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Influence of light intensity and salinity on growth and antioxidant machinery of *Thymus vulgaris* L.

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Thymus vulgaris L. commonly known as Thyme or Garden Thyme, is important medicinal herb used for their wide-ranging therapeutic properties. Agriculture practices of thyme plants influence their growth and biochemical composition. Here, we have evaluated the effects of two production systems and irrigation with saline water on growth, physiological characteristics and antioxidant capacity of *T. vulgaris*. Two levels of salinity stress (50 and 150 mM) were applied for 2 and 4 weeks under shade enclosure or open field. The results showed that NaCl-treated plants grown in shade enclosure showed reduced total dry weight and relative water content, photosynthetic characteristics and leaf pigments when compared to full sunny conditions. However, the shade conditions enhanced glucose and fructose accumulation mainly after a short period of NaCl stress application. The reduction of Ca^{2+} and K^{+} was lower in NaCl-stressed plants grown under open-field conditions. Besides, under sunny conditions, plants showed significant increase in malondialdehyde (MDA) and H_2O_2 contents. Our results demonstrated that these plants in open field have higher contents of reduced ascorbate (ASC) and reduced glutathione (GSH) than plants grown in shade enclosure, which could be related to enhanced activity of APX and GR. An increase in superoxide dismutase (SOD) and catalase (CAT) activity was also recorded. Moreover, activities of dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) were mainly dependent on the intensity of NaCl stress.

Keywords: Antioxidant enzymes, Ascorbate glutathione, Culinary, Herbal, Salinity, Stress, Thyme

Light is one of the most important environmental factors and plays a critical function in plant development and metabolism[1]. Additionally, light is indispensable for photosynthesis and photo-morphogenesis. Besides, the changes in irradiance have an impact on plant growth, morphology and anatomy, various aspects of physiology and cellular biochemistry, and also flowering time and plant productivity[2]. Furthermore, light influence the response of plants to several stresses such as salinity. Light and salinity stresses activate common responses, which mostly operate at the biochemical level, to electively counter the oxidative damage[3]. High sunlight and root-zone salinity may also alter photosystem II (PSII) photochemistry and photosynthetic pigment composition[3]. Thus, salt stress, as rated through ecophysiological metrics, is reduced by low light or shading. The low light (shade) may improve the physiological response of plants to salinity compared to unshaded plants, which could be due to the energy channeled for regulation of the internal ionic conditions i.e. for a higher uptake of Ca^{2+} and Mg^{2+}, while, the high light improved the salt tolerance in several plants[4].

Response mechanisms of plants to either excess soil salinity or high solar radiation have been investigated in great details over the past three decades. It is important to know how plants survive when exposed to the concomitant action of root zone salinity stress and high solar irradiance. One of the serious causes was the oxidative stress and ion imbalances, which results in osmotic stress, and generation of reactive oxygen species (ROS). Plants have evolved complex mechanisms that reduced oxidative and osmotic stress generated by salinity[1]. These mechanisms included ionic and osmotic balances adjustments and the turgor maintenance by synthesizing osmoprotectants. Such

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osmoprotectants include low molecular weight, highly soluble compounds, such as soluble sugars and proline[2]. Furthermore, these soluble compounds play a role in the stabilization of enzymes and/or proteins and in protecting cell membrane integrity against the denaturing effect of stress conditions on cellular functions. Plants also develop an efficient antioxidative system including enzymatic and non-enzymatic mechanisms[4] for protecting plants against negative effects of excess light energy[4] and salinity stress[5]. The non-enzymatic mechanisms involve low molecular weight antioxidants (ascorbate, glutathione, carotenoids), whereas the enzymatic antioxidant mechanisms involve the activity of superoxide dismutase (SOD, EC 1.15. 1.1), guaiacol peroxidase (GPX, EC 1.11.1.9), catalase (CAT, EC 1.11.1.6) and enzymes of ascorbate glutathione (AsA-GSH) cycle, such as ascorbate peroxidase (APX, EC 1.11.1.11), mono-dehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.8. 1.7)[5]. The first line of defence against ROS was the scavenging of superoxide radicals (O^{2-}) by SOD, which results the formation of H_2O_2 and O_2. CAT then transforms H_2O_2 in the peroxisomes, and by APX in the chloroplasts into H_2O and O_2[4]. Many works reported APX and POD activities were much highest in sun leaves under unstressed conditions. However, they increased in response to salt stress more in shade leaves[5]. On the other hand, the activity of CAT decreased in response to salt stress more than under high light[6].

Thymus vulgaris L. (Thyme or Garden Thyme), a medicinal plant from Fam. Lamiaceae family, is cultivated as an aromatic for culinary uses[7]. It has been shown to have beneficial expectorant properties, as well as others, such as antispasmodic, antibacterial, antifungal, antiviral, antiprotozoal and antioxidant[8]. Many factors *viz*. harvest time, seasonal variations, drying conditions, etc. may affect the composition of thyme. It was reported that plants grown in 'shade-enclosure' or 'open-field' conditions showed a varying growth and different responses, partly due to differences in the microclimates[9]. Moreover, thyme plants were cultivated in many countries such as Tunisia under greenhouse conditions and irrigated with low quality water with elevated salinity. Thus, in the current study we explored the response of thyme plants to salinity under different agriculture systems. Also, we tried to illustrate the combined effects of salt stress and light conditions on growth, photosynthetic apparatus and the antioxidant systems and to understand the interaction between stress enhancing the carbohydrate and the antioxidants enzymes.

Material and Methods

Experimental condition and plant material

The present study was performed on one year old *Thymus vulgaris*. The plants were cultivated in perforated 4-L plastic pots containing desert dune-sand. The plants were obtained from a commercial nursery. In the nursery, generally plants species were propagated by rooting the plant cutting from the wild species. The plant species was first identified by botanists from the Herbarium of the University of Gabes. Later, a dry pressed voucher of the plant was compared with deposited herbarium specimen at the same University. A set of voucher specimen was deposited in the herbarium of the Faculty of Sciences of Gabes, University of Gabes, Tunisia. The plants then cultivated for two months (April-May) with daily maximum air temperatures ranging between 29 and 31°C, and relative air humidity ranging between 49 and 60%. They were irrigated every 4 days with a complete nutrient solution (N, 1.8 mM; P, 0.35 mM; K, 0.64 mM; Ca, 1.0 mM; Mg, 0.35 mM; S, 0.35 mM; Fe, 0.03 mM; Zn, 0.4 μM, Mn, 5.0 μM; Cu, 0.1 μM and B, 0.02 mM)[11]. The salinity stress was obtained by adding NaCl to the nutrient solution to obtain 50 and 150 mM of total ion concentrations. Control treatment did not have NaCl. To avoid osmotic shock, concentration of the nutrient solution was increased by 50 mM per day till reaching the target salinity levels. Plants in full sun received a daily dose of 1800 to 2000 μM m^{-2} s^{-1} in the photo-synthetically active radiation (PAR). Shade plants were exposed to a daily dose of 500 to 700 μM m^{-2} s^{-1} in the PAR. Temperature maxima/minima were averaged 29/14 and 25/19°C in full sun and shade, respectively. From the moment when the final concentration of NaCl was obtained for the most severe stress level, the concentrations of nutrient solutions were kept constant for the following four weeks. Eight plants of each treatment were harvested during morning time (between 9 to 11 a.m. local time) after two and four weeks. Plants were immediately frozen in liquid nitrogen and stored at -80°C for biochemical analyses.

