Effect of NaCl and Phenylalanine on the Production of some Secondary Metabolites in *In Vitro* Cultures of *Mentha longifolia*

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ABSTRACT

The present study was conducted at the Tissue Culture and Plant Micropropagation Laboratory of Floriculture, Ornamental Horticulture and Landscape Gardening Department, Faculty of Agriculture, Alexandria University, during 2014 and 2015, to study the effect of five different concentrations of phenylalanine (0, 0.5, 5, 10, and 15 mg/l) and four different concentrations of sodium chloride (0, 2000, 4000 and 6000 mg/l) on the production of some secondary metabolites in *in vitro* cultures of *Mentha longifolia* using shoot tips and leaves.

Leaf segments about 1 cm^2 and shoot tips consisting of 3-4 cm long stem segments containing three auxiliary buds (as explants) were planted on MS medium supplemented with NAA (0.25 mg/l) + BAP(2.5 mg/l) + 30 g/liter sucrose + 7 g/liter agar for shoot regeneration from shoot tips, while MS medium contained 2,4-D (4 mg/l) + BAP (0.5 mg/l) for callus induction from leaf segments, the pH was adjusted to 5.8±0.02. Plantlets were subjected to 16 hours light and 8 hours darkness (2000- 2500 lux for 16 h/d provided by fluorescent tubes), the temperature was adjusted at 25º C ± 2 and 60-70% humidity. The cultures were incubated for 60 days in case of shoot tips and 100 days in case of leaves for callus induction. Chemical analysis was performed on callus tissues after 60 and 100 days from planting and shoot tissues after 60 days from planting which were produced under salinity levels, phenylalanine levels and their interaction to determine the contents of total chlorophyll, total phenolic, rosmarinic acid and proline in both tissues.

From the obtained results in this study, using shoot tips for the production of phenolic acid and rosmarinic acid was superior than the callus. Low concentration of sodium chloride (2000 mg /l) enhanced the production of some secondary metabolites. However high concentrations of sodium chloride (6000 mg /l) leads to slow growth, yellowing of cells and sometimes the deterioration of plants. Uses of intermediate concentrations of phenylalanine (5 and 10 mg /l) enhanced the production of chlorophyll and some secondary metabolites where as high concentration (15 mg /l) does not stop growth and cell division.

Key words: *Mentha longifolia* – Amino acids – *In vitro* – Secondary metabolites – Salt stress – Lamiaceae.

INTRODUCTION

Medicinal plants are an important source of drugs that had been used thousands of years ago (Clark, 1996). With the advent of synthetic drugs and replacing herbal medicines with them, the medicinal plants gradually faded. Then chemical drugs and antibiotics in the treatment of a wide variety of diseases due to the high speed of treatment were used. Due to harmful side effects of chemical drugs for human health, in recent years, attention and requesting of people to use herbal medicines to treat diseases has increased. As a third to a half of pharmaceutical products in United States of America are plant-derived (Molsaghi *et al*., 2014). In recent years, researches on the effects of natural inhibition against microorganisms showed that herbal products should be alternatives to synthetic drugs and may have a significant therapeutic effect (Nassiri and Hosseinzadeh, 2008).

Mentha longifolia (L) Hudson (wild mint, English horsemint) belongs to the genus *Mentha*, Lamiaceae family and is associated with medicinal and aromatic herbs. It is widespread throughout the Mediterranean, Central and Northern Europe, Asia Minor and Africa (Janković 1974 and Stamenković, 2005). Approximately 25 species belongs to Genus *Mentha*. Medicinal importance of *Mentha* is well known due to the presence of rosmarinic, the second most common ester of caffeic acid in the plant kingdom (Ellis and Towers, 1970). Herbal polyphenolic compounds are secondary metabolites with a characteristic aromatic structure, which can be classified into fifteen groups according to the basic part of their molecule, as for example phenols, phenolic acid, flavonoids, anthocyanins, quinones, catechins and tannins, just to name a few (Đilas *et al*., 2002). Rosmarinic acid (RA), exhibits various pharmacological activities including prevention of oxidation of low density lipoprotein and anti-allergic action. The biological activity of RA is described as antibacterial, antiviral, and antioxidative (Szabo *et al*., 1999).

