Scientia Horticulturae 299 (2022) 111027

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Effect of exogenous application of salt stress and glutamic acid on lettuce (*Lactuca sativa* L.)

Giulia Franzoni [a], Giacomo Cocetta [a,*], Alice Trivellini [b], Christian Garabello [c], Valeria Contartese [c], Antonio Ferrante [a]

[a] Department of Agricultural and Environmental Sciences, Università degli Studi di Milano, via Celoria 2, Milano 20133, Italy
[b] Institute of Life Sciences, Scuola Superiore Sant'Anna, Piazza Martiri della Libertà 33, Pisa 56127, Italy
[c] Green Has Italia SpA, c.so Alba 85/89, Canale 12043 Italy

ARTICLE INFO

Keywords:
Abiotic stress
Amino acid
Antioxidant
Salinity
Superoxide dismutase

ABSTRACT

Salinity is a serious environmental issue which can negatively affect crop growth and productivity worldwide. Lettuce is generally considered as a salt-sensitive crop; however, different cultivars may have different adaptive mechanisms to this environmental stress. The application of biostimulants has proven to be a strategic strategy to improve plant responses to abiotic stresses and to foster resilience of crops during cultivation. This study intended to explore the physiological mechanisms underlying Romaine lettuce plant responses to salt stress, also in combination with the exogenous application of glutamic acid. The glutamic acid treatment was applied as foliar spray for the first time before salt exposure, followed by three applications during the stress. To understand the effect of salinity and glutamic acid treatment, different physiological and molecular analytical determinations were performed. High salinity induced a general stimulation of PSII and chlorophyll content. In particular, the performance index (+102%) and the number of reaction centres per cross section (+75,7%) increased, whereas the energy dissipation as heat per reaction centres (-32,1%) and the net rate of the centres' closure (Mo) (-39.4%) decreased. Moreover, a reduction of yield (-26,5%) was observed in plants grown under high salinity. The concentration of proline was stimulated by salinity whereas ABA levels were reduced. The analyses of the genes encoding for ROS scavenging enzymes showed a general downregulation in response to salinity with the only exception of *LsSOD*. The application of the glutamic acid did not show a clear effect of the amino acid on lettuce plants, regardless the different growing conditions.

1. Introduction

Among environmental stressors, salinity is one of the most detrimental factors leading to severe losses in crops yield and quality (Aslam et al., 2017; Grieve et al., 2011). According to FAO (2015) more than 100 countries are affected by soil salinization and their extent is estimated at about 1 billion ha, worldwide. Several research studies report a constant increase in soil salinization due to both natural causes and improper agricultural practices (Adhikari et al., 2019; Annunziata et al., 2017; Aroca et al., 2013; Freitas et al., 2019; Molina-Montenegro et al., 2020). In addition to seawater intrusion, agriculture in coastal regions is further exacerbated by salt spray and salt deposition produced by saline aerosols during storms or high winds (Ferrante et al., 2011; Grieve et al., 2011). For these reasons, crop productivity is seriously jeopardized in Mediterranean areas, where more than 40% of soils are affected by salinity (Colla et al., 2010; Miceli et al., 2003; Nedjimi, 2014). Vegetables are generally considered more susceptible than staple crops to stressful environmental conditions, including salinity (Shahbaz et al., 2012; Shannon and Grieve, 1998) and the level of salt in the vegetable areas of cultivation, is generally higher than salt tolerance threshold levels (Colla et al., 2010).

Salt stress can alter the plant's physiological processes, altering

Abbreviations: ABA, Abscisic Acid; APX, Ascorbate Peroxidase; AsA, Ascorbic Acid; CAT, Catalase; DHAR, Dehydroascorbate Reductase; FW, Fresh weight; GA, Glutamic Acid; GR, Glutathione reductase; GSH, Glutathione; MDHAR, Monodehydroascorbate Reductase; PSII, Photosystem II; ROS, Reactive Oxygen Species; SOD, Superoxide Dismutase.

