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# Differential Responses of *Cakile maritima* at Two Development Stages to Salinity: Changes on Phenolic Metabolites and Related Enzymes and Antioxidant Activity

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## Abstract

Though halophytes are naturally adapted to salinity, their salt-tolerance limits are greatly influenced by endogenous (that is, physiological development stages) and exogenous factors (that is, salinity). In this work, the evaluation of oxidative stress, bioactive molecules contents, antioxidant activities and two enzymes involved in the synthesis of secondary metabolites were assessed in the edible halophytic species *Cakile maritima* as function of salinity (0, 100, 400 mM NaCl) and two physiological stages. Total phenols, flavonoids and tannin contents increased by 58, 80 and 18% in vegetative period and by 27, 28 and 31% in flowering one at 400 mM NaCl, respectively as compared to *C. maritima* shoots control. These data indicated that phenolic compounds played an important role in protecting this species from salinity. The stimulatory effect of the salt on the total phenols content leads to the investigation of the role of two regulatory shikimate/phenylpropanoid pathway enzymes in phenolic synthesis. Results indicated that increased treatment progressively stimulated positively the activities of phenylalanine ammonia lyase and shikimate kinase in the vegetative and flowering period. Increasing salinity is correlated to the accumulation of carotenoids, anthocyanin, proline and ascorbate contents. The antioxidant activity enhanced at the vegetative stage in salt condition as compared to the flowering one. These results strongly indicate that salinity induces the accumulation of secondary metabolites in *C. maritima* shoots by altering the phenolic synthesis enzymes, as well as for the up-regulation of antioxidant molecules defense.

**Keywords:** *Cakile maritima*; Antioxidant activity; Phenylalanine ammonia-lyase; Shikimate kinase; Salt treatment; Development stage

## Introduction

Abiotic stresses are considered to be serious threats to plants and vegetation since they decrease crop yield and quality. Among these, salinity is the major abiotic factor that causes important damages to plants. This phenomenon is more severe in the semi-arid and arid areas [1]. In fact, increased salinity induces specific changes at cell, tissue and organ levels and it disrupts different biochemical and physiological processes [2]. Salt stress leads to osmotic imbalance by limiting absorption of water from soil and to ionic stress resulting from high concentrations of potentially toxic salt ions within the cells [3]. It also generates oxidative stress in plant tissues which is manifested by reactive oxygen species (ROS) such as singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical [1]. Consequently, plants that grow well in high salinity have specific structural and physiological adaptations to cope with the altered processes, while maintaining their reproductive capacity [2]. Among them, halophytes have the ability to withstand the stressful environmental conditions using different mechanisms including their capacity to quench toxic ROS, since they are equipped with powerful antioxidant system that includes enzymatic and non-enzymatic components.

The biosynthesis and the accumulation of these secondary metabolites, such as polyphenol, are generally stimulated in response to biotic/abiotic constraints [4]. Consequently, one may hypothesize that optimal polyphenol yield would be obtained using stress-tolerant species [5]. These components are synthesized through aromatic amino acids starts with the shikimate pathway. Shikimate kinase (EC 2.7.1.71) is the enzyme responsible for converting shikimate to 3-phosphoshikimate, a committed step in the biosynthesis of chorismate [6]. The latter is the branching point metabolite and the major precursor of aromatic amino acids, folates, ubiquinones, and many other aromatic compounds. The aromatic amino acids formed in this pathway are then used for protein synthesis or converted, in the phenylpropanoid metabolism, into secondary metabolites such as lignin or phenolics and phytoalexins [7].

At the gateway from primary metabolism, L-Phenylalanine Ammonia Lyase (PAL, EC 4.3.1.5) plays a pivotal role in phenolic synthesis and thousands of reports emphasize the correlation between increases in the corresponding PAL gene/protein expression/activity and increases in phenolic compounds in response to different stimuli and stress factors [7]. PAL seems to be very sensitive to the physiological state of the plant like during pathological events and plays an important role in plant defense. It is also involved in the biosynthesis of the signaling molecules and salicylic acid which is required for plant systemic resistance [8,9]. In addition, there is an increasing interest for other antioxidant molecules like ascorbic acid (Vit C, ASC). In fact, ASC is crucial for plant defence against oxidative stress. It is a water-soluble antioxidant molecule, used as substrate for ascorbate peroxydase which catalyzes hydrogen peroxide detoxification. ASC acts directly to eliminate superoxide radicals, $^1O_2$, and as a secondary antioxidant during reductive recycling of the oxidized form of tocopherol [10] and protecting enzymes with prosthetic transition metal ions [11].

It was shown that the amount of bioactive compounds and antioxidant activities in plants are controlled by biological factors (genotype, organ and ontogeny), edaphic and environmental (temperature, salinity, water stress and light intensity) conditions. According to Lisiewska et al. [12], the evolution of phenolic content in

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higher plants may reflect their physiological status and developmental stages. In this context, Ksouri et al. [4] indicated that leaf and stem extracts of *Salsola kali* showed a significant decrease of their phenolic contents and consequently their antiradical activities at the reproductive stage, as compared to the vegetative one, while root extract showed the opposite tendency. Other studies reports that phenolic content varied as a function of plant growth in tomato and *Anethum graveolens* cultivars [12,13]. Studies have also found antioxidant properties to be influenced by various environmental factors for several plants. In fact, polyphenol synthesis and accumulation is generally stimulated in response to biotic/abiotic stresses such as salinity [14]. In this context, Oueslati et al. [15] showed an increase of total polyphenol content (2.41-8.17 mg gallic acid equivalent $g^{-1}$ DW) in the leaves of a medicinal plant *Mentha pulegium* L. under salt constraints.

In this context, we investigated the effect of salt treatment and development stage on the bioactive metabolite contents, antioxidant capacities and the enzymatic activities of the PAL and the shikimate kinase, in the halophytic species *C. maritima* (Brassicaceae), traditionally used for its purgative, diuretic and antiscorbutic properties and for culinary applications [3].