Morphometric measurements, ion contents and gas exchange

At the end of the experiment, eight plants were individually harvested and divided into roots, stems and leaves. After measuring their total fresh weight, all plant organs were oven-dried at 80°C for 48 h for

dry wt. analysis. Plant growth was estimated by determining the dry wt. of leaves, stems and roots. For ion analyses, dry leaves (1.0 g) were extracted with 20 mL of 0.1 M HNO_3. After filtration, Na^+, K^+, Mg^+, Ca^{2+}, Fe^{2+} contents were determined with an atomic absorption spectrometer (Avanta, GBC, Australia). Net photosynthetic rate (*A*), stomatal conductance (g_s) and transpiration rate (*E*) of four fully expanded leaves of each treated plants was determined with a portable photosynthesis system (LCp proþ, ADC, UK) under natural conditions (PAR was 800-1200 mM m^{-2} s^{-1} and air temperature was 20-30°C).

Leaf relative water content and water and osmotic potentials

To evaluate water status of plants, four fully exposed leaves of each plants were used to measure the leaf relative water content (%RWC)[10]. The RWC was calculated according the following equation % RWC = 100 × [(FW–DW)/(TW–DW)]. Where, FW is fresh weight, DW is dry weight, and TW is turgid weight determined after soaking the leaf samples in distilled water for 24 h at 4°C in a refrigerator. Dry weight was measured after oven-drying the samples for 48 h at 80°C. Pre-dawn leaf water potential (Ψw) was measured on four median leaves with petiole with a Scholander pressure chamber (PMS, Albany, OR, USA) using a standard methodology[11]. The osmolality was measured with a vapour pressure osmometer (Wescor 5520, Logan, UT, USA), the osmolality values were converted to osmotic potential (Ψπ) by the van't Hoff equation: Ψπ= –ciRT, where ci = weight concentration of solute, R is a gas constant = 8,31 $J.moL^{-1}.K^{-1}$, and T is temperature in °C[12]. Turgor potential (Ψp) was calculated as the difference between osmotic potential (Ψπ) and water potential (Ψw) values (Ψp= Ψw – Ψπ).

Extraction and analysis of carbohydrate

Sugars were extracted and analyzed using Alasalvar *et al*.[13] method. The extraction was done for 100 mg of frozen leaves in 2 mL acetonitrile/water (1:1, v/v) for 2 min. The extract was kept in a water bath at 55-60°C for 15 min and then filtered over a Whatman No. 541 filter paper. Samples filtrates were then used for the determination of the sugar contents using HPLC chromatography. A mixture of acetonitrile and HPLC grade water at a ratio of 75:25 (v/v) was used as the mobile phase. Column temperature and injection volume were set at 30°C and 20 µL, respectively. The mobile phase (filtered through a 0.45 µM Millipore filter and degassed prior to use) was a mixture of acetonitrile and HPLC grade water at a ratio of 75:25 (v/v) at 1 $mL \cdot min^{-1}$. Identified sugars were quantified on the basis of peak areas and comparison with a calibration curve obtained with the corresponding standards ranging 1-10 mg/100 mL of acetonitrile/ water (1:1, v/v). Sugars were expressed as milligrams per 100 g of fresh weight (mg/100 g FW).

Determination of chlorophylls and carotenoids

Leaf chlorophyll and carotenoids contents were determined according to Arnon[14]. Briefly, fresh leaves (0.5 g) were ground to a fine powder in liquid nitrogen and homogenized for 30 s in 5 mL of 95.5% acetone. The pigments' concentrations were estimated from absorbance at 647 nm and 664 nm and a solution of 95.5% acetone was used as a blank. Pigment concentrations were calculated as follows: Chl a (mg/g FW) = [12.7 × (A664)-2.69 × (A647)] × (0.5×5), Chl b (mg/g FW) = [22.9 × (A647) -4.69 × (A664)] × (0.5×5).

Total carotenoids were extracted in duplicates[15]. Frozen leaf tissue (1.0 g) was briefly extracted with acetone and shaken with diethyl ether and 10% NaCl. Two phases were obtained, the lipophilic phase was washed with Na_2SO_4 (2%), saponified with 10% KOH in MeOH, and the pigments were subsequently extracted with diethyl ether, evaporated and then made up to 25 mL with acetone. Total carotenoids were estimated at 450 nm in a UNICAM Helios- spectrophotometer (Cambridge, UK), and expressed as mg of β-carotene equivalent per g fresh weight, taking into account the molar absorption coefficient (ε1% cm) of 2560.

Quantification of oxidative stress markers

MDA (lipid peroxidation) was assayed according to Hodges *et al*.[16]. Leaves tissue was homogenized in 80% ethanol (MagNALyser, Roche, Vilvoorde, Belgium) and incubated with thiobarbituric acid (TBA) to produce pinkish red chromogen TBA-malondialdehyde (TBA-MDA). Absorbance at 440, 532 and 600 nm was measured, and MDA content was calculated and expressed as nmoL/g fresh tissue. For quantification of H_2O_2, 50 mg of leaves tissues were homogenized in 50 mM phosphate buffer (pH = 6.5) and was measured by the FOX1 (ferrous oxidation-xylenol orange) method[17]. NADPH oxidase was assayed according to Sarath *et al*.[18], measuring NADPH-dependent super-oxide generation as the reduction rate of NBT into monoformazan (ε530= 12.8 $mM^{-1}cm^{-1}$)

Determination of antioxidants levels

Reduced ascorbate (ASC) and reduced glutathione (GSH) were quantified by HPLC (Reversed-Phase HPLC of Shimadzu, Hai Zhonglu, Shanghai) following

the methodology described by Sinha *et al*.[19]. Leaf tissue was homogenized under liquid nitrogen using a mortar and pestle. The resulting powder was thawed on ice in a 6% metaphosphoric acid (MPA) solution (0.5 mL MPA/100 mg wet tissue). After homogenization, the samples were centrifuged at 14000 rpm for 12 min. About 100 µL of the supernatant was added to 300 µL eluent (2 mM KCl, pH 2.5). The antioxidants were separated on HPLC by injecting 10 µL onto a Polaris C18-A column with a 1.0 mL/min flow rate[20].

