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Effect of Salinity and Proline Application on The Chemical Content of *In Vitro* Regenerated Shoots of *Mentha Piperita.*

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ABSTRACT

The effect of salt stress and proline treatments on *in vitro* produced *Mentha piperita* was investigated in order to find out their effect on proline, charbohydrates and rosmarinic acid accumulation in the shoots tissue of regenerated plants. Apical portions with axillary buds were obtained from mother plants grown in the Experimental Garden of the Department of Floriculture and Garden Design, Alexandria University and were cultured in MS medium supplemented with 3% sucrose, 8 g/l agar and 0.25 mg/l NAA + 2.5 mg/l BAP in addition to five concentrations of NaCl (0.0, 0.5, 1.0, 1.5 and 2.0 g/l) and proline (0.0, 25, 50, 75 and 100 mg/l). Results showed that increasing NaCl concentration in the medium decreased the total soluble carbohydrates and increased free proline content, whereas, rosmarinic acid (RA) content decreased with the highest NaCl concentrations.

INTRODUCTION

The genus *Mentha* conatines approximately 25 species, belongs to the family *Lamiaceae*. Medicinal importance of *Mentha* is well known due to the presence of rosmarinic acid (RA), the second most common ester of caffeic acid in the plant kingdom and used as antioxidant (Ellis and Towers, 1970). As all species of this genus contain high amount of secondary metabolites, *in vitro* rapid propagation for production of improved clones is desirable for conservation and commercial exploitation. Plant tissue culture technology offers means to produce economically important secondry metabolites at an enhanced level within controlled laboratory environments (Tisserat and Vaughn, 2008).

Recently, it is commonly known that plants with high phenolic levels are natural source of antioxidant compounds and could be an important natural solution for problems currently encountered within the food industry and food nutritive value. Therefore, plant breeders are striving to produce such economically valuable crops. Nowadays, natural antioxidants are used in various industries such as; facial creams and sun blocks enhancement, wine stabilizing, meat processing and deep-fat frying (Chen and Ho, 1997; Jaswir *et al.,* 2000 and Katiyar *et al.,* 2000). One of the principle antioxidative constituents in extracts used for food processing is rosmarinic acid (Chen and Ho, 1997). 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). This acid was detected in shoot cultures of *Mentha piperita* and the synthesis of rosmarinic acid using shoot segments of plants was reported (Park, *et al.,* 2008).

Application of elicitors to plant cell and organ cultures is very useful for enhancing the biotechnological productivity of valuable secondary metabolites *in vitro* such as proline (Chen, 2000). Proline is a proteinogenic amino acid with an exceptional conformational rigidity, and is essential for primary metabolism. Kemble and MacPherson (1954) were the first to report the accumulation of proline in wilting perennial rye grass (*Lolium perenne*). Since then numerous studies have shown that proline content in higher plants increases under different environmental stresses. Proline accumulation has been reported during conditions of drought (Choudhary *et al*., 2005), high salinity (Yoshiba *et al*., 1995), high light intensity and UV irradiation, high heavy metals levels, oxidative stress. As well as, in response to biotic stresses (Fabro *et al*., 2004; Haudecoeur *et al*., 2009).

Therfore, the current study was conducted in order to investigate the effect of different NaCl and proline concentrations applied in the culture media on carbohydrates contents, proline and rosmarinic acid of *Mentha piperita* regenerated shoots.

MATERIALS AND METHODS

The experiment was carried out in the Tissue Culture Lab of the Faculty of Agriculture, Alexandria University in 2013. Six-month-old plants of *Mentha*

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piperita were collected from the experimental garden of the Floriculture and Garden Design Department, Alexandria University. Apical portions with axillary buds were separated and about 2-3 cm length were taken as explants. The explants were surface sterilized by immersing in 70% ethanol containing two drops of Tween 20, for one min., at room temperature and rinsed 3 times with distilled water and were then treated with an aqueous 0.1 % (w/v) $HgCl_2$ solution for 5 min., and washed with six changes of sterile distilled water under aseptic condition. Thereafter, these explants were subsequently cut into pieces carrying a node and internodal segments. Pieces were then incubated vertically in shoot proliferation medium composed of the basal MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 8 g/l agar and 0.25 mg/l NAA + 2.5 mg/l BAP (Debleena and Sandip, 2012).

The experiment was conducted as randomized complete design (RCD) and sodium chloride (NaCl) at 0, 0.5, 1.0, 1.5, 2.0 g/l and proline obtained from Sigma-Aldrich USA at 0, 25, 50, 75 and 100 mg/l were added to the basal medium either alone or in combinations. The pH of the medium was adjusted to 5.8. Media were prepared and poured in culture tubes (20 ml/tube) and tubes were autoclaved at 121°C for 15 min. Explants were inoculated in the culture tubes under aseptic condition in a laminar air flow chamber, each tube contained one explant and five tubes was considered one replicate. All the cultures were maintained in a culture room at 24±1° C under 16 h photoperiod. Cultures were maintained by sub-culturing the material every 4 weeks for 3 months under aseptic condition.