## Materials and Methods

### Plant material, culture conditions and preparation of extract

*Cakile maritima* seeds were harvested in August 2016 at Raoued, a locality close to the Mediterranean seashore, 20 km north of Tunis. Germination of seeds was conducted for one week in the dark and at room temperature. After that, seedlings were sown in pots (four seedlings per pot) filled with inert sand and irrigated daily with Hewitt nutrient solution (pH 7.3, electrical conductivity 2.7 mS $cm^{-1}$) [16] for 4 weeks. Seedlings were partitioned into three lots of plants grown of four individuals each and NaCl was added to the solution. The final salt concentration (0, 100 or 400 mM) was progressively adjusted with increasing NaCl concentrations (50 mM step per day). The culture was conducted in greenhouse under controlled environment (15-25°C temperature and 70-90% relative humidity, 16/8 h day/night photoperiod). Aerial parts of the plant were collected at two different developmental stages, vegetative and flowering. Shoots were dried at 35°C for one week. Plant extracts were obtained by magnetic stirring of 2 g of dry matter powder with 20 ml of methanol 80% for 30 min. Then, extracts were kept at 4°C for 24 h, filtered through a Whatman No. 4 filter paper and stored at 4°C until analysis. Besides, fresh shoots samples from each plant were used for performing the biochemical analysis.

### Evaluation of stress indicators

**Lipid peroxidation:** Lipid peroxidation was estimated by determining the malonyldialdehyde (MDA) contents [17]. Amount of 200 mg of fresh samples were homogenised in 1 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 15000 *g* for 10 min at 4°C. The supernatant was mixed (v/v) with 0.5% (w/v) thiobarbituric acid (TBA) prepared in TCA 20%, and incubated at 90°C for 30 min. After stopping the reaction in an ice bath, samples were centrifuged at 10000 *g* for 10 min. The supernatant absorbance at 532 nm was then measured. After subtracting the non-specific absorbance at 600 nm, MDA concentration (three replicates per treatment) was determined using the extinction coefficient (155 $mM^{-1}$ $cm^{-1}$).

**Electrolytes Leakage (EL):** Leaf disks (100 mg) of uniform size (5 mm diameter) were placed in test tubes containing 10 ml of double distilled water. The tubes were incubated in a water bath at 32°C for 2 h and the initial electrical conductivity of the medium (EC1) was measured. The samples were incubated again in a water bath at 95°C for 20 min to release all electrolytes then cooled to 25°C before measuring the final electrical conductivity (EC2). The EL percentage was calculated using the formula:

$$EL=(EC1/EC2)\times 100$$

**Pigments analyses:** Carotenoid and anthocyanin contents (µg/g Fresh Weight) in the aerial parts were measured based on the method described by Gould et al. [18].

The extraction of carotenoid was realized with 200 mg of plant material frozen in liquid $N_2$, ground to a fine powder (on ice) and then mixed with two milliliters of acetone: $H_2O$ (4:1, v/v). The mixture was agitated and left in the dark at 4°C for 72 hours. The extracts were centrifuged and the absorbance was measured with UV/visible spectrophotometer at three different wave lengths. Measurements of $A_{470}$, $A_{647}$ and $A_{663}$ were taken to calculate the concentrations of total carotenoids according to the following equation:

$$\text{Carotenoid (CAR, µg/g)}=5\,A_{(470)}+2.846\,A_{(663)}-14.876\,A_{(647)}$$

Anthocyanin content was assayed with 200 mg of plant material frozen in liquid $N_2$, ground to a fine powder in a mortar placed on ice, then two milliliters of a HCl: $H_2O$: MeOH (1:3:16, v/v/v) solution were added. The mixture was agitated and kept in the dark at 4°C for 72 hours. The extracts were centrifuged and the absorbance was measured with UV/visible spectrophotometer. Anthocyanin levels were estimated using the formula: $A_{530}$ -0.24 $A_{653}$. The subtraction of 0.24 $A_{653}$ compensated the small overlap in absorbance at 530 nm by the chlorophylls [19]. Triplicate measurements were taken for all samples.

**Ascorbic acid quantification:** The assay is based on the reduction of $Fe^{3+}$ to $Fe^{2+}$ by AsA and the spectrophotometric detection of $Fe^{2+}$ complexed with 2,2'-dipyridyl method [20]. DHA is reduced to AsA by preincubation of the sample with dithiothreitol (DTT). Subsequently, the excess of DTT is removed with *N*-ethylmaleimide (NEM) and total AsA is determined by the 2,2'-dipyridyl method. The concentration of DHA is then calculated from the difference of total AsA and AsA (without pre-treatment with DTT). Aerial parts of treated and untreated plants were shock frozen in liquid $N_2$. Aliquots of frozen plant material (0.4 g) were ground to a fine powder in a mortar (placed in ice), prechilled with liquid $N_2$ before adding 0.8 ml 6% (w/v) TCA. After grinding in the frozen TCA, the mixture was continually homogenized until completely thawed and then allowed to stand further on ice for 15 min. The homogenate was adjusted to a volume of 2 ml with 6% (w/v) TCA, then centrifuged for 5 min at 15600 g (4°C) and the supernatant was immediately assayed for total vitamin C and AsA. The following solutions are used: standard solution of AsA dissolved in 6% (w/v) TCA, 10 mM DTT dissolved in 0.2 M phosphate buffer (pH 7.4), 0.5% (w/v) NEM, 10% (w/v) TCA, 42% (w/v) $H_3PO_4$, 4% (w/v) 2,2'-dipyridyl dissolved in 70% (v/v) ethanol, and 3% (w/v) $FeCl_3$. The absorbance was read at 525 nm. A standard curve covering the range of 0-25 nmol AsA was used. Triplicate measurements were taken for all samples.

