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Hormonal priming and salt treatments effects on germination percentage and antioxidant activities in *O. majorana* (Majoram) seedlings

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Abstract – The aim of this study is to investigate the effect of salt on the antioxidant activity, the total phenolic flavonoid and tannins contents and the possible protective effects of marjoram seedling on lipid peroxidation GPX and Catalase activity.
Thus, antioxidant activity was evaluated by using different assays, total antioxidant, 2,2-diphenlyl-1-picrylhydrazyl (DPPH) radical by bleaching of the purple-colored solution of DPPH radical. Seeds of Marjoram (*O. majorana* L.) showed a difference of salt sensitivity with different NaCl concentrations (0, 50, 100, 150 mM) for a period of 15 days. Germination percentage was improved at 50 mM, decreased at 100 mM and absence at 150 mM.
Many research studies have shown that seed priming is an efficient method for increasing plant growth and yield in saline condition. For this reason, this experiment was conducted to evaluate the effect of priming with (1, 5, 10 mM) and non-primed seeds in presence and absence of salt, we conclude that proline decreased germination percentage. So, concentration of 75 mM NaCl, kept for the continuation of these experiences for determination of antioxidant capacity. The overall results showed that salinity could influence the performance of polyphenols and flavonoids by increasing the concentration of these metabolites in their tissues. An increase in phenolic contents, flavonoids, tannins, total antioxydant activity and DPPH were observed. Salt increased protein content, GPX and Catalase activity. There are advise to germinate marjoram seeds at NaCl 75 mM, because it richness in secondary metabolites and enzymatic antioxidant.

Keywords: Antioxidant activity, marjoram, germination, phenolics, flavonoids

1. Introduction

Salinity is considered as a major abiotic stress affecting crop production in arid and semi-arid regions in the world (Munns and Tester 2008). In Tunisia, salinity affects about 10% of the total land area. Crops are more exposed to this problem with increasing climate aridity (Hachicha et al. 1995). Salt stress leads to suppression of plant growth and development at all growth stages; however, depending upon plant species, certain stages such as germination seedling stage could be the most critical stages for salts stress in Tunisia.
For this reason, the culture of marjoram in Tunisia requires more studies on their tolerance to major environmental constraints. Seed germination can be a major factor limiting the establishment of plants under saline conditions (Ghavami and Ramin 2007). Seed germination is first critical and the most sensitive stage in the life cycles of plants (Ahmad et al. 2009) and the seeds exposed to unfavorable environment conditions like salts stresses may have to compromise the seedlings establishment (Albuquerque and Carvalho 2003). The germination and seedling growth can be affected by salinity either by creating an osmotic pressure that prevents water uptake or by toxic effects of sodium and chloride ions (Bajehbaj. 2010).
Priming allows the metabolic processes necessary for germination to occur without actual germination. Many research studies have shown that seed priming is an efficient method for increasing plant growth and yield under normal as well as stress condition (Farooq et al. 2007).
To prevent water loss from the cell due to salinity stress, plants synthesis and accumulate a number of compatible solutes called "osmolytes" include proteins and amino acids like proline. It is assumed that

under stress condition, these osmolytes scaveng reactive oxygen species (ROS). ROS are highly reactive and can seriously disrupt normal metabolism of the plant through oxidation of membrane lipids (Arafa et al. 2009). Several studies mentioned that under stress condition, exogenous proline application regulates osmotic potential and plays a vital role in sustaining plant growth under osmotic stress (Hoque et al. 2007). In the present study, we used a method for improving the germination capacity of seeds is the pretreatment, by proline. It is an amino acid that in some plants, reduce the time of seed germination and seedling establishment permit, under conditions of salinity. Marjoram is glycophyte sensitive to salinity at vegetative stage. However, the information pertaining to the role of exogenous proline on germination and early seedling growth is limited. O. majorana has strong antioxidant activity, mainly because of its high content of phenolic acids and flavonoids, which is useful in health supplements and food preservation (Sellamia et al. 2009). View the importance of marjoram in medicinal, therapeutic and economic fields and its richness in secondary metabolites that defense plant against biotic and / or abiotic stress. Therefore, the use of its essence requires more studies on their installation conditions in Tunisia and tolerance to major environmental constraints.

In a previous study we investigated the effect of NaCl concentration on physiological and biochemical of O. majorana at vegetative stage (Baatour et al. 2012a, 2012b).

However, no effect on salt on germinative stage had been study. View the importance of abiotic stress on germination behavior and in the establishment of seedlings. The purpose of this research was to evaluate (1) the effect of increasing NaCl concentration on percentage of germination in order to observe their behavior germ, evaluation of growth vis-à-vis this stress and efficacy of pretreatment with 1 mM, 5 mM, 10 mM and of proline (2). Finally, to study the effect of salinity on antioxidant capacity including enzymatic (activity of POD and CAT) and non-enzymatic (polyphenol, flavonoid, tannins antioxidant activities and DPPH content) systems were investigated in marjoram seedlings.