* Corresponding author.
E-mail addresses: giulia.franzoni@unimi.it (G. Franzoni), giacomo.cocetta@unimi.it (G. Cocetta), alice.trivellini@gmail.com (A. Trivellini), c.garabello@greenhasitalia.com (C. Garabello), v.contartese@greenhasitalia.com (V. Contartese), antonio.ferrante@unimi.it (A. Ferrante).

https://doi.org/10.1016/j.scienta.2022.111027
Received 28 September 2021; Received in revised form 21 February 2022; Accepted 23 February 2022
Available online 7 March 2022

photosynthesis and respiration, impairing protein biosynthesis, phytohormones regulation, determining nutrient imbalance, and damaging cell organelles (Arif et al., 2020; Shahid et al., 2020; Yu et al., 2020). The negative effects of salt stress can be divided in two phases: the osmotic phase and the ion toxicity phase (Isayenkov and Maathuis, 2019). Moreover, salt stress can lead to the overproduction and accumulation of reactive oxygen species (ROS) in cells, causing oxidative damage to nucleic acids, lipids, and proteins (Das and Roychoudhury, 2014). Stomata closure during the osmotic phase limits the CO_2 uptake and results in the production of ROS such as superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) at chloroplasts level. Photorespiration increases and promotes electron leakage that stimulate ROS production, too. Likewise, Na^+ and Cl^- toxicity affect the electron transport chain, also leading to ROS overproduction. Negative effects of salt stress are often connected to damages in different sections of the photosynthetic apparatus (Mehta et al., 2010). Plants react to high salinity in different ways and at different levels: by accumulating compatible solutes and osmolytes such as proline, glycine betaine, sugars, and other low weight molecules, to avoid ion toxicity, maintain water uptake, and protect plants from excessive ROS accumulation (Chen and Jiang, 2010; Shahbaz et al., 2012). In addition, plants can reduce the excess of ROS through the action of enzymatic and non-enzymatic protective mechanisms. Enzymes with antioxidant ability include superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), ascorbate peroxidase (APX; EC 1.11.1.11), and glutathione reductase (GR; EC 1.8.1.7), while glutathione (GSH) and ascorbic acid (AsA) are the main non-enzymatic antioxidants, followed by carotenoids, tocopherols, and phenolic compounds. AsA and GSH are the substrates involved in the ascorbate-glutathione cycle, allowing the detoxification from H_2O_2 through a series of reactions, involving APX, MDHAR, DHAR, and GR (You and Chan, 2015).

The effect of salinity on crops depends on several factors such as the level of salt concentration, the duration of the exposition, the plant phenological stage and the genotype. These aspects vary among species and varieties of a given crop (Machado and Serralheiro, 2017; Xu and Mou, 2015).

Lettuce (*Lactuca sativa* L.) is considered as a moderately salt sensitive crop with a threshold limit to 1.3 dSm^{-1} (Shannon and Grieve, 1998). Among leafy vegetables, lettuce is one of the most important species cultivated in the Mediterranean area. Spain, Italy, and France are the producers of lettuce in the Mediterranean basin, reaching a production of about 2.2 million tonnes in 2019 (FAOSTAT).

The application of biostimulant products containing a single amino acid or a combination of amino acids has been shown to have beneficial effects on plant growth and quality, in particular under adverse environmental conditions (Alfosea-Simón et al., 2020; Botta, 2012; Matysiak et al., 2020; Rai, 2002; SH SADAK et al., 2014). In plants, amino acids are a source of nitrogen, they are a constituent of proteins and precursors of several metabolites involved in plant growth and in plant eco-physiological interactions, such as pigments (Cho et al., 2009), vitamins (Asensi-Fabado and Munné-Bosch, 2010), secondary metabolites, and phytohormones (Westfall et al., 2013). Amino acids can act as osmolytes, regulating stomatal closure and ion transport (Rai, 2002). Glutamic acid plays a key role in plant defense, it is a precursor of proline, and it takes part in the biosynthesis of chlorophyll (Schön et al., 1986).

The present study aims to investigate the responses of lettuce plants subjected to salinity and the efficacy of the application of a glutamic acid solution in counteracting the negative effects of salt stress exposure.

The chlorophyll content and chlorophyll *a* fluorescence have been measured to assess the impact of salinity on the physiological status and quality of lettuce. Nitrate, proline, and osmolytes levels have been measured since they are considered as biochemical markers of plants responses to stress. Moreover, the expression of some of the key genes encoding for the enzymes involved in ROS scavenging has been analysed to underline the molecular responses induced by salt exposure and glutamic acid.

2. Materials and methods

2.1. Plant material, treatments and experimental plan

Two-week old Romaine lettuce (*Lactuca sativa* var. 'longifolia') plantlets (5-6 leaves) were supplied from a local nursery. A total of 36 plants was transplanted into 2.5 L plastic pots filled with a commercial peat substrate. The experiment was carried out in a greenhouse at the Faculty of Agricultural and Food Science of Milan from April 5 to May 14, 2018. The temperature inside the greenhouse (24 ± 2 °C) and the relative humidity level (79 ± 12%) were maintained by a fan-pad cooling system.