**Proline quantification:** Proline was extracted using dried plant material. The experiment was conducted on ice and 10 mg of plant samples were dissolved in 750 µl of 3% (w/v) aqueous sulfosalicilic acid and centrifuged at 14000 *g* for 10 min. The supernatant was kept and the pellet was redissolved in 750 µl sulfosalicilic acid for a second extraction. The amount of proline was measured by the colometric method described by Bates et al. [21] using ninhydrin, acetic acid and orthophosphoric acid complex. 1 mL of the plant extract was mixed with 1 mL of the ninhydrin reagent and 1 ml of glacial acetic acid and

immediately incubated at 100°C for one hour. Tubes were cooled (on ice) and 2 mL of pure toluene were added to the mixture which was strongly shaken for 40 to 45 seconds. The upper layer of the coloured solution is used for the absorbance lecture at 520 nm. Proline final concentrations were determined referring to a standard curve (0-10 µg proline/mL). Each sample was measured three times.

## Colorimetric quantification of phenolics

***Determination of total polyphenol content:*** Phenolic content was assayed using the Folin-Ciocalteu reagent, following Singleton's method slightly modified [22]. An aliquot (0.125 mL) of appropriately diluted sample extract was added to 0.5 ml of distilled water and 0.125 mL of the Folin-Ciocalteu reagent. After 3 min, 1.25 mL of $Na_2CO_3$ (7%, w/v) solution were added and the final volume was brought to 3 ml with distilled water. The absorbance was measured at 760 nm, after incubation for 90 min at room temperature in dark. Total polyphenol content of plant extracts was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g DW) through the calibration curve with gallic acid. The calibration curve range was 0-400 µg/mL. Triplicate measurements were taken for each sample.

***Estimation of total flavonoid content:*** The amount of flavonoid content was measured using the method described by Dewanto et al. [22]. An aliquot of suitable diluted sample or standard solution of (+)-catechin was added to a $NaNO_2$ solution and kept for 6 min before adding 0.15 ml of a freshly prepared $AlCl_3$ solution (10% w/v). After 5 min, 0.5 ml of 1 M NaOH solution was added. The final volume was adjusted to 2.5 ml with distilled water and thoroughly mixed. Absorbance of the mixture was determined at 510 nm. Total flavonoids were expressed as mg (+)-catechin equivalent per gram dry weight (mg CE/g DW), through the calibration curve of (+)-catechin.

***Total condensed tannins content:*** Contents of condensed tannins were carried out according to Sun et al. [23]. 50 µL of properly diluted sample were mixed with 3 ml of vanillin-methanol solution (4% w/v) and 1.5 ml of concentrated hydrochloric acid. The mixture was left for 15 min and the absorption was measured at 500 nm. The concentration of condensed tannins was expressed as mg (+)-equivalent catechin/g DW. The calibration curve range of catechin was established between 0 and 400 µg/mL. All samples were analysed in triplicate.

## Shikimate/Phenypropanoid pathway enzymes

***Assay of the shikimate kinase (SK):*** Fresh tissue was homogenized at 4°C in a grinding medium containing 0.1 M K-phosphate buffer (pH 7.4), 0.5 mM DTT, 2 mM l-cysteine, 2 mM EDTA, 8 mM β-mercaptoethanol and 0.5 g PVPP. The homogenates were filtred individually through four layer of gauze and centrifuged at 19000 g for 20 min at 0-4°C [24].

The shikimate kinase (EC 2.7.1.71) was assayed at 25°C by coupling the release of ADP to the oxidation of NADH using pyruvate kinase (EC 2.7.1.40) and lactate deshydrogenase (EC1.1.1.27) as coupling enzymes according to Krell et al. [25]. Shikimate dependent oxidation of NADH was monitored at 340 nm. The assay mixture contained 50 mM triethanolamine hydrochloride/KOH buffer at pH 7.0, 50 mM KCl, 5 mM $MgCl_2$, 1.6 mM shikimic acid, 5 mM ATP, 1 mM phosphoenolpyruvate, 0.1 mM NADH, 3 units/ml pyruvate kinase, 2.5 units/ml lactate dehydrogenase and 1 ml of the enzyme crude extract. The enzymatic activity was calculated using the extinction coefficient $\varepsilon$=6,22 $mM^{-1}$ $cm^{-1}$.

***Assay of the phenylalanine ammonia lyase (PAL):*** The extract enzyme preparation for phenylalanine ammonia lyase (PAL; EC 4.3.1.5) was obtained by homogenizing 5 g of fresh material in 10 ml of the extraction medium containing 0.1 M sodium borate buffer (pH 8.8), 20 mM β-mercaptoethanol and 5% PVPP (m/v). The homogenate was centrifuged at 16000 *g* at 4°C for 20 min. The enzyme activity was measured by adding 250 µl of the supernatent to 1 ml L-phenylalanine (0.1 M) and 1.75 ml sodium borate buffer (pH 8.8). After 1 hour incubation at 37°C, the reaction was stopped by adding 100 µl 6 N HCl and the absorbance was read at 290 nm [26].

## Determination of antioxidant activities

***Total antioxidant capacity:*** Total antioxidant activity of methanolic extracts was evaluated according to the method described by Prieto et al. [27], based on the formation of a green phosphate/$Mo^{5+}$ complex. An aliquot (0.1 mL) of appropriately diluted samples was added to 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Methanol (80%) was used instead of sample for the blank. The tubes were incubated in a boiling water bath for 90 min. Then, samples were cooled to room temperature and the absorbance was measured at 695 nm using a UV-Visible spectrophotometer. Antioxidant capacity was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DW). All samples were analyzed in triplicate.

***DPPH scavenging activity:*** The scavenging activity of *C. maritima* extracts was measured in term of hydrogen donating or radical scavenging ability using the DPPH method [28]. One milliliter of various concentrations (100-1000 µg/mL) of the methanolic extracts was mixed with 0.25 mL of a 0.2 mM $DPPH^{\cdot}$ methanolic solution. The mixture was vigourously shaken and placed in the dark at room temperature for 30 min. The absorbance of the resulting solution was then read at 517 nm. The antiradical activity was expressed as $IC_{50}$ (µg. $mL^{-1}$). The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

Where $A_0$ is the absorbance of the control at 30 min, and $A_1$ is the absorbance of the sample at 30 min. All samples were analyzed in triplicate.