Enzyme assay

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione-s-transferase (GST), dehydroascorbate reductase (DHAR) and (MDHAR) were extracted in 50-mM potassium phosphate buffer (pH 7.0) containing 10% polyvinyl pyrrolidone (PVP), 0.25% Triton X-100, 0.001M polymethyl sulfonyl fluoride (PMSF) and 0.001M ascorbate. SOD activity was determined according to Dhindsa *et al*.[21] by measuring the inhibition of nitroblue tetrazolium (NBT) reduction. CAT activity was assayed according to Aebi[22] by monitoring the rate of decomposition of H_2O_2 at 240 nm. APX, DHAR and GR activities were measured according to Murshed *et al*.[23]. GST enzyme activity was measured by the conjugation of GSH with excess 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm. GPX activity was assayed by measuring the decrease in NADPH absorbance (340 nm)[24]. The soluble protein content was estimated according to Lowry *et al*.[25].

Statistical analyses

Variance of data was analysed with the GLM procedure of SAS software for a Randomized Complete Block design with eight replicates. Where applicable, means were separated by Duncan's Test ($P \leq 0.05$).

Results

Growth and photosynthetic reactions

Thyme plants grown in open-field showed less decrease by 0.09% in total dry wt. (TDW) as compared to those grown in shade-enclosure system after a short term salt treatment (Table 1) and this reduction rises to 30% after 4 weeks. In shade-enclosure system, salinity treatment resulted in significant reduction in TDW by 11 and 31% compared to unstressed plants at 50 and 150 mM NaCl, respectively. In open-field, it was 13 and 34%, at 50 and 150 mM NaCl, respectively. After 4 weeks, the reduction in TDW decreased between control and NaCl treated plants. In addition, our result showed that the net photosynthetic was higher under open-field conditions with (1.5 and 4 µmolCO_2, after 15 and 30 days, respectively) compared to shade enclosure conditions. Whereas, the high salinity decreased net photosynthetic rate (*A*), stomatal conductance (g_s) and transpiration rate (*E*) by 34, 46 and 34%, respectively after 15 days of treatment in plants grown under shade enclosure conditions. While the reduction in photosynthetic parameters in stressed plants was less pronounced under open-field conditions (about 50%). Likewise, at 30 day, the less decrease in *g*s and *E* was observed in leaves of plant grown in open-field system. In contrast, a high drop in photosynthesis (74%) in plants grown under the same growth condition, as compared to control.

The applied growth environments significantly affected chlorophyll contents. The chlorophyll content was higher in plants grown under shade enclosure conditions than open-field conditions (Fig. 1). Short term of mild salinity stress (50 mM NaCl) reduced Chl a, Chl b and total chlorophyll contents in plant grown under shade enclosure by 66, 33 and 50%, respectively. In contrast, Chla, Chlb and total chlorophyll raised by

Table 1 — Total Dry Weight (TDW), Relative Water content (RWC), Water potential (Ψw), Osmotic potential (Ψs), turgor potential (Ψp), Photosynthetic rate (*A*), Stomatal conductance (gs) and transpiration rate (*E*) of *Thymus vulgaris* leaves as influenced by Shade enclosure, Open-field and different NaCl levels

	2 weeks						4 weeks					
	Shade enclosure			Open-field			Shade enclosure			Open field		
NaCl (mM)	0	50	150	0	50	150	0	50	150	0	50	150
Total dry wt. (g)	41.23^a	36.46^b	28.24^c	37.51^a	32.43^a	24.55^b	47.3^a	41.8^b	28.1^c	37.5^a	33.7^a	26.4^b
RWC (%)	95.5^a	89.9^a	87.4^b	88.8^a	88.1^a	86.8^a	95.5^a	89.9^a	87.4^b	88.8^a	88.1^a	86.8^a
Ψw (MPa)	-0.016^a	-0.05^b	-0.08^c	-0.09^a	-0.16^b	-0.19^c	-0.01^a	-0.05^b	-0.08^c	-0.09^a	-0.16^b	-0.19^b
Ψs (MPa)	-1.24^a	-1.78^b	-1.84^b	-1.63^a	-1.86^b	-2.17^c	-1.24^a	-1.78^b	-1.84^b	$-.63^a$	-1.86^b	-2.17^c
Ψp (MPa	1.28^b	1.72^a	1.77^a	1.54^c	1.86^b	1.98^a	1.28^b	$1,.56^a$	1.66^a	1.70^c	1.85^b	2.07^a
A (mol Co_2m^{-2} s^{-1})	1.38^a	0.80^b	0.91^b	$1,59^a$	$1,13^b$	0.95^c	2.14^a	1.87^b	1.78^b	3.99^a	1.46^b	1.02^c
gs (mmol H_2O m^{-2} s^{-1})	0.155^b	0.0086^a	0.0083^a	0.011^a	0.0086^b	0.0081^b	0.0077^a	0.0041^b	0.002^b	0.0085^a	0.0025^c	0.0066^b
E (mmol H_2O m^{-2} s^1)	0.91^b	0.682^a	0.596^a	0.531^a	0.430^a	0.272^b	0.271^a	0.242^{ab}	0.221^b	0.891^b	0.682^a	0.596^a

[Different letters indicate significant differences between treatments (Duncan test, $P \leq 0.05$]

30, 12 and 25%, respectively in response to mild salinity stress in plants grown in open-field, as compared system. Additionally, a different pattern was observed for mild and severe salinity treatments under shade enclosure growth conditions, with a decisive, reduction at the last time point (at 30 day) in severe

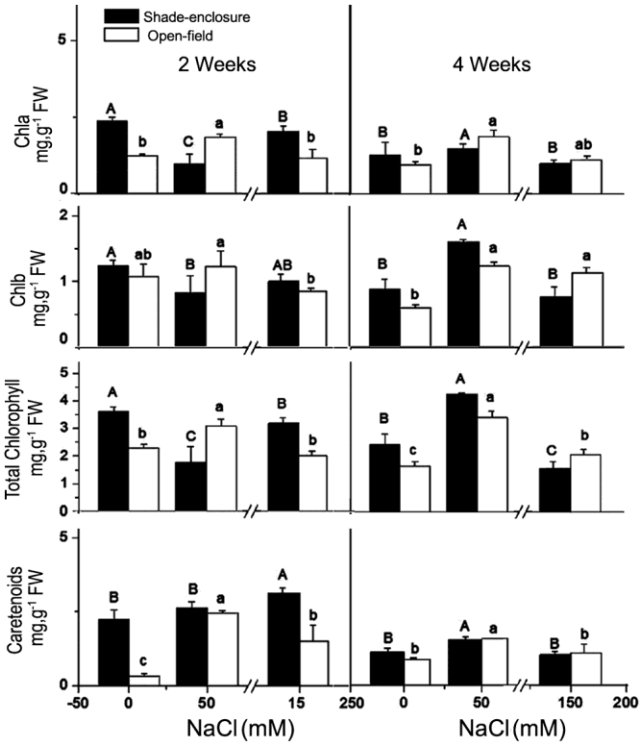

Fig. 1 — Effect of Shade enclosure, Open-field and different NaCl levels on leaf pigments in leaves of *T. vulgaris*. [Values are the means ± SE of eight replicates. Different letters indicate significant differences between treatments according to Duncan's Multiple Range Test at $P \leq 0.05$]

stressed plants compared to control. After 2 weeks, total carotenoid content increased more significantly in response to severe stress under shade enclosure growth conditions. While the amount rose to the higher level at mild salinity stress in open-field, thereafter it decreased by severe stress, but still higher as compared to control. Furthermore, after a long term of salinity exposure, total carotenoids increase only at moderate stress under both environmental conditions.