Mentha longifolia is used in the pharmaceutical, tobacco, food industries and particularly in cosmetology. Different parts of the plant including its leaves, flower, stem, and seeds have been also used widely in traditional folk medicine as antimicrobial, carminative, stimulant, antispasmodic and for the treatment of various diseases such as headaches and digestive disorders (Naghibi *et al*., 2005). In pharmacological research, there is enough indication for

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different biological effects of *M. longifolia* and the chemical compounds present in the essential oil of the plant.

Plant tissue culture, or the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro*, is an important tool in both basic and applied studies as well as in commercial application. It owes its origin to the ideas of the German scientist, Haberlandt, at the begining of the 20th century. The early studies led to root cultures, embryo cultures, and the first true callus/tissue cultures. The period between the 1940s and the 1960s was marked by the development of new techniques and the improvement of those that were already in use. It was the availability of these techniques that led to the application of tissue culture to five broad areas, namely, cell behavior (including cytology, nutrition, metabolism, morphogenesis, embryogenesis, and pathology), plant modification and improvement, pathogen free plants and germplasm storage, clonal propagation, and product (mainly secondary metabolite) formation, starting in the mid-1960s. The 1990s saw continued expansion in the application of the *in vitro* technologies to an increasing number of plant species. Cell cultures have remained an important tool in the study of basic areas of plant biology and biochemistry and have assumed major significance in studies in molecular biology and agricultural biotechnology (Thorpe, 2007).

Plants are usually exposed to different environmental stresses which limit their growth and productivity as well as cause considerable loss to worldwide agricultural production (Shao *et al*., 2009). One of the most important factors affecting plant growth and the production of secondary metabolites is the salt stress (Nikolova and Ivancheva, 2005). Salinity of soil or water is one of major stress obstacles to increase production in plant growing areas throughout the world and especially in arid and semi-arid regions it can severely limit plant production (Jamil *et al*., 2006). About 23% of the world's cultivated lands is saline and 37% is sodic (Khan and Duke, 2001).

Sodium chloride and some amino acids could promote *in vitro* production of some plant secondary metabolites. Secondary metabolites are often referred to as compounds that have no fundamental role in the maintenance of life processes in the plants, but they are important for the plant to interact with its environment for adaptation and defense.

Therefore, the objective of this study is enhancing some secondary products such as total phenolic compounds and rosmarinic acid in *Mentha longifolia* through salt stress and phenylalanine *in vitro* using leaves and shoot tips explants.

MATERIALS AND METHODS

The experiment was performed at the Tissue Culture and Plant Micropropagation Laboratory of Floriculture, Ornamental Horticulture and Landscape Gardening Department, Faculty of Agriculture, Alexandria University, during 2014 and 2015.

Source of plant material and explants sterilization:Two months old potted plants of *Mentha longifolia* L. were obtained from a commercial nursery. Explants consisting of 3-4 cm long stem segments containing three auxiliary buds and leaf segments (1 cm^2) were used. The explants were washed with tap water, they were surface sterilized with 70% ethanol for 1 min followed by soaking in 0.1% mercuric chloride and 2-3 drops of Tween 20 for 1-2 min and finally washed thoroughly 4-5 times in a sterile double distilled water to remove any traces of mercuric chloride. Explants were cut into small pieces and cultured in 200 ml jars containing 20 ml of culture medium.

Media preparation: Murashige and Skoog (MS) basal medium with vitamins + 30 g/liter sucrose + 7 g/liter agar was used. The pH was adjusted to 5.8±0.02 using 1N NaOH and 1N HCL. Medium was then poured in glass jars (200 ml) then autoclaved for 20 minutes at 121 ºC and 120 bar/cm^2 (Mehta *et al*., 2012).