The combination of two factors (salinity and glutamic acid (GA) treatment) each of them with two levels were evaluated in the experiment. After 1 week from the transplant, salinity was imposed by administrating 300 mL of a saline solution (100 mM NaCl) to a group of plants while tap water was dispensed to the other group (control). Irrigation was carried out in order to maintain a constant soil moisture in control plants. Treatments (10 mL) were applied by foliar spray 5 days after the transplant, every ten days for a total of four applications. Treatments consisted of water and a GA solution (2 mM).

Lettuce plants were harvested at commercial maturity stage (40 days after transplant). Non-destructive analyses were conducted the same day just before the harvest. Fresh weight (FW) was determined by cutting the plants at soil level and considering the whole lettuce head. Fresh leaf tissue was sampled and stored at −20 °C until used for biochemical analyses.

Leaf tissues were collected 3 and 6 h after the last treatment and stored at −80 °C until used for gene expression analyses.

2.2. Non-destructive analyses

2.2.1. In vivo chlorophyll estimation

A rapid and direct estimation of chlorophyll in lettuce leaves was performed using the portable chlorophyll content meter CL-01 (Hansatech Instruments, UK). The instrument estimates the chlorophyll content on the basis of the absorbance at 620 and 940 nm. The results are expressed as chlorophyll index (relative units).

2.2.2. Chlorophyll a fluorescence

The hand-portable fluorometer (Handy-PEA, Hansatech Instruments, UK) was used to measure the chlorophyll *a* fluorescence *in vivo*. The leaves were dark-adapted with leaf clips (4 mm diameter) for 30 min before the measurement. Afterwards, an array of three high-intensity light-emitting diodes produces a saturating light (3000 μmol m^{-2} s^{-1}) for 1 s that hits leaf tissues. JIP-test equations were applied to obtain derived parameters providing information about the photosynthetic machinery status (Table S1).

2.3. Analytical determinations

2.3.1. Abscisic acid

The concentration of abscisic acid (ABA) was determined by an indirect enzyme linked immuno-sorbent assay (ELISA) (Vernieri et al., 1989). Approximately 1 g of leaf tissue was homogenized (mortar and pestle) with 3 mL of distilled water. The mixture was centrifuged at 4000 rpm for 15 min at RT, the supernatant was collected and analysed using the Plant Growth Regulator Immunoassay Detection Kits (Sigma-Aldrich) according to manufacturer instructions.

2.3.2. Nitrate

The Cataldo's method (Cataldo et al., 1975) was used to measure the concentration of nitrate in lettuce leaves. Leaf samples were grinded

(mortar and pestle) with 3 mL of distilled water per gram of fresh tissue. The homogenate was centrifuged at 4000 rpm for 15 min at RT and the recovered supernatant was used for the colorimetric analysis. About 80 μL of 5% (w/v) salicylic acid in concentrated H_2SO_4 (SA- H_2SO_4) were placed in a tube and 20 μL of the plant extract were added. Tubes were stirred in order to mix the components. Finally, 3 mL of NaOH (1.5 N) was added and the samples were left on the bench to cool down to RT. The absorbance was measured at 410 nm with a spectrophotometer. The concentration of nitrate was calculated using a KNO_3 standard calibration curve as reference. Results are expressed as mg of NO_3-N per kg of FW.

2.3.3. Osmolytes

The same extract used for the determination of nitrate was analysed. The osmolarity of the extracts was measured using an automatic freezing point depression osmometer (Digital Osmometer, Roebling, Berlin, Germany) calibrated with sodium chloride solutions.

2.3.4. Proline

The ninhydrin-based colorimetric assay improved by Bates et al. (1973) was used to determine the content of proline. Approximately 1 g of leaf tissue was homogenized (mortar and pestle) with 10 mL of 3% sulfosalicylic acid. Samples were centrifuged at 4000 rpm for 5 min at RT. A reaction mixture was prepared with 3% sulfosalicylic acid (100 μL), glacial acetic acid (200 μL) and acidic ninhydrin (200 μL) and posed in a tube. Afterwards, 100 μL of the extract was added to the mixture. The tubes were stirred, and each lid was punctured with a needle to avoid high pressure during the incubation at 96 °C for 60 min in a water bath. The reaction was terminated by putting the tubes on ice. Finally, 1 mL of toluene was added to the mixture. The tubes were vortexed to mix the components and then let them rest for 5 min to allow the separation between the organic and water phases. The chromophore phase was read at 520 nm using toluene as reference. Proline concentration was calculated using a standard calibration curve and results are expressed as μg per g FW.