The effect of the different salt and proline treatments on total carbohydrate, proline and rosmarinic acid (RA) contents were measured. Total carbohydrates content was determined in the dried leaves according to the method of Dubios *et al*. (1956). Free proline content in the leaves as mg/g F.W. was estimated by the colorimetric method of Bates *et al.* (1973).

For rosmarinic acid determination, 300 mg of leaves was collected from *In vitro* regenerated plants after three months from the first culture, blended and placed in 10 ml of 50 % methanol and placed in water bath at 55 °C for 2 hours, then centrifuged for 10 minutes at 3500 rpm. One ml of the extract was diluted with 9 ml methanol (50%). Absorbance was measured at 333 η. The rosmarinic acid concentration was then calculated according to the equation reported by (Lopez-Arnoldos *et al*., 1995 and Komali and Shetty, 1998):

$A = \varepsilon bc$, where (A) is the absorbance at 333 η, (ε) is the extinction coefficient $\varepsilon = 19000$ L mol $^{-1}$ cm^{-1}, (b) is the width of cuvette b= 1 cm and (c) is the concentration of rosmarinic acid.

The effect of the different NaCl and proline concentrations and their interaction was studied by the comparison of means using the protected Least Significant Differences test (LSD) (Snedecor and Cochran 1995) at 0.05 probability using the SAS program (1985).

RESULTS AND DISCUSSION

Total carbohydrates content

All exogenous proline concentration caused an increase in total carbohydrates in the leaves in comparison with the control, with the highest increase reaching 19.14 mg/g D.W. when proline was added at100 mg/l (Table1). Proline application might have enhanced carbohydrates metabolism by hydrolyzing insoluble carbohydrates to the soluble state (Trembly and Trembly, 1991) which led to the increase in the total carbohydrate content.

Regarding the NaCl application, the total carbohydrates content decreased significantly with increasing NaCl concentration, with lowest carbohydrates content reaching 8.6 mg/g D.W. by 2g/l NaCl. The decrease in carbohydrates under salt stress condition might be related to the salty condition that makes cells spend more energy for osmoregulation to withstand the disturbance caused by salt accumulation inside the cells. Also, a high salt condition increases the respiration rate through the effect of sodium ions on the respiration cycle that leads to the decrement in carbohydrates (Maas, 1986; Wang *et al*., 1999; Huang and Liu, 2002).

In addition, a significant interaction influence between sodium chloride and proline on the total carbohydrates content was obtained (Table1). The lowest value of total soluble carbohydrates (3.45 mg/g D.W.) was resulted in from the application of 2gm/l NaCl and 0.0 mg/l proline, while, the highest value (25.32 mg/g D.W.) was resulted in from the application of 0.0 gm/l NaCl and 100mg/l proline.

Proline content

Data presented in Table (2) show that all proline applications significantly increased free proline content as compared with the control. The highest value (0.86 mg/l) of free proline was resulted in from the application of 100mg/l proline.

With regard to the effect of salt (NaCl) applications, the obtained results in Table (2) show significant increase in free proline content comparised with the control. The highest free proline value (0.77mg/l) was resulted in from applying 2g/l NaCl.

Table 1. Main effect of sodium chloride and proline treatments on the total carbohydrates content of *M. Piperita* shoots.

NaCl (gm/l)	Total carbohydrates (mg/g) dry weight					Mean
	Proline (mg/l)					
	0.0	25	50	75	100	
0.0	12.05	15.70	20.65	23.34	25.32	19.41a
0.5	9.11	13.61	16.21	20.12	23.08	16.43b
1.0	8.07	11.04	13.40	17.05	18.22	13.56c
1.5	5.32	8.46	10.50	15.06	16.54	11.18d
2.0	3.45	7.35	9.36	10.31	12.54	8.60d
Mean	7.60e	11.23d	14.02c	17.18b	19.14a	69.18

L.S.D $_{NaCl= 3.50}$, L.S.D $_{proline= 2.67}$, L.S.D $_{inter= 5.46}$

Table 2. Main and interaction effect of sodium chloride and proline on free proline content of *M. Piperita* shoots.

NaCl (g/l)	Free Proline(mg/g) fresh weight					Mean
	Proline (mg/l)					
	0.0	25	50	75	100	
0.0	0.12	0.22	0.53	0.59	0.76	0.44d
0.5	0.32	0.35	0.58	0.68	0.79	0.54c
1.0	0.35	0.58	0.64	0.75	0.87	0.64b
1.5	0.42	0.63	0.73	0.89	0.91	0.72a
2.0	0.54	0.68	0.78	0.90	0.98	0.77a
Mean	0.35d	0.49c	0.65b	0.76a	0.86a	

L.S.D $_{NaCl= 0.10}$, L.S.D $_{proline=}$ 0.18, L.S.D $_{inter= 0.26}$

Also, a significant interaction effect among NaCl and proline treatments on free proline content was obtained. The lowest value of free proline (0.12 mg/l) ocuured from the media void of NaCl and by applying 0.0 g/l NaCl and 25mg/l proline (0.22 mg/l), wherase, the highest value of free proline (0.98 mg/l) was obtained by applying 2g/l NaCl and 100mg/l proline (Table2).