***Iron reducing power:*** The ability of the extracts to reduce $Fe^{3+}$ was assayed by the method of Oyaizu [29]. Briefly, 1 ml of *C. aritime* extracts at different concentrations (500-10000) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1% w/v). After incubation at 50°C for 20 min, 2.5 ml of trichloroacetic acid (10% w/v) was added and the mixture was centrifuged at 650 *g* for 10 min. Finally, 2.5 ml of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of aqueous ferric chloride (0.1% w/v). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increasing reducing power. $EC_{50}$ value (µg. $ml^{-1}$) is the effective concentration at which the absorbance was 0.5 obtained from linear regression.

***β-Carotene bleaching test (BCBT):*** A slightly modified Koleva et al. [30] method was employed to estimate *Cakile* aerial parts capacity to inhibit the bleaching of the β-carotene. Two milligram of β-carotene was dissolved in 20 ml chloroform and to 4 ml of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Under vacuum at 40°C, the chloroform was evaporated and 100 ml of oxygenated water was added, then the emulsion was vigorously shaken. An aliquot (150 µl) of the β-carotene/linoleic acid emulsion was distributed in each of the wells of 96-well plate and methanolic solutions of the test samples (10 µl) were added. Three replicates were prepared for each of the

samples concentration. The plate was incubated at 50°C for 120 min, and the absorbance was measured using a model EAR 400 microtitre reader (Labsystems Multiskan MS) at 470 nm. Reading of all samples were performed immediately (t=0 min) and after 120 mn incubation. The antioxidant activity (AA) of the extracts was evaluated in term of inhibition of the bleaching of the β-carotene using the formula below and the results were expressed as inhibition percentage values.

β-carotene bleaching inhibition (%)=$[(A_{c(120)}-A_{s(120)})/(A_{c(0)}-A_{c(120)})] \times 100$

where $A_{c(0)}$ and $A_{C(120)}$ are the absorbance values of the control at 0 and 120 min, respectively, and $A_{s(120)}$ is the sample absorbance at 120 min. The results were expressed as $IC_{50}$ values ($\mu g.ml^{-1}$).

**Statistical analysis:** A two-way analysis of variance (ANOVA), with the physiological stage (D) and salt treatment (S) as factors, was performed for the whole data set using the STATI-CF statistical program. Means were compared using the Newman-Keuls test at the $p<0.05$ level when significant differences were found. Values were the means of three replicates.

## Results

### Lipid peroxidation and electrolytes leakage

The effect of salinity on *C. maritima* at both development stages, measured using the MDA formation and the electrolytes leakage (EL), seem to be more dependent on the salt treatment (S). These observations are confirmed by statistical analysis where the effect of salinity on the plants leads to significant differences. The two indices of oxidative stress studied have a similar response to salinity levels (Figure 1). The lipid peroxidation in the aerial parts of the plant, during the vegetative stage, increased significantly by 56% and 41% at 100 and 400 mM NaCl as compared to the control. In flowering stage, MDA accumulation was significantly lower than that in vegetative one. Similarly, electrolytes leakage was significantly correlated with salinity (Figure 1). It increased in all treatments, and was higher in vegetative period (2.2 and 2.9 times higher respectively at 100 mM and 400 mM) than in the flowering one (1.5 and 2 times higher at 100 mM and 400 mM, respectively) as compared to the untreated plants.

| Dependent variables | (D) | (S) | (S × D) |
|---|---|---|---|
| **MDA** | 3.052 | 155.425*** | 9.810** |
| **EL** | 6.071 | 64.691*** | 1.229 |
| **Carotenoids** | 7.839 | 104.261*** | 0.000 |
| **Antocyanins** | 10.406* | 8.101** | 0.092 |
| **Total Vit. C** | 13.977* | 81.271*** | 9.350** |
| **AsA** | 3245.673*** | 663.429*** | 486.544*** |
| **DHA** | 3186.497*** | 800.409*** | 459.351*** |
| **Proline** | 742.755*** | 545.868*** | 186.903*** |
| **Polyphenols** | 0.001 | 22.875*** | 2.737* |
| **Flavonoids** | 134.331*** | 19.049*** | 0.375 |
| **Tannins** | 26.810*** | 6.346 | 0.549 |
| **PAL** | 444.609*** | 425.014*** | 52.938*** |
| **SK** | 0.009 | 11.981** | 33.852*** |
| **DPPH** | 1309.688*** | 126.140*** | 64.204*** |
| **TAA** | 1003.913*** | 37.142*** | 1.862 |
| **Reducing power** | 533.929*** | 399.617*** | 63.832*** |
| **BCBT test** | 20.152** | 25.822*** | 0.693 |

Results of ANOVA: *F* values for the proportion of total variance attributed to the physiological development stage (D), salt treatment (S) and their interaction (S × D). Numbers represent *F* values: *P<0.01, **P<0.001, ***P<0.0001.

**Table 1:** Comparison of the development stage and the salt treatment on antioxidant compounds and activities (antioxidant and enzymatic).

### Change of amounts of antioxidant pigments, proline and ascorbic acids as function of salinity and physiological development stage

Carotenoid and anthocyanin contents of *C. maritima* showed a wide range (11.5 to 31.7 $\mu g\ g^{-1}$ FW and 1.1 to 2.5 $\mu g\ g^{-1}$ FW, respectively) (Figure 2a and 2b). Results displayed that salt treatment (S) seems to be the determinant factor in the amount of these molecules as it was depicted by the analysis of variance and as it is shown by results presented in Table 1. In fact, there is a significant increase in the amount of carotenoids as function of the salt dose rising for plants collected in the both periods of growth. Many previous works showed that the amounts of these molecules increased with salinity [31].