Mineral contents and water relations

Furthermore, the full sun light in open growth decreased the RWC, potentials (Ψ_w, Ψ_s and Ψ_p) and this decrease was less in thyme plants grown under shade enclosure. Water (Ψ_w) and osmotic potential (Ψ_s) were relatively lower in stressed thyme plants grown in open-field for long term than stressed thyme plants grown in shade enclosure system. In contrast, the turgor potential (Ψ_p) was relatively higher in thyme plants grown in open field system and exposed to salinity stress for 4 weeks (Table 1).

Salinity stress increased Na^+ concentration and decreased noticeably K^+, Ca^{2+}, Fe^{2+} and Mg^{2+} concentrations in thyme leaves grown in shade-enclosure system after 15 days (Table 2). Thereafter, the reduction in K^+, Ca^{2+}, Fe^{2+} and Mg^{2+} contents were getting less in leaves of thyme plants grown under open-field conditions. However, after 30 days, the Na^+ content was higher by 72% at 150 mM NaCl under open-field condition as compared to plants grown in shade-enclosure. Moreover, Fe^{2+}content declined sharply by 78% in response to severe salinity (150 mM NaCl) in plants grown under shade-enclosure

Table 2 — Ion concentrations in the leaves of thyme plants grown in Shade enclosure or Open field and fed with increasing concentrations of NaCl (50 and 150 mM NaCl)

		2 Weeks		4 Weeks	
	Treatment	Shade enclosure	Open field	Shade enclosure	Open field
	0	326,20±14.9b	1162,44±211,8c	385,80±35,7c	966,37±222,5c
Na	50	364,48±5,1b	2566,81±328,2b	1410,62±22,9b	1190,39±105,2b
(µeq.g-1DW)	150	474,06±131,9a	3873,79±62,9a	6495,19±372,3a	3573,50±1342,1a
	0	535,03±13,0a	660,10±40,1a	443,09±43,9a	594,25±7,3a
K	50	253,87±88,6b	645,65±10,9a	383,89±16,5b	399,87±24,9b
(µeq.g-1DW)	150	197,76±42,5c	464,71±69,9b	349,74±31,9b	347,95±15,6b
	0	4408,95±99,84a	972,42±10,64a	1087,10±10,27a	1002,75±196,63a
Ca	50	1303,22±266,09b	580,98±153,10b	850,51±0,73b	895,43±87,80b
(µeq.g-1DW)	150	1178,06±171,56b	360,49±17,49c	796,61±46,22b	788,00±105,04b
	0	995,68± 63,11a	701,85± 58,68a	1028,81± 246,41a	595,47± 10,08a
Mg	50	677,16± 98,20b	694,44± 52,63b	575,93± 19,56b	591,15± 3,83a
(µeq.g-1DW)	150	410,43± 94,92c	556,38± 39,93c	462,80± 5,40b	432,92± 20,16b
	0	1783,75±213,10a	844,80±97,47a	781,27±28,36a	208,78±4,57a
Fe	50	1159,32±160,42ab	815,77±195,47a	204,84±4,92b	99,64±4,39b
(µeq.g-1DW)	150	564,96±124,96b	549,55±0,97b	165,41±2,63c	35,75±2,55c

[Values are the means ± SE of eight replicates. Different letters indicate significant differences between treatments (Duncan test)]

conditions after 30 days and then it raised to 82% under open-field growth conditions. Whereas, the decrease in Ca^{2+} leaf content did not rise more than 30% in both environmental conditions. Sever salinity stress decreased the K^+ (41%) and Mg^{2+} (55%) in plants grown in open-field and in shade enclosure, respectively.

Carbohydrate contents

After 2 weeks of treatments, the concentration of glucose increased by mild and severe salinity stress by 55% and 53%, respectively, under shade-enclosure growth conditions (Fig. 2). While, under open field, both stress levels exhibited pronounced decrease in the glucose content as compared to control. After 4 weeks, the amount of glucose increased sharply especially in plants grown in open field. After a short-term exposure to salinity, mild stress did not affect the fructose content under both environment growth conditions. However, severe stress decreased fructose concentration in shade enclosure, while it was increasing in open field. However, after 4 weeks, both salt levels significantly increased the concentration of fructose in leaves of plants gown in shade-enclosure system but were lower than open-field growth conditions. We observed that the salinity stress did not significantly affect sucrose content after 2 weeks of the treatment in leaves of plants grown in shade enclosure system, but it had increased in those grown in open field by14 and 20% for mild and severe salinity stress, respectively. After 30 days of treatment, salinity had no effect on sucrose content. The concentration of starch increased sharply by severe stress (80%, compared to control) in shade enclosure system, while, it decreased in open field system. After 4 weeks, the starch leaf content decreased with increasing salinity level in both environmental conditions.

Oxidative stress markers

The malondialdehyde (MDA) content is routinely used as a measure of membrane lipid peroxidation and of membrane injury (Fig. 3). The plants treated with salinity stress showed a progressive decrease of MDA, after 2 weeks of treatment while it increased at open-field. Whereas, after 4 weeks of treatments, mild salinity stress treatment led to a gradual increase in the MDA levels under shade enclosure growth conditions, which raised to 28% at severe stress. However, under open-field condition, mild stress had no effect on MDA, and it was increased at 150 mM NaCl. In shade enclosure system, a concomitant increase in H_2O_2 content was recorded for mild stressed-plants (Fig. 3) and then decreased by 150 mM NaCl after 2 weeks. H_2O_2 content decreased in plant grown in open field system regardless the level salinity stress. After 4 weeks, H_2O_2 content increased at 150 mM NaCl in plants grown in shade enclosure and at open-field.