2. Materials and methods

Seeds of marjoram (Origanum majorana L.) were obtained from the Nursery of Nabeul. Seeds were surface-sterilized with 95% sodium hypochlorite solution for 10 min, and rinsed thoroughly with distilled water. The seeds were placed in Petri dishes; on two layers of filter paper moistened with 10 ml of treatment solution under aseptic conditions for 15 days in the dark at 25°C. The experiment was conducted in two subsets. In one set, the marjoram seeds were allowed to germinate in different concentration of NaCl (0, 50, 100 and 150mM). In the other set, the seeds were pretreated in two levels of proline (1, 5 and 10 mM) for 12 h and transferred to Petri dishes. Primed seeds were air dried at room temperature. Aliquots of 25 seeds were then placed in 10 cm Petri dishes with double-layer filter paper in the absence or presence of different concentrations of NaCl. Each treatment was replicated four times with 25 seeds for four consecutive days. The germinated seeds were counted every 24 h to 15 days, and the germination test was ended after four days. The following traits were measured; germination percentage.

Based to result above, 75 mM NaCl was kept to measure polyphenols, flavonoids, MDA, protein, catalase, ascorbate peroxydase and gaaicol peroxydase contents.

2.1. Plant material and germination percentage

Germination Percent (GP) was calculated based on following equation. GP% =100 × (total germinated seeds/total number) of seeds. Fresh weights (FW) and Dry weight (DW) of all samples treated with (0, 50, 100 and 150 mM) NaCl were recorded. Four seedlings were cut into radicle, hypocotyl and cotyledons and then the sum of the different organs were put in 60°C for two days. The dry weight (DW) was then determined using a precision balance. Tissue water content was obtained from the (FW - DW/FW) ratio.

2.2. Total protein extraction and enzyme assays

Leaves from separate plants were ground in a mortar with liquid nitrogen, and the powder was mixed with 50 mmol l−1 of pH 7.5 phosphate buffer containing 1 mmol l−1 EDTA, 1 mmol l-1 dithiothreitol (DTT), 50ml l−1 glycerol and 50 g l−1 polyvinylpyrrolidone (PVP), and centrifuged (15 000 × g, 4ºC, 20 min). For APX, 5 mmol l−1 ascorbic acid was also included in the extraction buffer. After extraction, protein concentration was determined according to Bradford (1986). Catalase (EC 1.111.1.6) activity was measured according to the modified method of Chance and Maehly.24 The reaction mixture consisted of 25 mmol l−1 potassium phosphate buffer (pH 7.0), 30 mmol l-1 H2O2 and enzyme extract.

The decomposition of H2O2 was followed by measuring the decrease in absorbance at 240 nm. Total peroxidase activity (EC 1.111.1.7) was assayed using guaiacol as an electron donor, with a reaction mixture containing 50 mmol l−1 potassium phosphate (pH 7.0), 0.1 mmol l−1 EDTA, 5 mmol l−1 H2O2 and 10 mmol l−1 guaiacol a method derived from Fielding and Hall. (1978). The increase of absorbance, due to tetraguaiacol formation, was recorded at 470 nm.

2.3. Membrane permeability (electrolyte leakage)

Electrolyte leakage (EL) was determined as described by Quartacci et al. (2002). Fresh seedlings were placed in test tubes containing 25 ml double-distilled water at room temperature. After 1 h, the initial electrical conductivity of the medium (EC1) was measured using a digital conductimeter (Model: Metrohm). The samples were placed in liquid nitrogen and then returned in the same tube for one additional hour of shaking and the final electrical conductivity (EC2) was measured. EL was calculated using the formula: EL = (EC1/EC2) *100.

2.4. Lipid peroxidation

The level of lipid peroxidation was determined by a procedure based on the method of Du and Bramlage (1992) 0.5 g fresh leaves were ground in 5 ml ice-cold phosphate buffer solution (0.05 mmol L−1, pH 7.8) containing 10 g L−1 PVP. The homogenate was centrifuged at 10 000 × g for 30 min. 2 mL supernatant was mixed with 2 mL thiobarbituric acid (TBA) (5 g L−1 TBA, 0.2 mg L−1 trichloroacetic acid). The mixture was heated at 100º C for 30 min, chilled on ice, and then centrifuged at 1000× g for 10 min. Absorbance of the supernatant was measured at 532 nm and adjusted for non-specific absorbance at 510 and 560 nm.

2.5. Determination of antioxidant assays

2.5.1. Dpph and superoxide anion radical-scavenging activity

The electron donation ability of the obtained methanol extracts was measured by bleaching of the purple-colored solution of DPPH radical according to the method of Sun et al.,1988 ethanolic extracts (2 mL, 10–1000 µgmL^{-1}) were added to 0.5 mL of 0.2 mmol L^{-1} DPPH. After an incubation period of 30 min at room temperature, the absorbance was measured against a blank at 517 nm. The antiradical activity was expressed as IC50 (µgmL^{-1}): the concentration required to cause 50% DPPH inhibition.

2.5.2. Evaluation of total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH.28 An aliquot of sample extract was combined in an Eppendorf tube with 1 ml reagent solution (0.6 mol l^{-1} sulfuric acid, 28 mmol l^{-1} sodium phosphate and 4 mmol l^{-1} ammonium molybdate). The tubes were incubated in a thermal block at 95º C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm (Anthelie Advanced 2, SECOMAN) against a blank. The antioxidant capacity was expressed as g GAE kg^{-1} DW). All samples were analysed in three replications.