Ascorbic acid content (reduced ascorbic acid (AsA) and dehydroascorbate (DHA)) in the aerial parts of *Cakile maritima* was found to be significantly variable (Figure 3). Our results showed a particular wealth of this species in vitamin C when compared to other plants. Its content is about 400 mg/100 g FW and this was recorded within the control plants. The variation of the total ascorbic acid content was proportional to the salt doses and it is the highest at 400 mM NaCl (Figure 3a) where it is 445.3 and 444.8 mg/100 g FW during the vegetative and the reproductive stages, respectively. This result corroborate with those reported by Hafsi et al. [32] and Telesinski et al. [33], where they showed that a salt treatment increases the production of vitamin C and is salt-dose dependent.

The oxidized fraction (DHA) increased as function of salinity while the reduced fraction (AsA) decreased, especially at 400 mM NaCl (Figure 3b and 3c). These results corroborate with those found by Ben Amor et al. [34] in two different localities of *C. maritima*. These authors found that prolonged exposure to high salinity (200 and 400 mmol/L NaCl) caused a decrease in reduced ascorbate and an increase in oxidized ascorbate, which was matched by a substantial decrease in the ASC/DHA ratio. This can indicate that the reduced form of the ascorbic acid interfere in the protection of the plant in the case of salt stress by being oxidized.

A highly significant effect of salinity (S), development stage (D) and their interaction (S*D) on shoot proline was found (Table 1). The quantification of this amino acid indicated that its amount increased with salt doses for both stages particularly the vegetative one (Figure 4), suggesting the positive role of proline in the salt tolerance.

### Change in phenolic metabolism as function of salinity and physiological development stage

As shown in Table 2, salt-challenged plants of *C. maritima* exhibited a significant increase in polyphenol accumulation (up to 58.3% at 400 mM in the vegetative stage), while there is no significant variability in the total phenolic content between the two development stages. These results were corroborated by ANOVA statistical analysis (Table 1) which showed that no significant effect was described by (D). However, (S) contributed by the highest proportion of total variance, followed by (S × D). As an important fraction of total phenolic, flavonoid content of *C. maritima* shoots showed significant differences (Table 2). Results displayed that (S) and (D) significantly influenced flavonoid content (Table 1). With similar tendency to phenolics, flavonoid content was remarkably higher in salt-challenged plants with the highest amounts found during the flowering period of the plant. Concerning the

| Development stage | Vegetative period | | | Flowering period | | |
|---|---|---|---|---|---|---|
| NaCl (mM) | 0 | 100 | 400 | 0 | 100 | 400 |
| Total polyphenol content (mg GAE/g DW) | 4.8 ± 0.04 d | 5.7 ± 0.01 cd | 7.6 ± 0.02 a | 5.3 ± 0.03 cd | 6.1 ± 0.00 bc | 6.7 ± 0.01 ab |
| Total flavonoid content (mg CE/g DW) | 0.5 ± 0.0 d | 0.6 ± 0.00 d | 0.9 ± 0.00 c | 1.1 ± 0.01 b | 1.2 ± 0.01 b | 1.4 ± 0.00 a |
| Tanin content (mg CE/g DW) | 1.1 ± 0.00 c | 1.2 ± 0.00 c | 1.3 ± 0.00 bc | 1.3 ± 0.00 bc | 1.5 ± 0.01 ab | 1.7 ± 0.00 a |

mg GAE/g DW: milligram gallic acid equivalent per gram dry weight; mg CE/g DW: milligram catechin equivalent per gram dry weight

**Table 2:** Variation of total polyphenol, flavonoid and condensed tannin contents in aerial parts of *Cakile maritima* as function of salinity (0, 100 and 400 mM NaCl) and physiological development stage (vegetative and flowering). Means (three replicates) followed by at least one same letter are not significantly different at P <0.01.

| Development stage | Vegetative period | | | Flowering period | | |
|---|---|---|---|---|---|---|
| NaCl (mM) | 0 | 100 | 400 | 0 | 100 | 400 |
| TAA (mg GAE/g DW) | 29.6 ± 0.03 b | 32.1 ± 0.01 a | 34 ± 0.01 a | 11.6 ± 0.02 e | 15.1 ± 0.06 d | 18.5 ± 0.04 c |
| DPPH test $IC_{50}$ (µg/ml) | 310 b | 260 ab | 240 a | 330e | 650 d | 480 c |
| Iron reducing power $EC_{50}$ (mg/ml) | 4.44 b | 4.38 b | 2.38 a | 6.19 d | 4.61 c | 4.26 b |
| BCBT test $IC_{50}$ (mg/ml) | 9.15 c | 5.68 ab | 3.73 a | 12.6 d | 7.44 bc | 7.35 bc |

mg GAE/g DW: milligram gallic acid equivalent per gram dry weight

**Table 3:** Variation of the antioxidant activities of methanolic extract in *Cakile maritima* leaves as function of salinity (0, 100 and 400 mM NaCl) and physiological development stage (vegetative and flowering). Means (three replicates) followed by at least one same letter are not significantly different at *P* <0.01.