MS medium supplemented with NAA (0.25 mg/l) and BAP (2.5 mg/l) was used for shoot tips regeneration (Roy and Mukhopadhyay, 2012). Five concentrations of phenylalanine (0, 0.5, 5, 10, and 15 mg/l) and four concentrations of NaCl (0, 2000, 4000 and 6000 mg/l) were added to the growing media. The cultures were subjected to 16 hours light and 8 hours darkness (2000-2500 lux for 16 h/d provided by fluorescent tubes), the temperature was adjusted at 25º C ± 2 and 60-70% relative humidity. Callus was induced on MS medium containing both 2, 4-D (4 mg/l) and BAP (0.5 mg/l) (Darvishi *et al*. 2014).

Collected data: Chemical analysis was performed on both shootlets and callus tissue after 60 days and 100 days only in callus tissue, to determine:

Total chlorophyll content: Total chlorophyll was determined as (µg/g FW) according to Moran and Porath (1980).

Total phenolic compounds: The free bound phenolic contents in shootlets and callus were determined using folin- ciocalteu's method (AOAC, 2000) as (mg/g FW).

Rosmarinic acid (RA): The method of RA extraction used through this study was reported by Lopez-Arnoldos *et al*. (1995) and Komali and Shetty (1998) as (µmol/g FW).

Proline content: Proline was estimated using the acid ninhydrin method as described by Bates *et al.* (1973) as (µg/g FW).

Statistical analysis:The experiment was designed as factorial in a split plot design with two factors, where phenylalanine treatments (0, 0.5, 5, 10, and 15 mg/l) were arranged as main plots and sodium chloride treatments (0, 2000, 4000 and 6000 mg/l) were set as sub plots over two explants (shoot tips and leaves) each with 3 replicates. Data were analyzed for

significance by analysis of variance according to Steel and Torrie (1980). Statistical analysis was done by ANOVA, F-test, and L.S.D procedures available within the SAS software package (Version 9.13 2007).

RESULTS AND DISCUSSION

Effects of phenylalanine on chlorophylls content of *Mentha longifolia* explants

Generally, data presented in Table (1) revealed that adding phenylalanine alone to the medium caused significant increasing in chlorophylls (a, b and total) content of the used explants i.e. callus and shootlets, compared with the control treatment (medium free from phenylalanine). Also, using phenylalanine alone at 5 mg/l gave the highest significant average mean of total chlorophyll of callus and / or shootlets, compared with the other concentrations. The previous treatment led to increase the total chlorophyll content of callus with 64.09 % after 60 days and with 90.56 % after 100 days and with 51.40 % for shootlets after 60 days over the control treatment. Besides, increasing the concentration of phenylalanine over 5 mg/l in the media led to significant decrease the total chlorophyll content of the used explants.

These results were probably attributed to the direct role of the amino acid phenylalanine in the biosynthesis of the green pigments, which considers as a source for nitrogen and carbon as structural components of chlorophyll formation, hence adding phenylalanine at a proper concentration to the medium led to activate chlorophyll synthesis and protect its decomposition, consequently its content in the used explants could be increased.

Our results are similar to the findings of Ghai *et al*. (2002) on *Brassica napus,* Khodary (2004) on *Zea mays*, Agarwal *et al*. (2005) on wheat, El-Tayeb (2005) on barely, and Hayat and Ahmad (2007).

Effects of sodium chloride on chlorophylls content of *Mentha longifolia* explants

Generally, data presented in Table (2) showed that adding sodium chloride at (4000 and 6000) mg/l to the medium gave the highest significant average value of total chlorophyll content of *Mentha longifolia* callus had a high resistance for salt stress, thus increasing sodium chloride in the medium led to increase the total chlorophyll content in their tissues.

Marcelis and Hooijdonk, (1999) reported that salinity reduces the chlorophyll content in salt susceptible plants and increases it in salt tolerant plants as for callus where increased salinity levels caused an increase in chlorophyll a and b contents and total chlorophyll.

Besides, data in Table (2) revealed that using sodium chloride at any concentration in the medium led to significant decrease the average mean of total chlorophyll content of *Mentha longifolia* shootlets compared with the control treatment (medium free of sodium chloride).