2.6. Colorimetric quantification of phenolics

2.6.1. Phenolic compounds analysis

Leaves were dried in a dark and aerated room with a temperature of 25-30ºC, for two weeks. etalonic leaf extracts, used to determine the polyphenol content and antioxidant activities, were obtained by magnetic stirring for 30 min of 1 g dry organ powder with 10 mL Acetone 80 %. Extracts were kept at 4ºC for 24 h and filtered through Whatman filter paper. This final solution was stored at 4ºC and used to determine phenolic, flavonoid and tannin contents and to estimate non-enzymatic antioxidant activities (total antioxidant activity and Scavenging ability of DPPH radical).

2.6.2. Total phenolic content

Total phenolics were assayed using the Folin-Ciocalteu reagent, following Singleton et al. (1999) method; the method was based on the reduction of a phosphowolframate-phosphomolybdate complex by phenolics to blue reaction products and slightly modified by Dewanto et al (2002). An aliquot of 125µl of diluted extract (20% (v/v)) was added to 500 µL of deionized water and 125 µl of F-C reagent. After shaking, the mixture was incubated for 3 min at room temperature. Then, 1250 µl of 7% Na2CO3 solution was added. The volume obtained was adjusted to 3 ml using distilled water, mixed vigorously,

and held for 90 min at ambient temperature. The absorbance of the solution was then measured at 760 nm against a blank. The sample was analysed in triplicate and the total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram of dry weight through a calibration curve range of 50 to 400 µg ml-1(R2 =0.99).

2.7. Flavonoid quantification

Total flavonoids were measured using a colorimetric assay developed by Dewanto et al. 2002 An aliquot of diluted sample of leaf extract or standard solution of (+)-catechin was added to 75 µl NaNO2 solution (70 g l-1) and mixed for 6 min, before adding 0.15 mL AlCl3 (100 g L-1). After 5 min, 0.5 ml of 1 mol l-1 NaOH solution was added. The final volume was adjusted to 2.5 ml, thoroughly mixed, and the absorbance of the mixture was determined at 510 nm. Total flavonoids were expressed as grams of (+)-catechin equivalent per kilogram dry weight (g CE kg-1 DW), through the calibration curve of (+)-catechin (50 -500 mg mL). All samples were analysed in three replications.

2.8. Tannin quantification

0.05 ml of fenugreek samples extracted in non diluted acetone was added to 3 ml of vanilline (4%), and 1.5 ml of concentrated H2SO4. Absorbance of resulting pink-coloured solution was read at 500 nm against extract solvent as a blank (Zhishen et al. 1999). The amount of total condensed tannins is expressed as mg (+)-catechin /g DW.
The calibration curve range was 0 à 500 µg.ml-1 de catéchine (R2=0.99). All samples were analysed in three replications.

3. Results and discussion

3.1. Effect of salinity treatments on germination and priming seedling growth

Final germination percentages (FG%) are characterised by a decrease with salt addition in solution medium (Figure 1). In fact, under control conditions and in the presence of NaCl 50 mM, FG% max were respectively 86.7% and 88 % (Figure 1). Therefore, FG% was significantly higher at 50 mM NaCl and decreased about 52% and absence at 100 and 150 mM NaCl, respectively.
This result corroborates several other studies, revealing that glycophytes, are sensitive to salt during germination stage (Gorai and Neffati 2007)

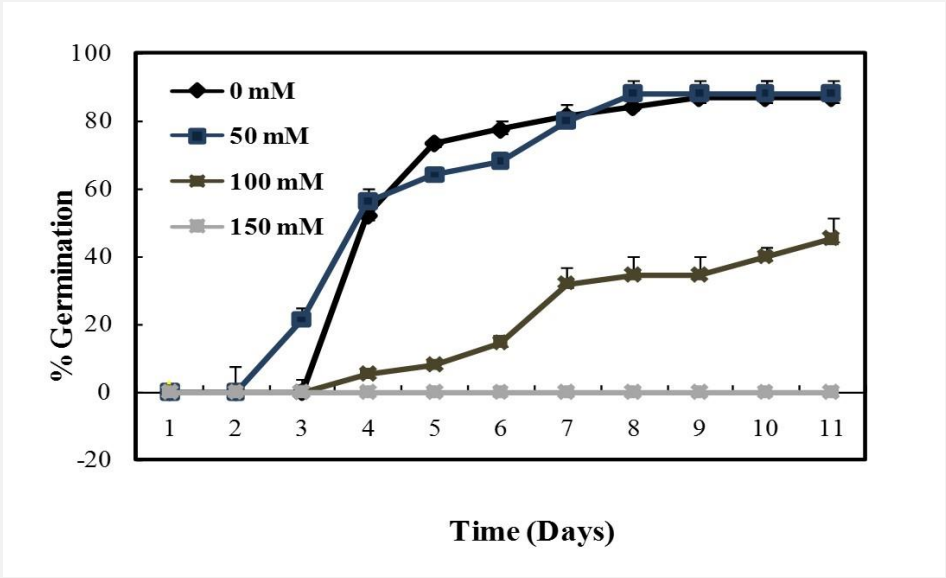

Figure 1. Germination seeds, treated with 0, 1, 5 and 10 mM proline for 15 days. Data are the mean of four samples of 25 seedlings each.

A decrease of germination of marjoram seeds with increased level of salinity, can be confirmed by Shahbazi et al. (2011) that suggested inhibition of seed germination could be attributed to osmotic stress or to specific ion toxicity by salt.