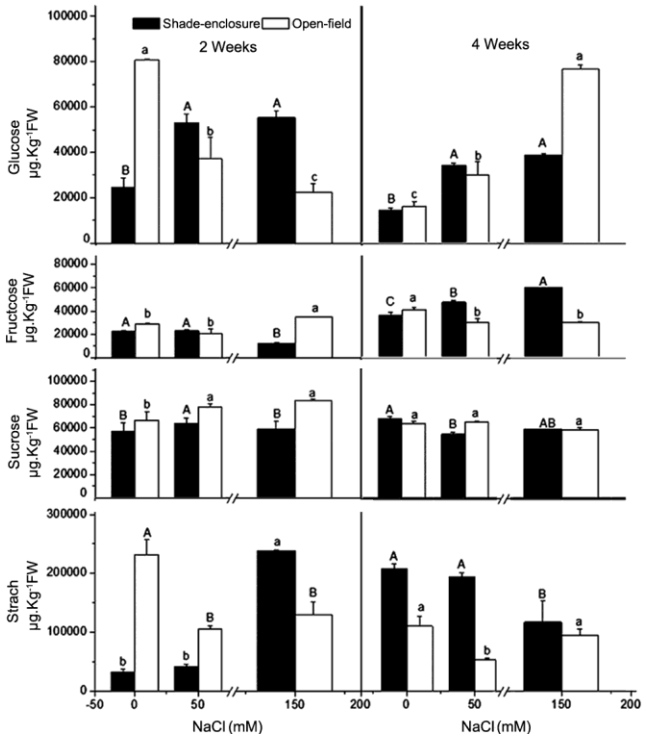

Fig. 2 — Effect of Shade enclosure, O*P*en-field and different NaCl levels on carbohydrate content in leaves of *T. vulgaris*. [Values are the means ± SE of eight re*P*licates. Different letters indicate significant differences between treatments according to Duncan's Multi*P*le Range Test at $P \leq 0.05$]

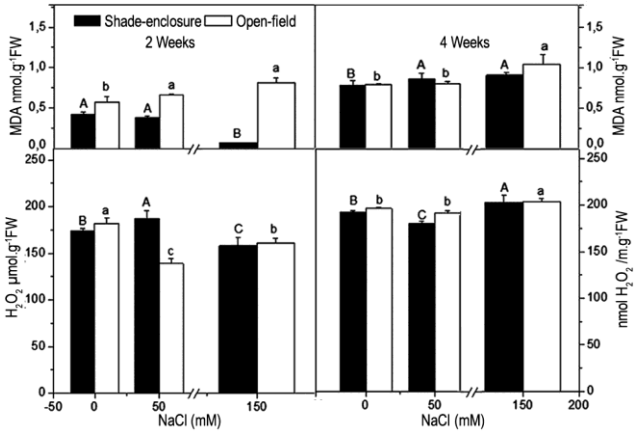

Fig. 3 — Effect of shade enclosure, O*P*en-field and different NaCl levels on MDA and H_2O_2 content in leaves of *T. vulgaris*. [Values are the means ± SE of eight re*P*licates. Different letters indicate significant differences between treatments according to Duncan's Multi*P*le Range Test at $P \leq 0.05$]

Changes in ROS-scavenging antioxidant enzyme activity

The enzyme activities displayed significant fluctuations in response to salinity and environment conditions and showed an obvious time-dependent change. In shade enclosure, mild stress imposed high level of SOD enzyme activity and it increased by two folds after 2 weeks (Fig. 4), relative to that of control. While, after 4weeks, the activity of SOD decreased with salinity as compared to control. Though the induction at enzyme level was relatively less in sunny conditions as compared to shade enclosure, a much profound increase in SOD activity at severe stress level was observed. Thus, the activity of SOD showed a further increase relative to what in shade enclosure. Similarly, after 2 weeks, the activity of CAT was higher in plants grown in shade enclosure system than in those grown under sunny conditions. More increase in CAT enzyme activity was observed at severe stress level. In contrast, open field conditions markedly increased the activity of CAT enzyme in severe stressed plants more than shade enclosure condition (4 folds, as compared to untreated samples) after 4 weeks (Fig. 4). Likewise, the activity of POX was time-dependent change (Fig. 4). Compared to shade conditions, sunny conditions reduced the activity of POX enzymes regardless the level of salinity. $NAPH_{OX}$ oxidase increased significantly by 50 mM NaCl under shade enclosure conditions and in sunny conditions. After 4 weeks of treatment, the activity of $NAPH_{OX}$ oxidase increased with increasing salinity (36 and 78%, for mild and severe stress, respectively) in shade enclosure system, but no effect of stress was observed in thyme plants grown in open field system (Fig. 5)

Ascorbate-glutathione related enzymes

The APX activity decreased by NaCl stress in short term under shade enclosure system, relative to what in control. While, APX showed the highest increase by 35% in 50 mM NaCl in long term (Fig. 5). The 150 mM NaCl treated plants grown under open filed exhibited higher activity of APX compared to those grown in shade enclosure.

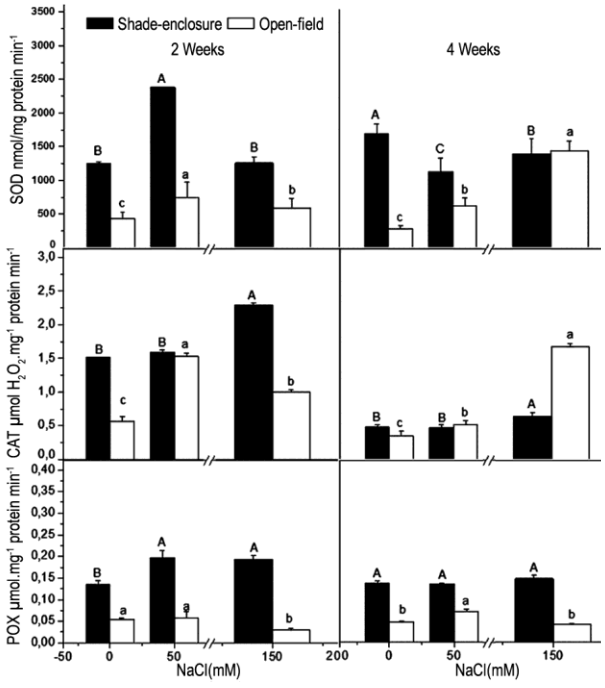

Fig. 4 — Effect of shade enclosure, O*P*en-field and different NaCl levels on SOD, CAT and *P*OX activity in leaves of *T. vulgaris*. [Values are the means ± SE of eight re*P*licates. Different letters indicate significant differences between treatments according to Duncan's Multi*P*le Range Test at $P \leq 0.05$]