Weimberg (1975), Munjal and Goswami (1995), Soussi *et al.* (1998), Jenifer and Franklin- Janus (2002) and Xu *et al.* (2008) reported that salinity caused a decrease in chlorophyll and carotenoids level in other plants. Decrease in chlorophylls level under salt stress may be due to reduction in pigment biosynthesis or enzymatic chlorophyll degradation (Xu *et al*., 2008 and Yang *et al.,* 2009). The loss of chlorophyll under salt stress could be related to photoinhibition or reactive oxygen species formation. The reduction in photosynthesis under salinity can also be attributed to a decrease in chlorophyll content. (Kato and Shimizu, 1985). The observed decrease of chlorophyll content in the plants grown under saline conditions may be attributed both to the increased degradation and the inhibited synthesis of that pigment (Garsia-Sanchez *et al*., 2002). Moreover, the reduction in photosynthetic pigments (chlorophyll a, chlorophyll b, and total carotenoids) due to salinity effect which attributed to the disturbance of ions absorption involved in chloroplast formation and protein synthesis and/or plastid breakdown (Abd El-Wahab, 2006). However, it was observed that the degradation of chlorophyll pigments was increased as a result of exposure to NaCl (Eryilmaz, 2006).

The present results are in agreement with those of Siler *et al.* (2007), on centaury, Koocheki *et al*. (2008), on *Teucrium polium*, *Thymus vulgaris*, *Zataria multiflora*, and *Ziziphora clinopodioides,* and Najafi *et al.* (2010), on *Satureja hortensis*.

Effect of the interaction between phenylalanine and sodium chloride on contents of *Mentha longifolia* explants of chlorophyll a, chlorophyll b and total chlorophyll

Data in Table (3) showed that medium with 5 mg/l Phenylalanine and free from sodium chloride gave the highest values of total chlorophyll content (390.79) in

Table 1. Means of chlorophyll a (Chl.A), chlorophyll b (Chl.B) and total chlorophyll content (T.Chl) of callus and shootlets of *Mentha longifolia* as affected by phenylalanine concentrations

Phenylalanine mg/l	Callus 60 days			Callus 100 days			Shootlets 60 days		
	Chl.A µg/g	Chl.B µg/g	T.Chl µg/g	Chl.A µg/g	Chl.B µg/g	T.Chl µg/g	Chl.A µg/g	Chl.B µg/g	T.Chl µg/g
0	11.276 c	8.132 c	19.408 c	10.217 b	5.346 b	15.563 c	145.34 c	37.610 b	182.95 bc
0.5	14.628 b	11.162 ab	25.790 b	16.043 a	10.360 a	26.403 ab	189.77 b	51.694 a	241.464 a
5	19.594 a	12.253 a	31.847 a	17.453 a	12.204 a	29.657 a	224.06 a	52.932 a	276.992 a
10	4.297 e	12.033 a	16.330 d	10.444 b	6.117 b	16.561 c	146.65 c	40.681 b	187.331 b
15	8.198 d	9.496 bc	17.694 cd	11.602 b	10.425 a	22.027 b	111.87 d	37.479 b	149.349 c
L.S.D $_{(0.05)}$	1.685	1.701	3.0199	2.390	2.838	4.387	28.956	8.2172	36.586

L.S.D: least significant differences at 0.05 of probability.
Means followed by the same letter(s) in each column are not significantly different according to L.S.D at 0.05 level of probability.

Table 2. Means of chlorophyll a (Chl.A), chlorophyll b (Chl.B) and total chlorophyll content (T.Chl) (µg/g fresh weight) of callus and shootlets of *Mentha longifolia* as affected by sodium chloride (NaCl) concentrations

NaCl mg/l	Callus 60 days			Callus 100 days			Shootlets 60 days		
	Chl.A µg/g	Chl.B µg/g	T.Chl µg/g	Chl.A µg/g	Chl.B µg/g	T.Chl µg/g	Chl.A µg/g	Chl.B µg/g	T.Chl µg/g
0	8.732 c	7.714 b	16.446 d	11.340 c	7.275 b	18.615 c	259.82 a	75.577 a	335.40 a
2000	10.103 c	8.783 b	18.886 c	14.223 b	7.171 b	21.394 b	145.33 b	38.115 b	183.42 b
4000	15.688 a	13.417 a	29.105 a	9.674 c	7.566 b	17.240 c	152.81 b	35.682 b	188.49 b
6000	11.870 b	12.547 a	24.419 b	17.369 a	13.547 a	30.916 a	96.20 c	26.944 c	123.14 c
L.S.D $_{(0.05)}$	1.422	1.312	2.321	1.845	1.474	2.468	27.017	8.138	34.051

L.S.D: least significant differences at 0.05 of probability.
Means followed by the same letter(s) in each column are not significantly different according to L.S.D at 0.05 level of probability.