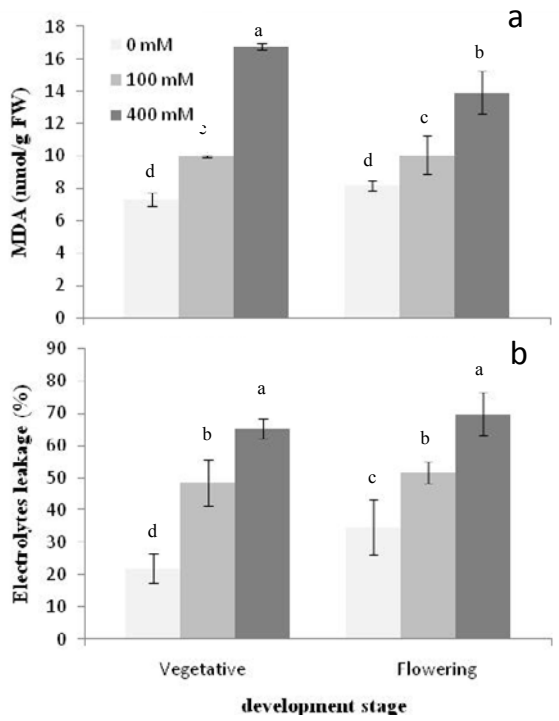


**Figure 1:** Variation of MDA (a) content and electrolyte leakage (b) in aerial parts of *Cakile maritima* as function of salinity (0, 100 and 400 mM NaCl) and physiological development stage (vegetative and flowering). Means (three replicates) followed by at least one same letter are not significantly different at P<0.01.

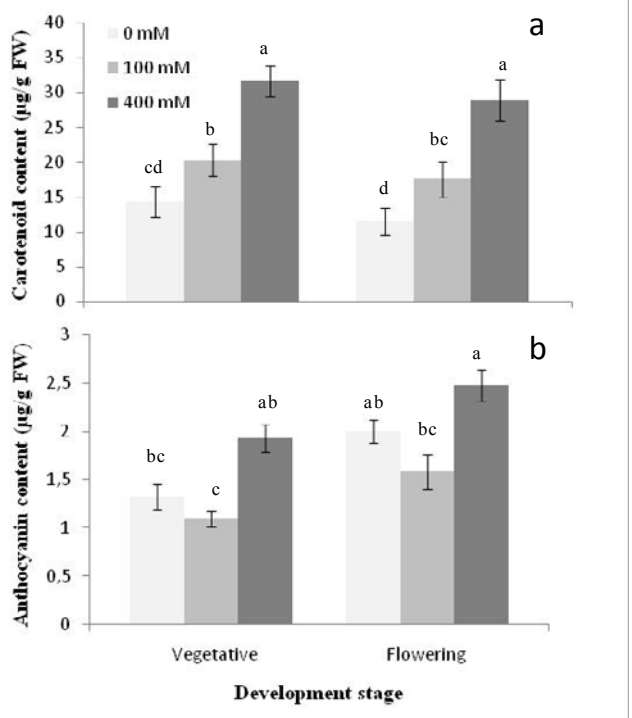


**Figure 2:** Variation of carotenoid (a) and anthocyanin (b) contents in *Cakile maritima* aerial parts as function of salinity (0, 100 and 400 mM NaCl) and physiological development stage (vegetative and flowering). Means (three replicates) followed by at least one same letter are not significantly different at P<0.01.

condensed tannins, these compounds seem to be not affected by salinity at the vegetative period (Table 2). This result was confirmed by ANOVA statistical analysis (Table 1) which showed that (D) contributed with the highest proportion of total variance.

The observed stimulatory effect of salinity on phenolic contents led us to investigate on the activity of two enzymes belonging to the shikimate and phenylpropanoid pathways, involved in phenolic synthesis: SK and PAL.

As shown in Figure 5a, PAL activity increased with salinity regardless the development growth. The analysis of variance (ANOVA) showed the high influence of each factor (S) and (D) and their interaction (S × D). This activity is as much higher as the salt treatment is stronger. In fact, at the vegetative stage, the PAL activity increase by 19% at 400 mM as compared to the control, however, it is more pronounced during the

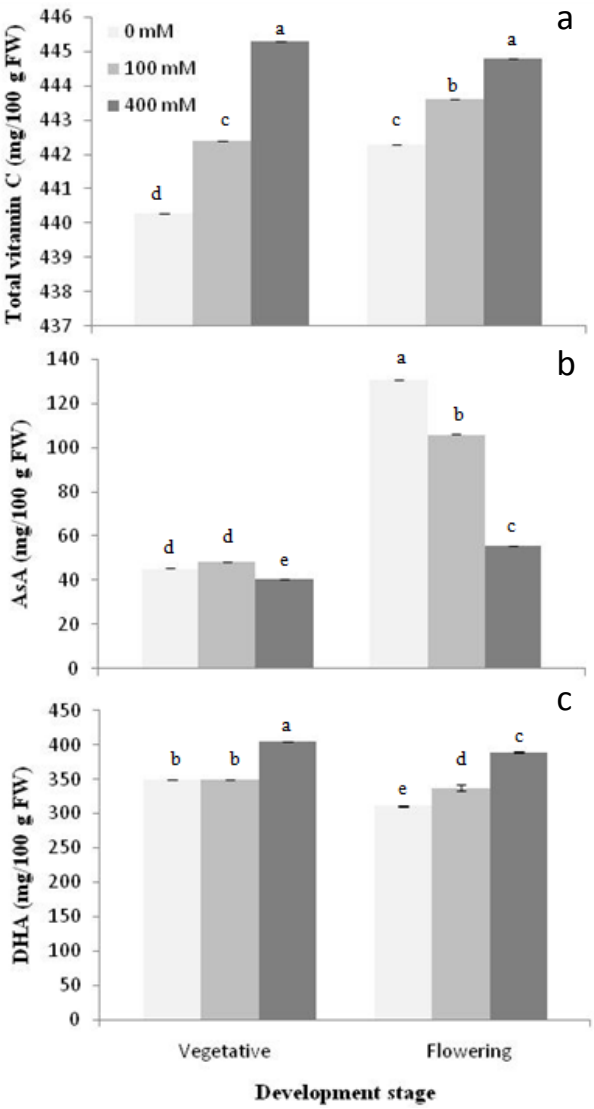


**Figure 3:** Variation of ascorbic acid (total (a), reduced ascorbate (b) and dehydroascorbate (c)) contents of in aerial parts of *Cakile maritima* as function of salinity (0, 100 and 400 mM NaCl) and physiological development stage (vegetative and flowering). Means (three replicates) followed by at least one same letter are not significantly different at P<0.01.