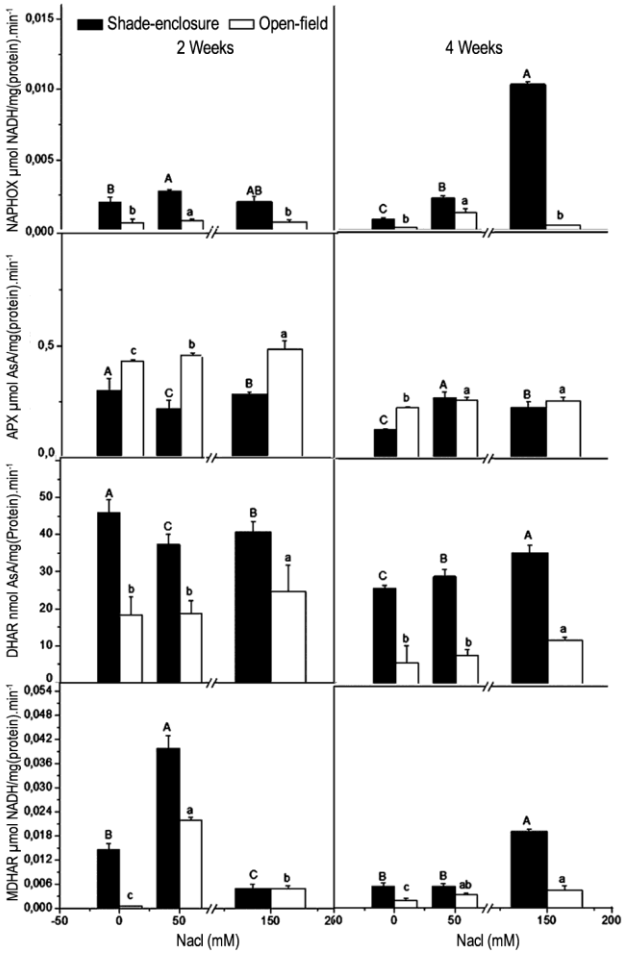

Fig. 5 — Effect of shade enclosure, O*P*en-field and different NaCl levels on $NAPH_{ox}$, A*P*X, DHAR and MADHAR activity in leaves of *T. vulgaris*. [Values are the means ± SE of eight re*P*licates. Different letters indicate significant differences between treatments according to Duncan's Multi*P*le Range Test at $P \leq 0.05$]

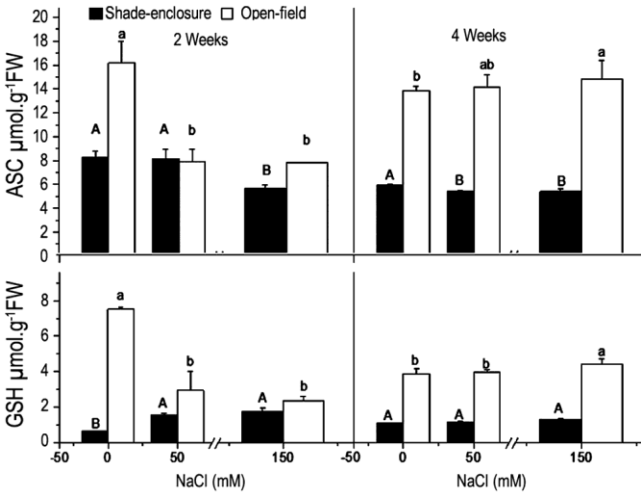

Fig.6 — Effect of shade enclosure, O*P*en-field and different NaCl levels on G*P*X, GR and GST activity in leaves of *T. vulgaris*. [Values are the means ± SE of eight re*P*licates. Different letters indicate significant differences between treatments according to Duncan's Multi*P*le Range Test at $P \leq 0.05$]

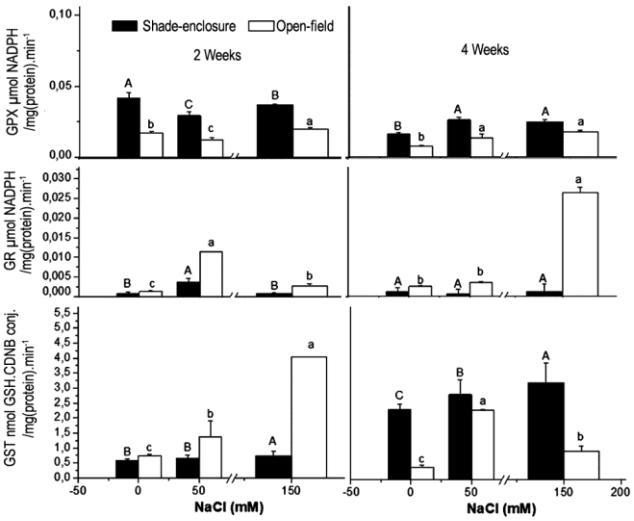

Fig7— Effect of shade enclosure, O*P*en-field and different NaCl levels on AsA and GSH content in leaves of *T. vulgaris*. [Values are the means ± SE of eight re*P*licates. Different letters indicate significant differences between treatments according to Duncan's Multi*P*le Range Test at $P \leq 0.05$]

In shade enclosure system, stressed thyme plants exhibited a higher activity of DHAR and MADHAR enzymes compared to those grown in open field system. After 2 weeks, DHAR enzyme activity decreased with the increase in salinity under shade enclosure, but it increased in severe stressed plants by sunny growth condition. Short-term exposure of mild stress also increased sharply the activity of MDHAR by 2 and 4 folds in shade enclosure and in sunny conditions, respectively. Furthermore, after 4 weeks, severe salt stress increased the activity of DHAR and MDHAR enzymes mainly in shade enclosure (Fig. 5).

Sunny growth conditions decreased the GPX enzyme activity independent on applied salinity stress (Fig. 6). At short term, 150 mM NaCl increased the activity of GPX enzyme in both environmental conditions, as compared to control. After long term, the highest level of NaCl increased the activity of GPX by 50 and 60% under shade enclosure and open field, respectively, relative to control. Moreover, the activity of GR was high under open field compared to shade enclosure. This activity was increased sharply in sunny conditions by 4 folds at 150 mM NaCl relative to what in control plants. Furthermore, the activity of GST enzyme was higher in sunny conditions at short term of treatment, thereafter; it decreased at 30 days of treatment relative to what in plants grown under shade enclosure (Fig. 6). Our data showed that the high salinity (150 mM) affected the activity of GST enzyme in shade enclosure system after 15 days. This activity was increased sharply in sunny conditions by 2 and 4 folds at 50 and 150 mM NaCl, respectively. In contrast, after 4 weeks, GST enzyme activity increased with the increase in salinity stress in thyme leaves grown under shade enclosure, but it reduced by severe stress in sunny conditions.

Effect on non-enzymatic antioxidants

The variation in light intensity elicited distinctive non-enzymatic responses of ascorbate (ASA) and glutathione (GSH) (Fig. 7) in thyme leaves. For instance, the plants that experienced full sunlight exhibited a higher ASA and GSH content than plants grown in shade enclosure. Furthermore, the salinity effect was depending on time course of experiment, since after short term, salinity decreased the ASA content and increased the GSH content under shade enclosure. After 4 weeks, the sunny conditions further increased the contents of ASA (62%) and GSH (78%) of the 150 mM salt-treated plants when compared to plants grown in shade enclosure.