Salt decreased (radical, cotyledon and hypocotyle) biomass, hydratation and elongation compared to control ones (Table 1). This decrease was significant at NaCl 100 mM.
The reduction in growth seedling under salt stress of marjoram may be attributed to the osmotic effect that cause disturbances in the water balance, reduction of photosynthesis and consequently an inhibition of growth (Shahbazi et al. 2011).
The percentage of seed germination decreased in response to NaCl in primed (1, 5 and 10 mM proline) and non-primed (0 mM proline) seeds.
Meanwhile the most improved germination was observed in non-primed than in presence of 5 mM, 1mM and 10 mM of proline (Figure 2).

Table 1. Effect of NaCl treatment (0, 50 and 100 mM) in dry weight (mg), the water content (ml.g^{-1}DW) and length (cm) in radicle, hypocotyl and cotyledons of seedlings of *Origanum majorana* L.

	Organs							
	Radicle			**Hypocotyl**			**Cotyledon**	
	DW	H_2O	Length	DW	H_2O	length	DW	H_2O
0	0.13±0.08^a	8.23±1.38^a	1.20±0.09^b	0.50±0.08a	3.25±1.08^a	3.2±1.12^a	1.02±0.05^a	1.97±0.08^a
50	0.11±0.06^a	7.27±1.04^a	1.65±0.16^a	0.46±0.08a	3±0.16^b	0.78±0.11^b	0.96±0.02^b	1,08±0.04^b
100	0.09±0.01^b	0.10±0.04^b	0.45±0.01^b	0.40±0.08a	1.5±0.15^c	0.56±0.13^b	0.85±0.11^b	0.2±0.01^c

Means of six replicates ± SE. Means followed by different letters are significantly different (P≤0.05) as determined by analysis of variance (ANOVA).

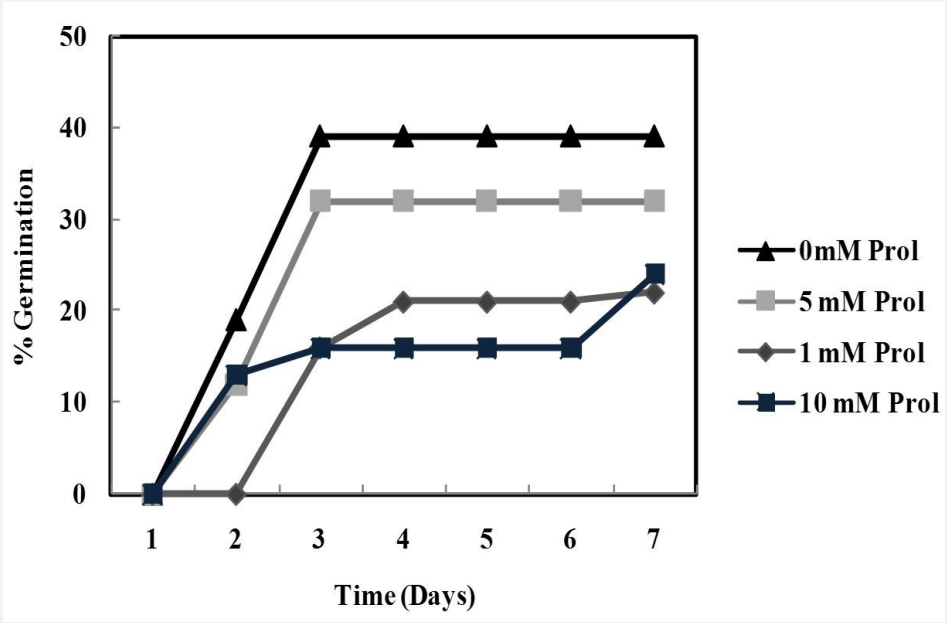

Figure 2. Germination seeds, treated with 0, 1, 5 and 10 mM proline for 15 days. Data are the mean of four samples of 25 seedlings each.

The results were in contradiction with Soughir et al. (2012) who suggested that seed priming is a successful method that has been proved to improve seed germination and emergence of seedlings. In addition seed priming, especially with NaCl, have improved germination and growth of many crops under stressed conditions (Basra et al. 2005).
Like asparagus plants (Pill et al. 1991) and cucumber plants (Passam and Kakouriotis 1994).
Therefore, considering the result above, we chosen to analyze the effect of salt in absence of proline and presence of 75 mM NaCl on growth and productivity of marjoram.

3.2. Effect of nacl treatments on electrolyte leakage and membrane integrity

The extent of membrane damage in marjoram seedling was estimated by MDA and EL content. MDA levels of seeds were determined to evaluate lipid peroxidation (Figure 3A). In the absence of salt, MDA level was (0.9 µmol.g^{-1}FW). In the presence of NaCl 75 mM, MDA content decreased by 1.9-fold as compared to control. The EL index decreased significantly after seven days of germination (Figure 3B). These results suggest that salt might have alleviated the membrane damage induced by salt.