Table 3. Means of total chlorophyll, phenols, rosmarinic acid and proline contents of callus and shootlets of *Mentha longifolia* as affected by phenylalanine (Phe) and sodium chloride (NaCl) concentrations

Explant type	Total chlorophyll content (µg/g fresh weight)					Total phenols (mg/g fresh weight)				Rosmarinic acid content (µmol/g fresh weight)				Proline content (µg/g fresh weight)			
	Phe	NaCl mg/l				NaCl mg/l				NaCl mg/l				NaCl mg/l			
	mg/l	0	2000	4000	6000	0	2000	4000	6000	0	2000	4000	6000	0	2000	4000	6000
Callus	0	14.59	17.69	25.63	19.70	5.43	6.22	6.30	4.79	0.730	0.812	0.916	0.757	1303.82	545.42	68.56	71.68
	0.5	19.69	23.53	24.14	35.78	1.72	2.38	1.63	4.47	0.435	0.288	0.192	1.095	61.29	46.74	25.966	430.10
	5	25.75	27.21	33.46	41.18	2.40	1.26	2.98	4.42	1.026	0.097	1.390	1.028	281.54	109.08	172.45	398.93
	10	10.83	12.40	16.67	25.40	4.76	4.87	5.26	5.55	0.750	0.893	0.935	1.146	338.85	713.72	243.10	1106.43
	15	11.34	13.81	45.61	0000	5.92	5.58	5.56	0000	1.074	1.060	0.967	0000	102.85	211.93	1386.93	261.80
Shootlets	0	310.06	142.11	175.73	103.90	4.81	7.69	3.06	3.97	0.669	1.908	0.468	0.583	70.29	129.63	83.19	87.06
	0.5	381.61	195.24	280.59	108.33	4.60	2.89	5.66	3.23	0.848	0.371	0.970	0.515	72.23	96.09	70.29	107.70
	5	390.79	300.30	248.69	168.19	6.03	6.49	3.72	4.61	1.168	1.131	0.595	0.642	87.06	75.45	64.49	34.82
	10	327.52	147.56	154.32	119.91	6.35	6.17	5.48	4.73	1.354	1.282	1.009	0.731	111.57	142.53	162.52	98.03
	15	267	132	83.09	115.28	6.37	6.61	5.22	4.83	1.484	1.477	0.757	0.834	75.45	67.07	74.81	80.61
$L.S.D_{(0.05)}$			52.847				1.6548				0.4802				194.6157		

L.S.D: least significant differences at 0.05 of probability.
N S: Not significant at 0.05 level of probability.
Means followed by the same letter(s) in each column are not significantly different according to L.S.D at 0.05 level of probability.

shootlets, while medium with 10 mg/l Phenylalanine and free from sodium chloride gave the lowest value of total chlorophyll content (10.83) in callus.

Generally, data presented in Table (3) showed that adding phenylalanine at 0.5 and / or 5 mg/l to the medium in absence of sodium chloride gave the maximum significant content of total chlorophyll in the shootlets of *Mentha longifolia,* compared with the other explant (callus) and / or concentrations.

These results may be due to that the shootlets of *Mentha longifolia* are considered as salt sensitive and adding phenylalanine at a proper concentration to the medium led to activate the chlorophyll synthesis in their tissues.

Also, data in Table (3) indicated that adding sodium chloride at 2000 mg/l to the medium in absence of phenylalanine gave the highest significant values of total phenols and rosmarinic acid in the shootlets of *Mentha longifolia,* compared with the other explant (callus) and / or concentrations.

These results may be due to that the exposure of *Mentha longifolia* shootlets to salinity stress at a specific concentration led to force their tissues to form the antioxidant materials which are important for plant protection.