2.3.5. Total thiols

The concentration of total thiols in lettuce leaves was determined by Leão et al. (2014) method. About 0.5 g of leaf tissue was grinded with mortar and pestle with 6 mL of a reaction solution containing 0.1 M Tris-HCl buffer (pH 8.0), 1 mM EDTA, and 1% ascorbic acid. Samples were centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was collected and 1.5 mL potassium phosphate buffer (0.2 M, pH 8.2), 0.1 mL Ellman's reagent (0.01 M), and 7.9 mL of methanol were added. After 15 min of reaction at 37 °C, the absorbance at 412 nm was determined. Total thiols concentration was calculated using a molar extinction coefficient of 13,100 M^{-1} cm^{-1}.

2.4. Total RNA isolation and analysis of gene expression

Frozen leaves of lettuce were thoroughly grinded with liquid N in a cold mortar. Approximately 100 mg of powder was collected in a cryotube and stored at −80 °C. The Spectrum Plant Total RNA Kit (Sigma-Aldrich, Italy) was used to isolate total RNA. The absorbance of RNA samples at 230, 260 and 280 nm using a NanoDrop N-1000 spectrophotometer (NanoDrop Technologies) was used to assess RNA concentration and purity. The 260/280 ratio around 2.0 is generally accepted and expected 260/230 values are commonly in the range of 2.0–2.2, usually higher than the respective 260/280 value. Reverse transcription was carried out using the SuperScript IV cDNA Synthesis Kit according to the manufacturer's instruction (Invitrogen, Italy) and the SYBR® Green (Applied Biosystems) qPCR protocol was applied for the quantitative RT-PCR analysis. The reaction mix was prepared by adding 10 μL of SYBR Green, 0.4 μL of forward and reverse primers, 2 μL of cDNA diluted 1:20, and 7.2 μL of RNase free water. The total volume for each PCR reaction was 20 μL. Analysis was performed using the ABI7300 (Applied Biosystem) thermocycler and PCR program. Reactions were run in triplicate from two biological replicates. Gene expression analyses were assessed using gene-specific primers for: superoxide dismutase [Fe] 3, chloroplastic (*SOD* XM_023880725.1), catalase (*CAT* XM_023874935.1), L-ascorbate peroxidase 6, chloroplastic/mitochondrial (*APX* XM_023891707.1), monodehydroascorbate reductase, chloroplastic/mitochondrial (*MDHAR* XM_023896983.1), dehydroascorbate reductase (*DHAR* AB158512.1), glutathione reductase, chloroplastic (*GR* XM_023877582.1). Chloroplastic isoform of each gene has been chosen to focus the attention on the photosynthetic apparatus. Primers for these genes were designed using the program Primer-Blast (www.ncbi.nlm.nih.gov) (Table S2). Applied Biosystem software was used to analyze the gene expression and results were calculated using the 2^{-ddct} method described by Livak and Schmittgen (2001). According to this method, the data are presented as fold change in gene expression normalized to a housekeeping gene and relative to a calibrator. The Elongation factor 1 alpha (EF1α) was used as housekeeping due to the highest stability in its expression levels, whereas the non-stressed and non-treated sample after 3 h was chosen as internal calibrator.

2.5. Statistical analyses

Data were subjected to ANOVA and Tukey post-test ($P < 0.05$) was used to assess the differences among means. Analyses were performed using GraphPad Prism version 8 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Additional information is reported in each figure and table's caption.

3. Results

3.1. Growth, chlorophyll in vivo and chlorophyll a fluorescence

Lettuce yield was calculated considering the fresh weight of the entire head at harvest and a density of 10 plants m^{-2}. The two-way ANOVA showed that the interaction between salinity and treatment was not significant ($p < 0.05$). However, considering the effect of each factor, the stress condition shown a significant effect on plants growth for $p < 0.0001$, whereas the treatment did not affect the production in a significant way. The application of high salt solution induced a decrease (−26.5%) in lettuce fresh weight. In particular, the average yields were about 679 g m^{-2} and 499 g m^{-2} in plants grown under control and stressful conditions, respectively (Fig. 1A).

The levels of chlorophyll measured *in vivo* were not affected by the application of the glutamic acid solution, whereas the chlorophyll content measured in plants subjected to salt stress were significantly higher ($p < 0.0001$) if compared with those grown under control condition, regardless the treatment. Chlorophyll concentrations in lettuce plants grown under high salinity were about 2.0 points higher than those measured under control condition (Fig. 1B).