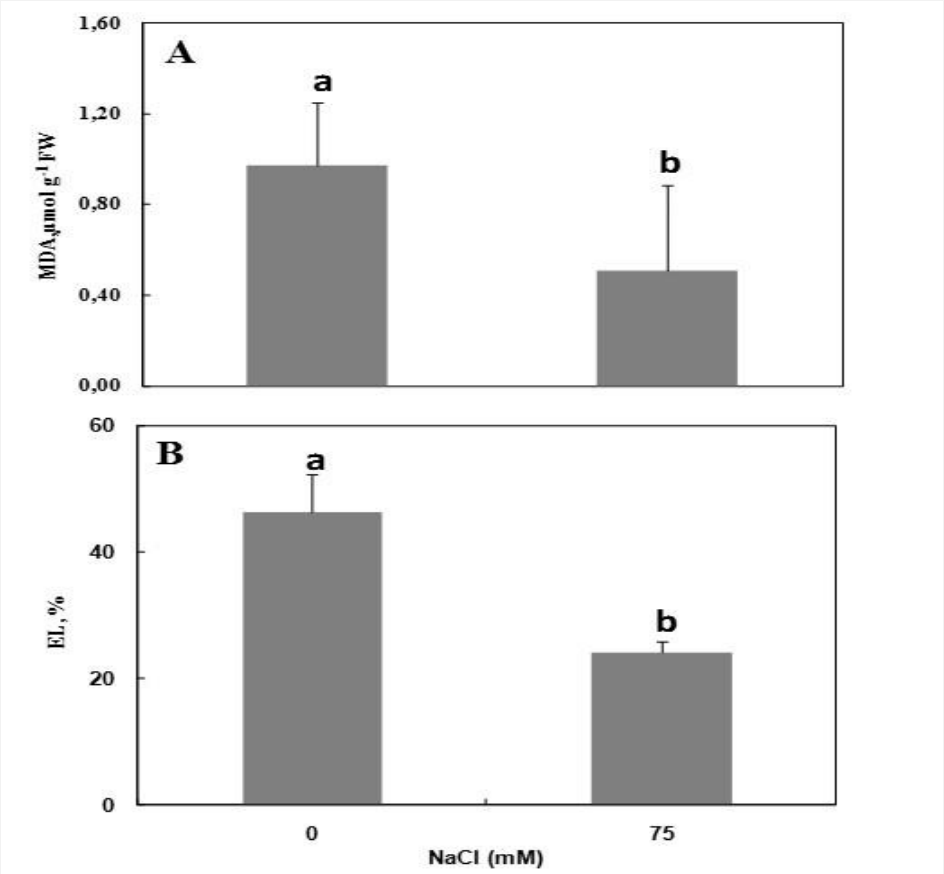

Figure 3. Effect of NaCl on MDA content (A) and electrolyte leakage levels (EL) (B) of marjoram germinating under 0 and 75 mM NaCl for 15 days. Means of six replicates ± SE. Means followed by different letters are significantly different ($P \leq 0.05$) as determined by analysis of variance (ANOVA).

3.3. Effect of nacl treatments on protein content and antioxidant enzyme activities

Protein contents increased about 26.19% after 15 day of NaCl treatment as compared to control (Figure 4A).

Protein content of marjoram under salt condition were similarly to (Shaddad et al., 2005), who suggested that total soluble protein usually increased under high salt concentrations in glycophytes like wheat (Farouk, 2011).

To determine the response of marjoram to salt-induced oxidative stress, GPX and Catalase (CAT) activities contents were measured in seedlings grown with or without 75 mM NaCl. Salt-stress caused an increase in GPX. This increase was 1.69 higher than control seedlings (Figutre 4 B). The NaCl treatment had no effect on CAT activity (Figure 4C).

Our results show that salt treatment significantly increased GPX activities in marjoram seeds in the presence of NaCl 75 mM. In addition to protect themselves against ROS toxic oxygen intermediates that causes phytotoxic reactions such as lipid peroxidation, protein degradation and DNA mutation (Pitzschke and Hirt 2006). Salt induced a stimulation of antioxidant system such as catalase and peroxidases.