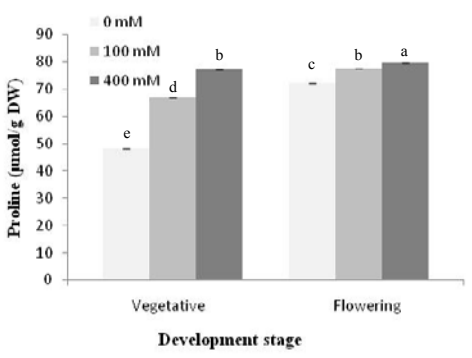


**Figure 4:** Variation of proline content (µmol/g DW) in the aerial parts of *Cakile maritima* as function of salinity (0, 100 and 400 mM NaCl) and physiological development stage (vegetative and flowering). Means (three replicates) followed by at least one same letter are not significantly different at P<0.01.

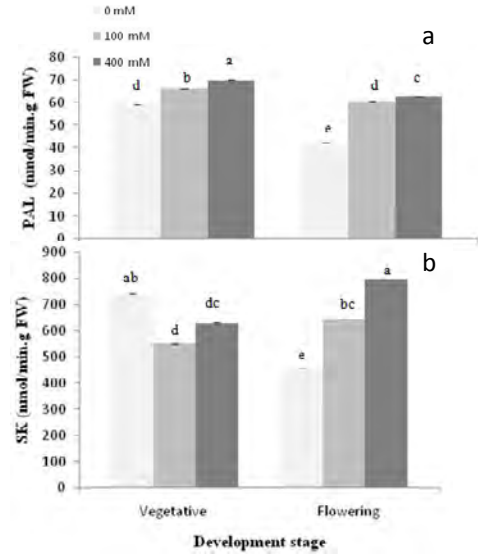


**Figure 5:** Variation of PAL total activity (nmol trans-cinnamic acid/min.g FW) (a) and shikimate kinase total activity (nmol NADH/min.g FW) (b) in the aerial parts of *C. maritima* as function of salinity (0, 100 and 400 mM NaCl) and physiological development stage (vegetative and flowering). Means (three replicates) followed by at least one same letter are not significantly different at P<0.01.

flowering period and goes from 42.3 (0 mM) to 62.8 nmol/min.g FW (400 mM) which is about 48%.

Moreover, the activity of shikimate kinase differs between the two periods of development. It was shown a slight decrease of this activity in first stage at 100 mM followed by an increase at 400 mM. However, shikimate kinase activity increased positively with salinity in flowering period (Figure 5b).

## Change in antioxidant as function of salinity and physiological development stage

Total antioxidant activity of the two developmental stages was extremely different (Table 3). Independently of the treatment, this ability was two-fold higher in the vegetative stage (between 29.6 and 34 mg AGE/g DW) than that in flowering one (between 11.6 and 20 mg AGE/g DW). In addition, the ANOVA analysis (Table 1) indicated that the significant influence on this activity is essentially based on the physiological stage than the salt treatment. As salinity progressed, the antioxidant activity increased in the shoots of *C. maritima* and was in proportion with the total phenols. Hence, the addition of NaCl in the culture medium increased the TAA of about 8.5% and 11.5% in the vegetative stage and of 30% and 59% in the reproductive one, at 100 and 400 mM, respectively. In this context, Petridis et al. [35] observed an enhancement of the antioxidant activity of shoots and roots of four olive cultivars under saline conditions. The same authors showed that this increase is usually correlated to the polyphenol contents.

The magnitude of DPPH· radicals quenching activity seemed to be related to the physiological stage and NaCl treatment too, as $IC_{50}$ values largely differed between the two periods (Table 3). The highest antiradical activity was recorded for the plants treated with 400 mM NaCl at both stages and it reached 240 µg/ml (vegetative period) and 480 µg/ml (flowering period) and it was even more important at the first period than in the flowering one. Our results corroborate with other authors, which have mentioned the positive correlation between the increase of the antioxidant activity and the salinity tolerance in many plants such as in pea, tomato and citrus [36].

The evaluation of the $Fe^{3+}$ reducing power of shoots extracts is shown in Table 3. The trend for ferric ions reducing activity of the different growth periods showed a similar tendency as compared to their DPPH radical scavenging activities, when a comparison between $EC_{50}$ and $IC_{50}$ propensities is made. In fact, vegetative stage showed relatively the stronger ferric ion reducing activity reflected by low $EC_{50}$ values, as compared to the flowering stage ones.

Furthermore, physiological development stage (D) followed by salt treatment (S) seem to be the determinant factors in the values of this activity as it was depicted by the analysis of variance (Table 1). This activity increased with salinity and the 400 mM treated vegetative plant extracts appeared to possess the highest reducing activity. The reducing power is an important aspect for the estimation of the antioxidant activity which might be due to the reduction of superoxide anion, inactivation of free radicals or complexion with metal ions or combination of the three [1].

In the β-carotene/linoleate system, free radicals arising from the oxidation of linoleic acid react with the insaturated molecules of β-carotene and leads to rapid discolouration of the orange solution. The presence of antioxidant reduces this discolouration by inhibiting the lipid peroxydes [37]. Methanolic extracts of *Cakile* prevented the bleaching of the β-carotene on a dose dependent manner. At 400 mM of NaCl, shoots displayed the highest capacity of inhibiting the lipid peroxides as compared to different treatment with the lowest $IC_{50}$ value (3.7 mg/ml) from vegetative stage. These findings corroborate with polyphenols contents. Moreover, $IC_{50}$ decreased as the salinity was increased in the culture medium, indicating that the antioxidant capacity of the shoots extracts was stimulated by salt treatment.

## Discussion

Electrolyte leakage and MDA level were commonly known as markers of oxidative stress and the antioxidant status. It is noteworthy that the magnitude of the oxidative stress increased with the salinity. It has been frequently reported that salt stress increased the level of lipid peroxidation and induces oxidative stress in plant tissues expressed as EL [34] and our results, agree with those of Ksouri et al. [14] and Ben Amor et al. [34].