Discussion

Compared to shaded leaves, sunny leaves showed less growth under stress condition

The combination of multiple environmental factors including light and salinity stress alters plant growth and metabolism although under cultivated conditions. The acclimation of cultivated plants under such conditions at short and long term is not well studied[1]. According to our data, the total dry weight was not

significantly affected by high light relative to plants grown under shade enclosure without salt treatment. Under open field conditions, certain plant species do not grow because light irradiance decreases photosynthetic rates. Therefore, all plants have their own optimal light intensity ranges for growth. Light intensity that is too high or too low impacts morphology, photosynthetic physiology, and secondary metabolite production. These characteristics are closely related to medicinal plant productivity such as *T. vulgaris*. Salt stress is one of the major environmental factors which limit thyme growth and production[6]. According to our data, 50 mM NaCl and 150 mM induced moderate (11%) and severe stress (31%) effects, respectively, on thyme plants biomass under shade enclosure growth condition. Interestingly, only after 2 weeks, severe salinity stress reduced the TDW of sunny thyme leaves more than shaded ones. In general, this reduction in plant growth could be an adaptive response to stress[26]. At fresh weight (FW) levels, stressed plants also showed less FW due to low water accumulation[27]. Reduced TDW of salt stressed leaves exposed to sunny conditions was related to photosynthetic assimilation. Photosynthesis is a major process in plant dry matter production[27]. Furthermore, the results of the present study showed that the *A* decreased under salt stress condition (Table 1). Before the salt treatment, the leaf photosynthetic parameters *A*, gs and *E* showed significant responses to low light under shade enclosure. Apparently, *A* and gs were decreased in plants grown under shade enclosure relative to those grown in open field. Whereas, *E* was lower in shade enclosure than in open field at 15 and 30 days. Our results showed that the thyme plants receiving shade treatment exhibited impaired photosynthetic capacity due to reduce *A* and *gs*. Under salt stress, the *A* declined further under sunny conditions and showed a further decrease after 4 weeks. Under such conditions, the treated plants are predisposed to suffer photoinhibition.

Salt stress increased influx of Na^+ which could damage chlorophyll content suggested that reduced rates of photosynthesis may be due to reduced levels of Chl, particularly Chla[3]. Moreover, low g_s in stressed plants reduced the availability of CO_2 at its fixation site[30]. The decrease in *A* in internal CO_2 concentration and finally inhibition of photosynthesis metabolism are generally attributed to stomatal limitation. This conclusion is consistent with the present study, where the *gs* values were lower in open-field than shade enclosure. When plants are exposed to high irradiation associated with a high vapour pressure deficit, plants show reduced g_s to prevent water loss, which results in decreased intercellular CO_2 concentration and depression of photosynthesis. Reduced photosynthesis increased oxidative stress when absorbed light energy exceeds energy use by metabolism. Impairment of photosynthetic electron utilization, especially when Calvin–Benson cycle is slowed down by stomata closure, is a major cause of this stress[31]. Decreases in Chl b content have been suggested to be an indication of Chlorophyll destruction by excess irradiance[31]. Plants grown under shaded conditions are known to optimize their effectiveness of light absorption by increasing pigment density per unit leaf area[32]. Chl b is usually the main component of light harvesting chlorophyll protein, therefore the marked increase in Chl b content in stressed and shaded leaves after a long-term exposure mild stress demonstrated the thyme could maximize the light harvesting capacity[32].

Under salinity, the increase of carotenoids indicated their potential to detoxify accumulated reactive oxygen species[33]. In the present study, it appeared that the salinity interacts with high light at short and long term of treatment enhanced the antioxidant role of carotenoids. Carotenoids are known to function as collectors of light energy for photosynthesis and as quenchers of triplet chlorophyll and O_2. Moreover, they dissipate excess energy via xanthophylls cycle and can act as powerful chloroplast membrane stabilizers that partition between light harvesting complexes (LHCs) and the lipid phase of thylakoid membranes, reducing membrane fluidity and susceptibility to lipid peroxidation[34]. Because the photosynthetic energy utilization in sunny growth species is lower than in shade species, it was assumed that higher antioxidant protection would be necessary to compensate for higher light-mediated oxidative stress[35].

Osmotic adjustment was a mechanism to adapt salinity stress in sunny and shaded leaves

To avoid water loss, our stressed plants decreased g_s, *E* and Ψ_w similar to other stressed species as was reported in the study of Tounekti *et al*.[36], however in our study, salinity did not affect RWC value in short term, highlighting an effective osmotic adjustment, as previously stated in rosemary plants[36]. Also, the large decrease in Ψ_w and Ψ_s under salt stress with few associated changes in tissue hydration has been considered as an adaptive strategy of the evergreen sclerophyllous trees and shrubs to stress. In agreement

with our results, it was demonstrated that the shade condition maintained suitable leaf turgor in stressed plants mainly after a short term of salt stress exposure[37].

Negative effects of stress on shaded plant leaves growth after a short period of treatments could be explained by osmotic effects and ionic imbalances resulting from nutritional deficiency cations[38]. The osmotic effects of salinity stress can reduce growth by inhibiting cell expansion and cell division. Plants accumulated higher levels of compatible solutes to improve salt resistance[38]. Soluble sugars, including sucrose, glucose and fructose, have been reported as compatible solutes, playing an important role in osmoregulation and as well as scavengers of free radicals during salinity stress. In the salt-stressed thyme plants, these solutes could largely contribute to the osmotic adjustment and ionic balance control[38]. Here, we propose that the induced sugars levels such as fructose, glucose, sucrose and starch allowed the stressed plants to keep a favourable water relation under shade enclosure. Besides, our results showed that the g_s and E were declined in response to high light and salinity levels. In fact, the highest decrease in both photosynthetic parameters was recorded at 150 mM under sunny growth conditions after a long term of treatment. Stomata give plants the ability to regulate transpiration but also the opportunity to control water use efficiency (transpiration efficiency)[38].

Shaded plants experienced less oxidative damages and more antioxidants level

The common biochemical response during both biotic and abiotic stress is enhanced ROS production[39]. Rapid and nonspecific reactions of ROS result in severe cell biochemical changes including lipid peroxidation and damage to proteins and DNA, which may lead to cell death. Salt stress is known to cause lipid peroxidation in several species, which has often been used as an indicator of stress induced damage at the cellular level[39]. Under shade enclosure, thyme plants can withstand severe salt stress without showing any hint of oxidative stress. It is worth noting that chlorophyll loss is an efficient photoprotection mechanism that finely modulates the amount of light intercepted by leaves[40]. Therefore, it is not surprising that the highest MDA accumulation observed in 150 mM NaCl treated plants in sunny conditions concomitant with lower A and chlorophyll. In any case, it becomes clear that with high MDA accumulation, thyme plants can withstand this salt-induced oxidative stress, by activating several antioxidants enzymes. Because the photosynthetic energy utilization in shade plants is lower than sunny species, it was assumed that higher antioxidant protection would be necessary to compensate for higher light mediated oxidative stress[33]. There are many potential sources of H_2O_2 in plant cells, including chloroplasts, mitochondria, peroxisomes, plasma membrane NADPH oxidase, cell wall peroxidases, apoplastic oxalate, and amine oxidase[41]. The plasma membrane located NADPH oxidase is mainly involved in H_2O_2 production in response to salt stress[41]. Our findings indicate that exposure of thyme to moderate salinity in shade enclosure, induced H_2O_2 accumulation in concomitance with higher MDA. To maintain a relatively low ROS concentration, plants have evolved highly regulated enzymatic and non-enzymatic mechanisms to keep a balance between ROS production and detoxification so that the cellular redox homeostasis can be maintained[42]. Increased levels of antioxidant enzymes activity in plant tissues as a mechanism of salt-adaptive trait has been reported[43]. Thus, by neutralizing the harmful effects of H_2O_2, SOD and CAT regulate the steady-state level of H_2O_2, thereby preventing the formation of extremely toxic hydroxyl radicals[44]. The decrease in H_2O_2 concentration in salt-treated plants was associated with an increase of CAT and SOD, likely as a consequence of the direct effect of NaCl or the indirect effect mediated via an increase in levels of H_2O_2. As POX has much stronger affinity for H_2O_2 than CAT, so it is believed that POX is associated with H_2O_2 scavenging under lower concentration, while the *en masse* H_2O_2 scavenging is facilitated by CAT[45]. It was reported that POX activity plays a major role in the scavenging of H_2O_2 at relatively low concentration and acted as fine regulator for ROS balance[45].