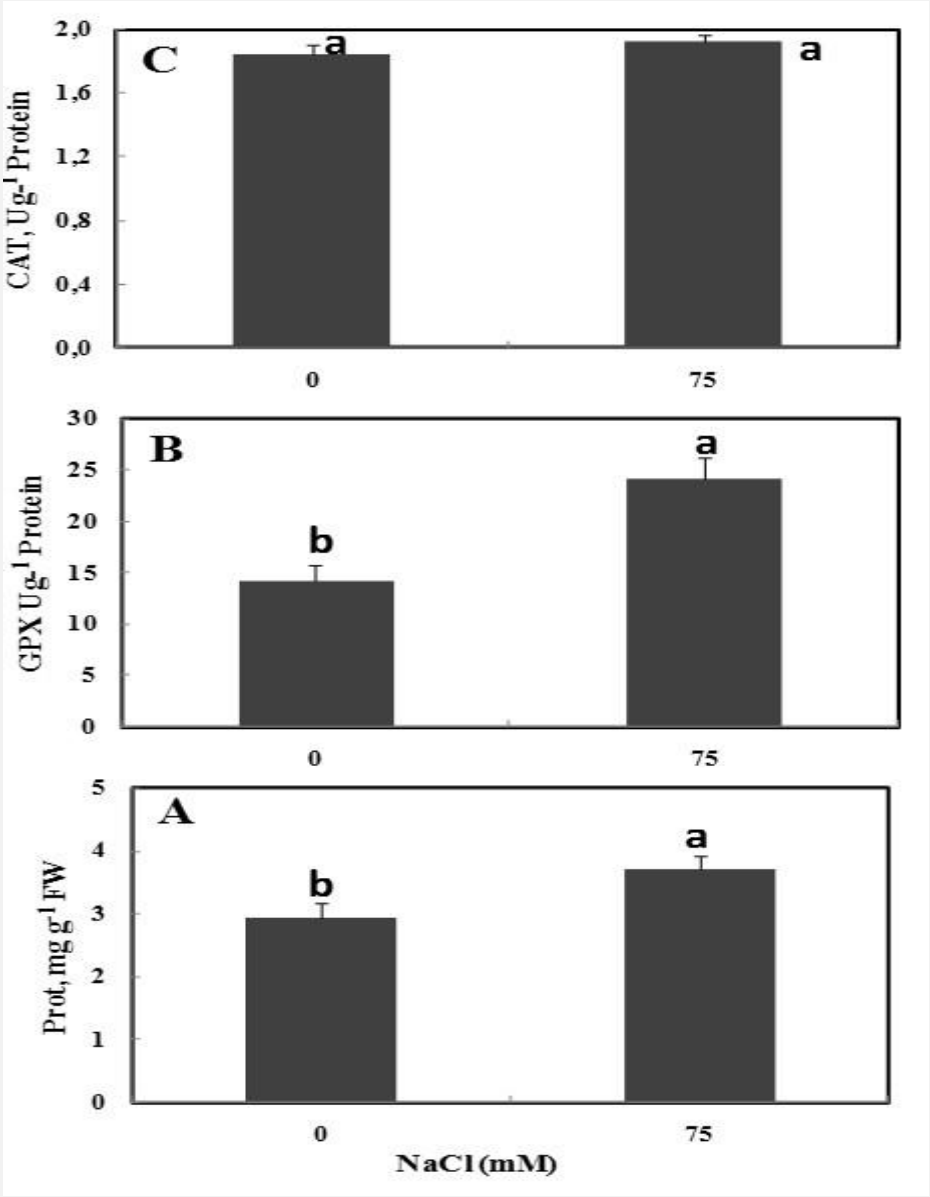

Figure 4. Effect of NaCl on total protein contents (A), catalase (B) and Guaiacol peroxidase (C) for 15 days of marjoram under 0 and 75 mM NaCl. Means of four replicates ± SE. Means followed by different letters are significantly different ($P \leq 0.05$) as determined by analysis of variance (ANOVA).

In our experiments, the induction of GPX in germination of seeds correlated with a decrease in EL and MDA contents, suggesting a membrane protection under salt stress conditions. Thus, MDA accumulation, which represents the level of lipid peroxidation and thus, the accumulation of ROS was also reduced during salt stress associated with an increase in activities of CAT and GPX. Collado et al. (2010) suggested that cell membrane stability trait could be used as a criterion for salt tolerance.

Salinity, like other environmental stresses causes an increase of ROS production (Rahimizadeh et al. 2007). Therefore, antioxidant enzymes such as GPX and CAT can protect plant cells from injury. Responses of GPX and CAT enzymes activate the essential component of the plant antioxidative defense system as they dismutases two O^{2-} to water and oxygen (Cakmak and Horst 1991).

Antioxidant activity: Antioxidant activity was evaluated by the effect of extract samples on total antioxidant activity, DPPH radical and superoxide radical anion scavenging activities at NaCl 75 mM. Antioxidant activity increased under salt treatment by 14.86% (Figure 5).

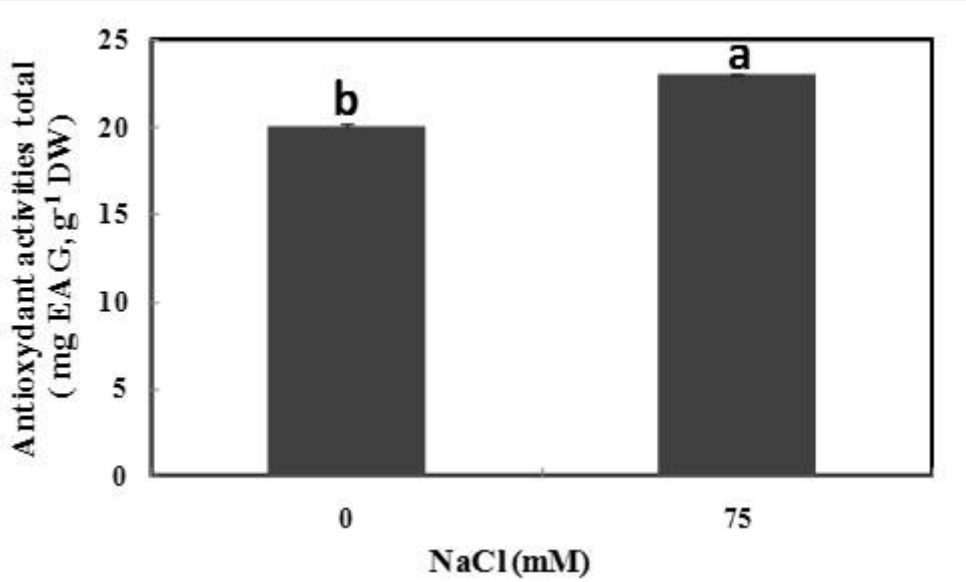

Figure 5. Effect of NaCl on antioxidant activities content for 15 days of marjoram under 0 and 75 mM NaCl. Means of four replicates ± SE. Means followed by different letters are significantly different ($P \leq 0.05$) as determined by analysis of variance (ANOVA).