Salinity differently affects the antioxidant molecules equipment of plants. The degree of oxidative cellular damages in plants exposed to this stress is controlled by the capacity of protection against oxidative agents. The involvement of the no-enzymatic system constituted by antioxidant molecules is important in the detoxification of several ROS. Salt tolerance seems to be favored by an increased antioxidative capacity to detoxify reactive oxygen species. Amount of antioxidant pigments, proline, ascorbic acids and phenolics were enhanced in the presence of salt particularly at 400 mM NaCl for both development stages. Our results are, in fact, in agreement with previous works reporting the seasonal variation of phenolic contents and antioxidant activities [4,38]. Carotenoids and anthocyanins are classified among the compounds having antioxidant activity [12]. In plant, carotenoids have a photoprotective role either by dissipating the excess of energy or by quenching the ROS that naturally occur during photosynthesis or by inhibiting the lipid peroxidation [39]. Besides, β-carotene cooperates with tocopherols in the radical scavenging capacity within the inner part of lipid membranes [40]. It has also been reported a positive correlation between antioxidant capacity and anthocyanins content in blackberries, red raspberries and strawberries [38]. Also, many studies confirmed the role of vitamin C as a powerful antioxidant and its implication in neutralizing the ROS [39-42]. In addition, the comparison of ascorbic acid content with other conventionally rich plant sources of this vitamin such as carrots (6 mg/100 g FW), bananas (10-30 mg/100 g FW), citrus (40-50 mg/100 g FW), spinach (51 mg/100 g FW) and tomato (20-25 mg/100 g FW) [43] indicated that *C. maritima* is an important source of this natural antioxidant potentially beneficial to human health. Besides, proline accumulation normally occurs in the cytosol where it contributes substantially to the cytoplasmic osmotic adjustment [3]. In addition to this role, it contributes to stabilizing sub-cellular structures, scavenging free radicals and buffering cellular redox potential under stress conditions [44].

Plants widely vary in their phenolic composition and contents, with both physiological periods and environments affecting the kind and level of phenolic compounds [38]. Our results corroborate with many works that reported the increase of phenolic compounds under increasing salinity [45,46] and showed that this increase mitigates the ionic effect of NaCl [47]. These compounds seem to possess a huge role in ameliorating the stress defense of the plant and interfere as efficient antioxidant substances.

The total phenolic content differed slightly among the two studied physiological stages of the plant. However, extracts with higher phenolic content did not always have higher flavonoid and tannin contents, as was evident for salt-challenged plant extracts from the flowering period which had a higher total flavonoid and tannin contents compared with those of vegetative plants (Table 2), although the total phenolic content was lower in the second developmental period compared to the first one (Table 2). These results suggest that different salt-stressed plant extracts of each period contain different levels of total flavonoids as a proportion of the total phenolic compounds [48]. Despite the fact that our results have shown that phenolic compounds content varies slightly depending on the development stages, those of the antioxidant activities exhibited a marked difference from the vegetative to the flowering periods. These data are in agreements with Ksouri et al. [4], where they found that leaves and stems of *Salsola kali* have an important antioxidant activity in the vegetative stage. This might suggest that these compounds play different roles depending on the state of the plant which could lead us to believe that, during flowering, these molecules are moving more toward a physiological role. While during the vegetative stage, they act preferentially as protector of plants by acting primarily as antioxidants. This proposal has been verified by many previous research works which confirmed the role of phenolics as major antioxidants in plants, especially within halophytic species [4,38].

The activity of enzymes involved in phenolic synthesis (SK and PAL) was also affected by salinity and showed significant differences as function of salt doses and development stages. The PAL activity can differ depending on the physiological stages, genotype and environmental factors [49]. Several studies reported that this activity is highly influenced by many abiotic and biotic factors [50]. Gao et al. [51] reported an increase in the PAL activity in *Jatropha curcas* leaves after a treatment with copper. Similarly, Kovacik et al. [52] had showed that a nitrogen deficiency in *Matricaria chamomilla* leads to a rise in the enzyme total activity of 61% compared to control. The induction of enzymes of the shikimate pathway under different kinds of abiotic stress was previously demonstrated. Msehli et al. [53] had shown that SK activity increased with Fe deficiency in the species *Medicago ciliaris*.

In fact, the induction of the PAL is associated with the accumulation of secondary compounds of the phenylpropanoids that provides specific protections to plants against stress [54]. Many studies indicated that the activation of the PAL and the increase of the phenolic content in plants represent a general response associated to the resistance to

stress [55]. Moreover, besides their role in the primary metabolism, enzymes of the shikimate pathway constitute an essential link with the secondary metabolism and the shikimate kinase plays a regulation role facilitating the orientation to specific secondary compounds [56]. Thus, the simultaneous activation of enzymes from the shikimate and the phenylpropanoid pathways in order to produce phenolic compounds can be considered as an efficient response to salt stress.

## Conclusion

No-enzymatic molecules contents and antioxidant activities depend on several factors, mainly environmental conditions, and their salt-tolerance limits are greatly influenced by endogenous (that is, physiological development stage) and exogenous factors (that is, salinity). Our results demonstrated the influence of salt conditions and period of growth on the evolution of bioactive compounds such as phenolics and ascorbic acid and antioxidant activity of *C. maritima*. It can be stated that both endogenous factor (D) and exogenous factor (S) and their interaction were highly implicated on the polyphenol biosynthesis and quality.

The increase of bioactive compound amounts and antioxidant capacities confirmed the stimulation of their synthesis in presence of salt constraint and their role as protectors of plant structures against the oxidative stress. In fact, the nature and the complexity of the plants bioactive substances influence their activities. The parallel induction of enzymes of the phenylpropanoid and shikimate pathways came also to confirm the tolerance of *C. maritima* to salinity.

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