment (mM)	trichome density
E.V.S	0	23 ± 0.01^{c}
E.V.S	75	36 ± 0.24^{a}
L.V.S	0	19 ± 0.34^{d}
L.V.S	75	34 ± 0.14^{b}
P.F.S	0	9 ± 0.14^{e}
P.F.S	75	25 ± 2.01^{c}

Evaluated area (0.6 × 0.46 mm) = 0.276 mm^2. Presented values = mean ± SE, n = 10; Means in each column are separated by Duncan's multiple range test at a 5% level of probability.

In our findings, the functional role of trichomes leaf maturity becomes less important and they therefore senesce or shrivel. Our results are in agreement with Gairola et al. (2009). Other contrasting works have reported that trichomes remain functional in mature

In our result the development of stages in leaves increase the content of terpene alcools at control treatment to reach 22.75, 42.93 and 52.04 (Table. 2). The controversy was observed with salt treatment except the P.F.S. In fact the values were respectively 9.16, 0.22 and 172.67 at E.V.S, L.V.S and P.F.S at 75 mM (Table 2).

Thus our result was contradictory or can't be explained to Meng et al. (2012), who showed that the greater number of glandular trichomes on the leaves was in relation with the higher amount of terpene substances. This is due to the fact that the glandular trichomes are the main leaf sites of terpene biosynthesis and possess a complete enzymatic equipment.

Glandular trichomes secrete various types of compounds. A growing body of experimental evidence shows that terpene biosynthesis takes place within these trichomes (SIEBERT, 2004). Terpenes usually constitute the major lipophilic components of these secretions. The secretions of glandular trichomes have been exploited by humans for a number of uses (Schilmiller et al. 2008). These secretions was previously studied with Baatour et al. (2012). There is also a general understanding that salt-stressed conditions could be favour the production of plant secondary metabolites such as essential oils and density of trichomes.

CONCLUSION

The trichome density, including non-glandular and glandular, gradually decreases with leaf maturity and increased with salinity. It seems that under salinity, young leaves are less involved in essential oil production than mature leaves. Thus to obtain favoured EO content *O. majorana* must be harvest at PFS and treated with salinity.

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RESUMO: O óleo essencial (OE) de *Origanum majorana* L. (Lamiaceae) foi extraído, através de hidrodestilação, de plantas cultivadas sob condições controladas e salinas. A composição do óleo essencial foi determinada por GC-MS. O material vegetal foi coletado em três estádios vegetativos: precoce (EVS), tardio (L.V.S) e floração precoce (PFS). O rendimento do óleo essencial foi de 0,11% e 0,071% para S.V.S, 0,19% e 0,37% para L.V.S, 0.23% e 0,47% para P.F.S, para o controle e na presença de NaCl 75 mM, respectivamente. O estresse salino e desenvolvimento vegetativo de rendimento afetou a formação dos principais compostos: *cis*-sabineno hidratado terpineno-4-ol. As folhas foram observadas em microscópio eletrônico de varredura (MEV), para determinar o número de tricomas. Os resultados mostraram que a densidade globular do tricoma diminui com a maturidade da folha mas aumenta com a salinidade.

PALAVRAS-CHAVE: Óleo essencial. *Origanum majorana* L Salinidade. Tricomas.

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