In our experiments antioxidant potential of seeds has been determined as the free radical scavenging ability using a stable DPPH radical. This ability was very high in absence and presence of salt (Table 2)**.** The assessment of antioxidant activity showed that *O. majorana* seeds were able to scavenge this radical (Table 2). DPPH radical scavenging activity was significantly increased at 75 mM NaCl. These findings showed that marjoram seeds had a great antioxidant capacity at salt addition. IC50 values presented in Table 2, which refer to the mass of flour at which DPPH radicals were scavenged by 50%, are negatively correlated to antioxidative ability. As for DPPH, at NaCl 75 mM, seeds extracts were more effective O_2^- scavengers than control ones with 34 and 44 µg ml^{-1}, respectively at 75 and 0 mM NaCl. *O majorana* seeds exhibited significant superoxide anion scavenging capacity (Table 2). According to the literature data, antioxidant ability of seeds is highly positively correlated with phenolics content (Verma et al. 2008).These results revealed that ethanolic extracts of *O. majorana* varieties were free radical scavengers, acting as primary antioxidants.

Table 2. Amounts of free radical scavenging activity and antiradical activity of *Origanum majorana* L. seeds cultured under NaCl 75 mM

Extract	DPPH scavenging activity (µg/ml)	IC5 Antiradical Activity (µg/ml)
0 mM	200±0.26[a]	44±0.14[a]
75 mM	100±0.15[b]	34±0.32[b]

Means of six replicates ± SE. Means followed by different letters are significantly different (P≤0.05) as determined by a variance (ANOVA).

3.4. Effect of nacl treatments on total polyphenols flavonoids and tanins content

Phenols and flavonoids play an important role in giving protection to the plants against deleterious effects of UV rays and also against certain microorganisms and are responsible for most of the medicinal properties of the plant.

So the present study also includes the estimation of total phenolic content and total flavonoid content of the extracts of marjoram seedling. Total phenolic content of marjoram seedling were increased by 17.16% (Figure 6). Similarly, previous studies suggested that salt constraint may increase phenolic accumulation of marjoram aerial parts, as a response to the oxidative stress (Baatour et al. 2012a).

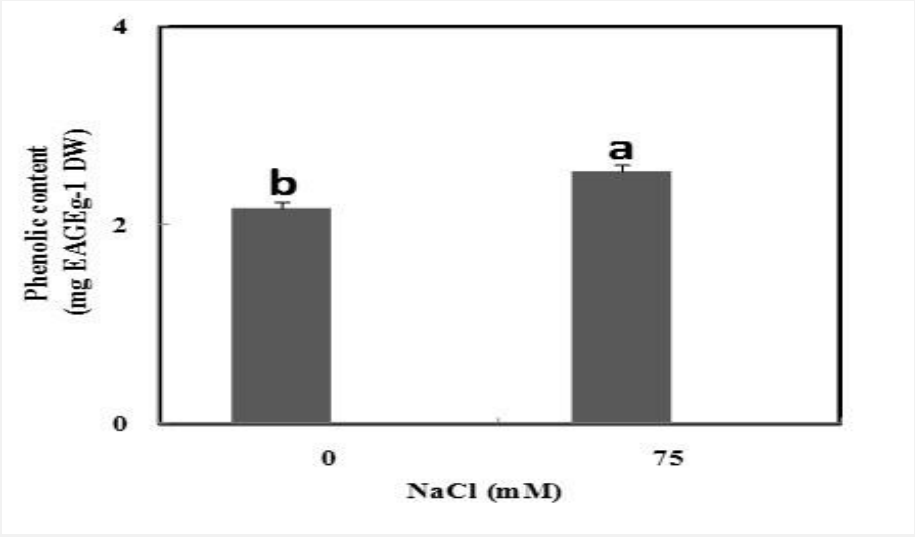

Figure 6. Effect of NaCl on phenolic content for 15 days of marjoram under 0 and 75 mM NaCl. Means of four replicates ± SE. Means followed by different letters are significantly different ($P \leq 0.05$) as determined by analysis of variance (ANOVA).

Likely, flavonoids contents increase under increasing salinity (Figure 7). A similar pattern was reported in phenols and flavonoids content. The seedling was shown to have least content of these two secondary metabolites as compared to vegetative stage (Baatour et al. 2012a). This might be due to the fact that the stored metabolites in the seeds have been utilized during germination.