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).

Rosmarinic acid content

The addition of proline at 25 mg/l resulted in the highest rosmarinic acid content (0.05mg/g) in comparison with the control (0.03 mg/g) and all other proline applications, whereas applying 100 mg/l proline resulted in lower rosmarinic acid content (0.02 mg/g) than the control. On the other hand, applying 50 and 75mg/l proline did not significantly affect the RA content (Table3)

The reduction in RA at high exogenous proline level may be due to the accumulation of proline which led to the inhibition of proline production by the plant tissues. It may be due to the limited amount of proline dehydrogenase inside the plant tissues which may not be able to oxidize the relatively large amount of proline as mentioned by Kwok and Shetty (1998).

Table 3. Main and interaction effect of sodium chloride and proline on RA (mg/g fw) content in *M. Piperita* shoots

NaCl (g/l)	Rosmarinic acid (mg/g) fresh weight					Mean
	Proline (mg/l)					
	0.0	25	50	75	100	
0.0	0.025	0.042	0.034	0.032	0.027	0.030b
0.5	0.032	0.067	0.032	0.030	0.025	0.040a
1.0	0.030	0.051	0.026	0.023	0.023	0.030b
1.5	0.027	0.043	0.024	0.021	0.020	0.030b
2.0	0.023	0.032	0.022	0.020	0.019	0.020c
Mean	0.030b	0.050a	0.030b	0.030b	0.020c	

L.S.D $_{NaCl= 0.015}$, L.S.D $_{proline= 0.018}$, L.S.D $_{inter= 0.043}$.

Similar results were obtained by the NaCl application. Applying 0.5 g NaCl resulted in higher (0.04 mg/g) RA and 2g/l NaCl gave lower (0.02 mg/g) RA content than the control (0.03 mg/g). Moreover, the obtained data showed a significant interaction effect occurring between NaCl and proline applications on rosmarinic acid content. The highest value of rosmarinic acid 0.067 mg/g was obtained by applying 0.5g/l NaCl and 25 mg/l proline, whereas, the lowest value of rosmarinic acid (0.019 mg/g) was resulted in from applying 2g/l NaCl and 100 mg/l proline (Table3).

Duval and Shetty (2001) found that proline at low concentrations stimulated plant growth but at high concentrations the proline analog inhibited it. They mentioned that the G6PDH activity was correlated with the proline content. The increase in RA concentration due to 25 mg/l proline application may be due to the accumulation of proline in the cytosol causing the increase in $NADP^+$ level and driving the pentose phosphate pathway toward the shikimate and the phenylpropanoid pathway as stated by Yang and Shetty (1998) and Perry and Shetty (1999).

It could be concluded that high salt stress had negative effects on *Mentha* shoots in the culture media and those effects can be decreased by the addition of proline which increased the soluble carbohydrates, free proline and RA under salty conditions. This might help in the selection of high salt resistant plantlets and regeneration of salt resistant *M. piperita* plantlets.

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الملخص العربي

تأثير الملوحة والبرولين على المحتوى الكيميائى للمجموع الخضرى للنعناع الفلفلى تحت الظروف المعملية

هدى السيد المقدم

فى تجربة معملية على نبات النعناع *M. piperita* لدراسة تأثير إضافة تركيزات من كلوريد الصوديوم وهى (٠.٠، ٠.٥، ١.٠و٢.٠جم/لتر) وإضافة الحمض الأمينى البرولين بتركيزات(٠.٠، ٢٥، ٥٠، ٧٥ و١٠٠ ملجم/لتر) على حمض الروزمارنيك والبرولين والكربوهيدرات الكلية الذائبة. أضيفت هذه التركيزات إلى بيئة موراشيج وسكوج MS المكونة من ٣% سكروز+ ٨ جم/لتر آجار + ٠.٢٥ ملج/لتر نفتالين حمض الخليك+ ٢.٥ ملجم/لتر بنزيل أدنين أمينو بيورين وبعد التعقيم تم صبها فى أنابيب الزراعة تحت ظروف معقمة. وتم زراعة البراعم الطرفية فى الأنابيب المحتوية على البيئة المعقمة. البراعم الطرفية تم الحصول عليها من مشتل قسم الزهور ونباتات الزينة التابع لكلية الزراعة جامعة الإسكندرية. وبعد مدة ثلاثة أشهر من الزراعة وإعادة الزراعة فى المعمل تحت ظروف متحكم فيها من درجة حرارة وإضاءة ورطوبة ،تم الحصول على النمو الخضرى المناسب لتقدير بعض المكونات الكيميائية مثل الكربوهيدرات الكلية الذائبة والبرولين وحمض الروزمارينك. وكانت أهم النتائج المتحصل عليها كالتالى: زيادة تركيز كلوريد الصوديوم فى بيئة الزراعة أدى إلى نقص فى الكربوهيدرات الكلية الذائبة وكذلك زاد البرولين الحر أما محتوى الخلايا من حمض الروز مارينك فقد تناقص بزيادة تركيز كلوريد الصوديوم.