In addition, results in Table (3) revealed that adding sodium chloride at 4000 mg/l combined with phenylalanine at 15 mg/l to the medium gave the highest significant value of proline in the callus tissues of *Mentha longifolia,* compared with the other explant and / or concentrations.

In the present study generally, sodium chloride caused an increase in proline content of *Mentha longifolia* shootlets and callus in comparison to the control. Proline is an amino acid that increases its value in tissue of plants, under environmental stress conditions (Aziz *et al.,* 2008). The increase in proline content could be attributed to a decrease in proline oxidase activity in saline conditions (Muthukumarasamy *et al.,* 2000). Plants under salt stress may present increased levels of certain compounds. Amino acids (alanine, arginine, glycine, serine, leucine, and valine, together with the amino acid, proline, and the non-protein amino acids, citrulline and ornithine) and amides (such as glutamine and asparagines) have also been reported to accumulate in plants subjected to salt stress (Mansour, 2000). On *Catharanthus roseus* and *Matricaria chamomilla* higher total free amino acids in plants subjected to salt stress conditions were reported by Osman *et al.* (2007) and Cik *et al.* (2009).

Proline may be act as radical scavenger and protects cells against salt induced oxidative stress (Hong *et al.,* 2000). The increase of free proline under salt stress may be due to the stimulation of its synthesis which is a useful defensive way of plants (Saliem, 2000). Also the increase of free proline is a result of osmotic disturbance within cells that leads to a decrease of the osmotic potential and proline is synthesized in the cytoplasm of stressed cells which keep the equilibrium between vacuole and cytoplasm (Delauney and Verma, 1993). Further, the accumulation of free proline under high sodium chloride and high exogenous proline may be related to the synergistic effect of both of them. Proline plays an essential role in the osmoregulation of plant cells when it accumulates at high concentration at the cytoplasm and decreases water potential of cytoplasm and this will cause a balance with the low water potential of the vacuole resulting from the accumulation of ions in it and that will keep a suitable turgor of cells and grow under salt stress condition (Greenway and Munns, 1980).

Our results are in agreement with those of Abraham *et al.* (2003) who reported that proline occurs widely in higher plants and accumulates in larger amounts than other amino acids. According to literature, proline accumulates in leaves as a response to salt stress by Hendawy and Khalid (2005) on *Salvia officinalis*, Ashraf and Orooj (2006) on *Trachyspermum ammi*, Al-Amier and Craker (2007) on spearmint, Ali *et al.* (2007) on chamomile and sweet marjoram, Osman *et al*. (2007) on *Catharanthus roseus,* Abd EL-Azim and Ahmed (2009) on *Achillea fragratissima*, Cik *et al.* (2009) on *Matricaria chamomilla,* Zaki *et al.* (2009) on sweet fennel and Najafi *et al.* (2010) on *Satureja hortensis*.

Effects of phenylalanine on total phenolic compounds and rosmarinic acid content of *Mentha longifolia* explants

Generally, data presented in Table (4) on the averages values of the total phenolic compounds and rosmarinic acid content of the periods (60 and 100 days) indicated that adding phenylalanine at 5 mg/l to the medium gave the highest values of the two compounds in *mentha longifolia* callus, compared with the other concentrations. While as for the shootlets, data in Table (4) showed that using phenylalanine at 15 mg/l gave the highest content of total phenols and rosmarinic acid in their tissues compared with the other concentrations.

These results were probably due to that each plant tissue requires a proper concentration from phenylalanine to stimulate of key enzyme Phenylalanine Ammonia Lyase (PAL) activity which is the gateway of shikimik acid pathway,