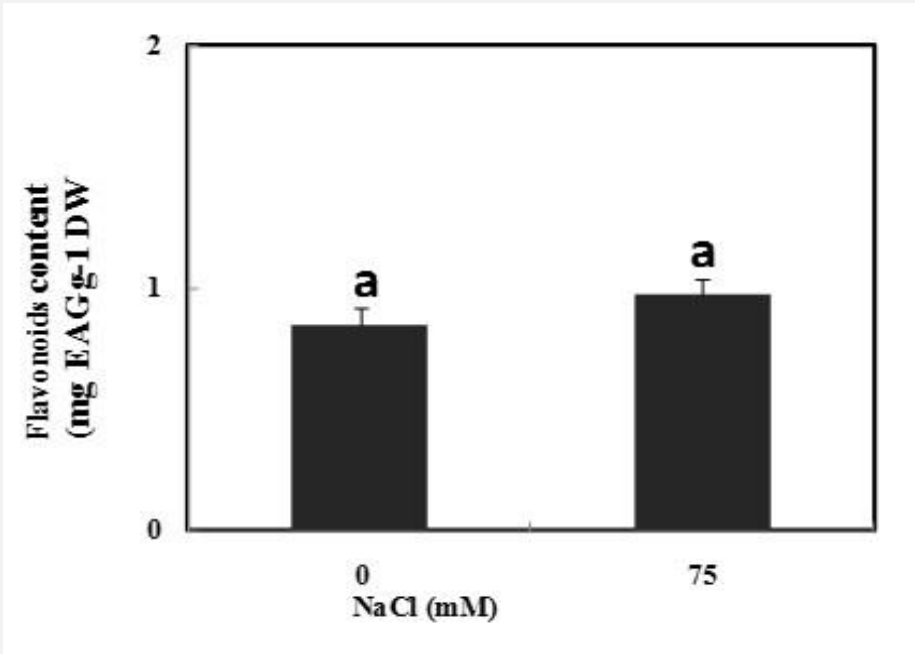

Figure 7. Effect of NaCl on flavonoids content for 15 days of marjoram under 0 and 75 mM NaCl. Means of four replicates ± SE. Means followed by different letters are significantly different ($P \leq 0.05$) as determined by analysis of variance (ANOVA).

Salt addition increased the tannin content in *Origanum* seeds (Figure 8). Therefore, tannins content is more important than flavonoids and polyphenols. Our result agree with those of Aisha et al. (2014), who found the ability of these plant samples to play a major role as antidiarrhoea and antihaemorrhagic agent.

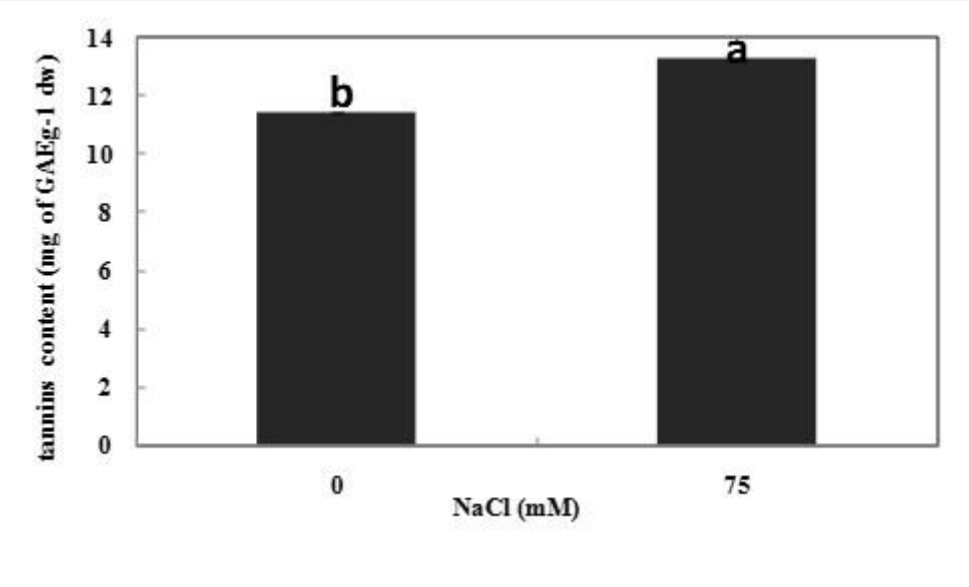

Figure 8. Effect of NaCl on tannins content for 15 days of marjoram under 0 and 75 mM NaCl. Means of four replicates ± SE. Means followed by different letters are significantly different ($P \leq 0.05$) as determined by analysis of variance (ANOVA).

The oxidation inhibiting activity of tannins have been known for a long time and it is assumed to be due to the presence of gallic acid (Khan et al. 2011). Baatour et al. (2012a), revealed an important content of this compounds in marjoram at vegetative stage. Inaddition the presence of tannins could also responsible for the astringent flavor of *O. majorana* seeds. The antiradical scavenging activity could be related to high content of tannins. Our result was similarly to Manian et al. (2008)**,** who suggested that this later had bigger ability to capture free radical. From the present investigation, we were found the presence of flavonoids and phenols which are known to possess antioxidant properties (Senthil Kumar et al. 2012)**.** So, marjoram can accumulate total phenolics under salt stress that protect plants from ROS due to their antioxidative properties like H-donating ability (DPPH). The increase of phenolics production under saline conditions is reported in various plants like *Catharanthus ruseus* (Jaleel et al. 2008) and wheat (Abd El-Baky 2009). A positive relationship had been reported between antioxidant activity and accumulation of total phenolic contents (Naciye et al. 2008).
Duenas et al. (2009) reported that germination caused significant changes in the phenolic composition (increasing) mainly due to endogenous enzymes activation and the complex biochemical metabolism of seeds during this process.

4. Conclusion

Finally, the richness of O. majorana seeds in secondary metabolites known for their antioxidant and anti-inflammation activities could support the utilization of seeds of this plant in a large field of application including cosmetic, pharmaceutical, agroalimentary and biological defense.

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