The results of this study reveal that in thyme plants, the POX activity was induced only under shade enclosure which concomitant with lower concentration of H_2O_2. While in sunny conditions, the activity of this enzyme declined at high salt level. Hence, it seems the thyme plants were able to induce an efficient antioxidant response protecting the photosynthesis of shaded plants after short term exposure to salinity. We assumed that the thyme exposed to full sunny condition will be exposed to high temperature than under shade enclosure. Previous study reported that SOD, CAT and POX activities decreased significantly at high temperatures in light, whereas non-enzymatic defences

increased[30], as ASC was observed in our study. This result suggested a possibility of direct ROS scavenging role of AsA and GHS in thyme grown high light through their synthesis by APX and GR, respectively. The ASC-GSH cycle plays a key role in this defence, and the components of this cycle are present in different organelles in plant cells[35]. One of these components is ascorbate ASC, a non-enzymatic antioxidant, which associated with H_2O_2 scavenging via APX[35].

In addition, GSH plays a key role in the antioxidative defence system in response to salinity. APX, GR, GSH, and AsA in ascorbate-glutathione pathway mediate removal of H_2O_2 by transferring electrons from NADPH to H_2O_2, utilizing GSH and AsA as mobile redox buffers. GSH and AsA are involved in many cellular processes, including their key roles in plant tolerance and in activation of enzymes involved in plant growth and development[44].

The outcomes of the present investigations showed that ASC and GSH concentrations in thyme grown in sunny conditions treatments were higher than plants grown under shade enclosure. We assumed that the decrease of H_2O_2 and MDA in sunny condition at long salt stress treatment is due to the important role of ASC and GSH. The ASC and GSH pool are generally determined by its rates of not only synthesis but also regeneration[43] and this depends on the different productions system in this work. According to our results, it appeared that in open field, the high pool of GSH content accumulated at short term was due to the high activity of GR induced by the high light. The high level of GSH helped in maintaining the levels of ascorbate by acting as reductant in dehydroascorbate reductase (DHAR) to ascorbate (AsA) as observed in the present outcome. Thus, in response to salinity, it is obvious that the amount of ASC was regenerated by DHAR under shad enclosure response to lower level of salt stress (50 mM NaCl). MDHAR and DHAR are two important enzymes related to the regeneration of ASA which are equally important in regulating AsA level and its redox state under oxidative stress condition. The findings of the present study suggested that improved GR activity in NaCl treated plants led to optimization in first time (2 weeks) of MDHAR by way of enhanced GSH production. Besides, 50 mM NaCl improved the maintenance of AsA by enhancing the activity of DHAR in plants grown under shade enclosure. Overexpression of MDHAR and DHAR has highlighted the roles of GSH-dependent ascorbate pools in responses such as stomatal regulation[23].

Interestingly, after a long term, AsA and GSH content was maintained regardless to their slight accumulation in shading and NaCl treated plants. In fact, a slight decrease in AsA content was recorded at 50 and 150 mM NaCl. Likewise, the content of GSH was not affected with the increase in salt stress in shading plants thyme. According to the present results, the thyme plants used the MDHAR and DHAR enzymes in the first line of defence against oxidative stress inducted by NaCl under shade enclosure by the regeneration of AsA at short term. Likewise, the NaCl treated plants involved both enzymes in salt tolerance by maintaining a high level of AsA and GSH at long term. After 4 weeks and in sunny conditions, the activity of APX and GR was enhanced and the content of AsA and GSH reached to its higher content mainly at highest level of salt stress (150 mM). It appeared that AsA are utilized by APX in ascorbate glutathione cycle to reduce H_20_2 to water. Likewise, increase in GSH concentration was further observed, which might have contributed to efficient H_20_2 scavenging, lowering lipid peroxidation through increased activity of GR and maintaining the optimal concentrations of GSH.

In the present investigation, we suggested that APX and GR activity were induced by the high salinity and this increase was enhanced by high light. Nevertheless, the high activity of GST in the current treatment appeared to be involved in catalysing the conjugation of GSH to a wide variety of hydrophobic and electrophilic compounds to form less- or non-toxic peptide derivatives. This conjugation reaction is involved in the detoxification and processing of various xenobiotics but also endogenous toxic and nontoxic metabolites, which after glutathionation are rapidly transported to vacuole[44]. At the same time, high GPX activity at long term as response to salinity in shading plants suggested better tolerance of thyme by decomposition of H_2O_2 using GSH and converts to GSSG. Importantly, the light seemed to not improve the activity of GPX. Such activity was reduced by light at short and at long term which may be substituted by APX activity at this time. In the present investigation, we observed that thyme plants manipulate differently the enzymes of ascorbate-glutathione pathway depending on time, salinity and shade or light. Nevertheless, this study revealed that long-term changes in foliar enzymes activities correlate more

with salinity than light, indicating that salinity is an important factor modulating antioxidants.

Conclusion

This study has demonstrated that Thyme (*Thymus vulgaris*) adapted differently to the shade enclosure and open-field with or without salinity. The tolerance of *T. vulgaris* to moderate and severe salinity stress was dependent on the concurrent light regime. Under shade enclosure, improved salt tolerance can be associated with marked osmotic adjustment by accumulating a higher level of compatible solutes. The results obtained suggest that the variation in environmental light conditions similarly modulated the activity of antioxidant enzyme and non-enzymatic activities. Maintaining a high level of antioxidative enzyme activities, in short and long term, may have contributed to better protection mechanisms, and to better growth under salt stress. Interestingly, it appeared that the high light enhanced the useful to the growers and gardeners to select best growth condition to maintain a good quality product of thyme plants, especially in the Mediterranean area, where low quality waters are very often used.

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Conflicts of interest

Authors have declared no conflict of interests.

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