Table 4. Means of total phenolic compounds (T.Phenols) (mg/g fresh weight) and contents of rosmarinic acid (RA) (µmol/g fresh weight) and proline of callus and shootlets of *Mentha longifolia* as affected by phenylalanine concentrations

phenols mg/l	Callus				Shootlets		Callus		Shootlets
	60 days		100 days		60 days		60 days	100 days	60 days
	T.Phenols mg/g	RA µmol/g	T.Phenols mg/g	RA µmol/g	T.Phenols mg/g	RA µmol/g	Proline content (µg/g)		Proline content (µg/g)
0	5.687 a	0.804 a	3.144 c	0.476 d	4.889 c	0.907 b	497.37 a	424.65 a	92.549 b
0.5	2.554 c	0.503 b	5.709 a	0.884 b	4.100 d	0.676 c	141.03 b	224.66 b	86.583 b
5	2.770 c	0.885 a	6.277 a	1.080 a	5.219 bc	0.884 b	240.50 b	123.10 b	65.461 c
10	5.116 a	0.931 a	2.903 c	0.597 cd	5.688 ab	1.092 a	600.53 a	221.28 b	128.666 a
15	4.269 b	0.775 a	4.659 b	0.778 bc	5.760 a	1.138 a	490.88 a	203.36 b	74.491 c
L.S.D $_{(0.05)}$	0.781	0.247	0.834	0.182	0.4831	0.1315	169.49	132.77	10.158

L.S.D: least significant differences at 0.05 of probability.
Means followed by the same letter(s) in each column are not significantly different according to L.S.D at 0.05 level of probability.

Table 5. Means of total phenolic compounds (T.Phenols) (mg/g fresh weight) and contents of rosmarinic acid (RA) (µmol/g fresh weight) and proline of callus and shootlets of *Mentha longifolia* as affected by sodium chloride (NaCl) concentrations

NaCl mg/l	Callus				Shootlets		Callus		Shootlets
	60 days		100 days		60 days		60 days	100 days	60 days
	T.Phenols mg/g	RA µmol/g	T.Phenols mg/g	RA µmol/g	T.Phenols mg/g	RA µmol/g	Proline content µg/g		Proline content µg/g
0	4.052 a	0.803 ab	3.988 b	0.654 bc	5.637 a	1.105 a	417.67 ab	160.19 b	83.326 c
2000	4.065 a	0.630 b	3.738 b	0.564 c	5.975 a	1.234 a	325.38 b	157.08 b	102.159 a
4000	4.351 a	0.880 a	5.016 a	0.740 b	4.635 b	0.759 b	379.40 ab	360.49 a	91.065 b
6000	3.849 a	0.805 ab	5.412 a	1.094 a	4.279 b	0.661 b	453.79 a	279.88 a	81.649 c
L.S.D $_{(0.05)}$	NS	0.225	0.513	0.135	0.622	0.213	125.53	113.42	6.284

L.S.D: least significant differences at 0.05 of probability.
N S: Not significant at 0.05 level of probability.
Means followed by the same letter(s) in each column are not significantly different according to L.S.D at 0.05 level of probability.

which in turn produced the phenolic compounds as reported by Razzaque and Ellis (1977).

Similar trend of results was reported by Phatak and Heble (2002), Kim *et al.* (2006), Namdeo *et al.* (2007) and Roy and Mukhopadhyay (2012).

Furthermore, data on proline content in Table (4) revealed that tissue callus contained more proline than that of shootlets, and adding phenylalanine alone at any concentration to the growing medium led to significant decrease the proline content of the used explants, compared with medium free from phenylalanine, with one exception of using phenylalanine at 10 mg/l.

These results may be due to that adding phenylalanine at a proper concentration to the medium led to activate the growth of the used explants and reduce their stress, consequently their proline content could be decreased.

Effects of sodium chloride on total phenolic compounds and rosmarinic acid content of *Mentha longifolia* explants

Generally, data presented in Table (5) indicated that adding sodium chloride to the growing medium at 4000 or 6000 mg/l for callus or at 2000 mg/l for the shootlets gave the highest average contents of total phenolic and rosmarinic acid, compared with the control treatment.

These results were probably due to that adding sodium chloride at a specific concentration for each plant tissue led to exposure the used explant to salt stress, consequently the explant tried to avoid harmful effect of salinity by increasing the formation of some organic materials such as phenols and rosmarinic acid.

Nikolova and Ivancheva (2005) reported that the content of some secondary plant products are significantly higher in plants grown under salt stress than in those cultivated under normal conditions. The present results are in agreement with those of Al-Amier and Craker (2007) on spearmint they found that phenolic acid concentration increased in salt stressed plants and Abd EL-Azim and Ahmed (2009) they indicated that the content of phenols on *Achillea fragratissima* significantly increased along with increasing salinity. Also, Cik *et al*. (2009) found that accumulation of phenolic acids (protocatechuic, chlorogenic and caffeic acids) on *Matricaria chamomilla* increased by salinity. Bourgou *et al*. (2010) showed increase of phenols with saline treatment on *Nigella sativa* and Queslati *et al*. (2010) on *Mentha pulegium.*

Effects of sodium chloride on proline content of *Mentha longifolia* explants

Data in Table (5) showed that increasing the added sodium chloride to the growing medium led to increase the proline content in each of callus and / or shootlets tissues of *Mentha longifolia,* compared with the control treatment (medium free from sodium chloride).

These results may be attributed to the importance of presence of proline at a proper level in plant tissues during salt stress, which in turn imparts stress tolerance by maintaining cell turgor, or osmosic balance, stabilizing memberanes thereby preventing electrolyte of reactive oxygen species within normal ranges, thus preventing oxidative burst in plants as reported by Hayat *et al*. (2012).

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الملخص العربي

تأثير كلوريد الصوديوم والفينيل الانين على إنتاج بعض المركبات الثانوية المُنتجَة معملياً في نبات النعناع طويل الأوراق

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أجريت هذه الدراسة بمعمل زراعة الأنسجة بقسم الزهور ونباتات الزينة وتنسيق الحدائق بكلية الزراعة (الشاطبي) جامعة الإسكندرية خلال الفترة ٢٠١٤-٢٠١٥م، بهدف تعزيز إنتاج بعض المركبات الثانوية المتراكمة في أنسجة نبات النعناع طويل الأوراق معملياً تحت تأثير كلوريد الصوديوم والحامض الأميني فينيل ألانين. تم أخذ النباتات والبالغ عمرها شهرين من مشتل تجاري وزراعتها معمليا ووضعها تحت ظروف إضاءة صناعية باستخدام لمبات الفلورسنت ١٦ ساعة إضاءة وعلى درجة حرارة ٢٥°م تم زراعة القمم النامية بطول ٣-٤ سم وزراعة الأوراق ١سم٢ على بيئة غذائية معقمة مضاف إليها السكروز ٣٠ جم/لتر، والآجار ٨ جم/لتر، وكلوريد الصوديوم (٠، ٢٠٠٠، ٤٠٠٠، ٦٠٠٠ مليجرام / لتر) وفينيل ألانين (٠، ٠,٥، ٥، ١٠، ١٥ مليجرام/ لتر)، في حالة زراعة القمم النامية استخدم الNAA (٠,٢٥ مليجرام /لتر)، والBA (٢,٥ مليجرام/لتر) معا، عند زراعة الأوراق استخدم الBA (٠,٥ مليجرام/لتر) وال 2,4-D (٤ مليجرام/لتر) معا،وتم ضبط رقم الحموضة 5.8±0.02. تم تحليل الأفرع الخضرية الناتجة بعد ٦٠ يوم من الزراعة، ونسيج الكالس تم تحليله بعد ٦٠ يوم مرة وبعد ١٠٠ يوم مرة أخرى لتقدير نسبة المركبات الفينولية الكلية وحمض الروزماري والبرولين المتراكمين داخل أنسجة النباتات. تفوقت الأفرع الخضرية في المحتوى الكلي للكلوروفيل والمحتوى الكلي للمواد الفينولية وحمض الروزمارينك مقارنة بمحتواهم في نسيج الكالس، استخدام التركيزات المنخفضة من كلوريد الصوديوم(٢٠٠٠ مليجرام/لتر) لتحفيز إنتاج وتراكم بعض المركبات والمواد الثانوية الفعالة يعتبر الحل الأمثل، مع تجنب التركيزات المرتفعة(٦٠٠٠ مليجرام/ لتر)، بينما التركيزات المتوسطة والمرتفعة من الحامض الأميني فينيل ألانين (٥، ١٠، ١٥ مليجرام/ لتر) تعتبر الحل الأمثل لتحفيز إنتاج وتراكم بعض المركبات الثانوية الفعالة.