The graph of chlorophyll *a* fluorescence parameters (2) shows the

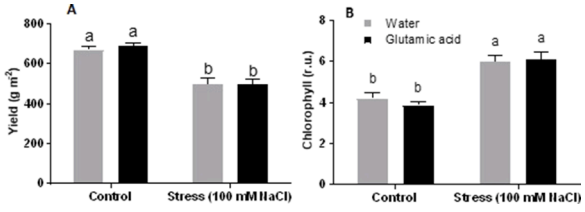

Fig. 1. Yield (A) and chlorophyll content (B) of lettuce plants grown under non-stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Measures were taken at the end of the growing cycle. Values are means ± SE (yield: $n = 6$; chlorophyll content: $n = 30$). Data were subjected to two-way ANOVA and Tukey's multiple comparison test was used for evaluating the differences among means. Different letters, where present, represent significant differences ($p < 0.05$).

overall response of photosynthesis to salinity and glutamic acid application. In this chart, parameters' values are normalized to 0 -gray solid line representing the non-stressed and non-treated plants. Salt stress strongly affected a great number of parameters, as shown by the distance of the circle and square symbols from the reference gray line. On the contrary, the treatment with glutamic acid did not induce any strong modification in the trends.

Salt stress induced an up-regulation of PSII function, as shown by the variation of several parameters. The ANOVA results for fluorescence parameters are shown in Table S3. A significant increase (+102%) in the performance index (PI) was observed in plants grown under salinity, regardless the treatment. In particular, the lowest and the highest values were measured in control (1.39) and stressed (3.14) plants treated with water, respectively.

Furthermore, the density of PSII active reaction centres at t_0 (RC/CSo) and at t_{max}, (RC/CSm) significantly increased by +68.0% and +75.7% in stressed plants. Similarly, the electron transport flux per cross section (ETo/CS) (+51.5%), and the energy needed to close all reaction centres (Sm) (+32.5%) were higher in stressed samples compared to control ones. A significant interaction between stress and treatment has been shown in Sm values. At the same time, salt exposition induced a significant decrease in the energy dissipation as heat per reaction centres (DIo/RC) (−32.1%), in the absorbed energy flux per reaction centres (ABS/RC) (−27.3%), in the trapped energy flux per reaction centres (TRo/RC) (−26.5%), and in the net rate of the centres' closure (Mo) (−39.4%).

On the contrary, salt stress and glutamic acid treatment did not affect the minimal fluorescence (Fo), maximal fluorescence (Fm), variable fluorescence (Fv) and maximum quantum efficiency of PSII (Fv/Fm) in a significant way. The Fv/Fm values in both growing conditions were about 0.86.

Fig. 2

3.2. Nitrate

The nitrate concentration in lettuce leaves was significantly ($p < 0.05$) affected by the salt stress. In particular, the lowest level was measured in untreated plants grown under high salinity (Fig. 3). However, no significant differences emerged comparing the treatments,

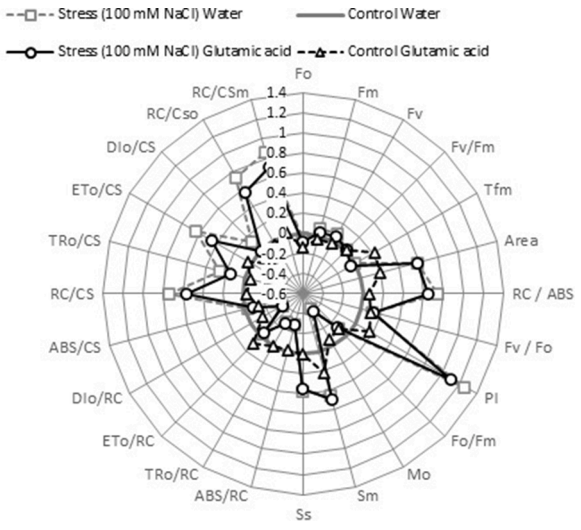

Fig. 2. Chlorophyll *a* fluorescence parameters of lettuce plants grown under non stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Measures were taken at harvest. Values are means ± SE ($n = 6$). (Ft – Fcw)/Fcw, was used to normalize the data. In the formula, "Ft" and "Fcw" represent the values of treated plants and control plants treated with water, respectively. Values of "Fcw" plants were normalized to 0 (control plants treated with water, gray circle = 0).

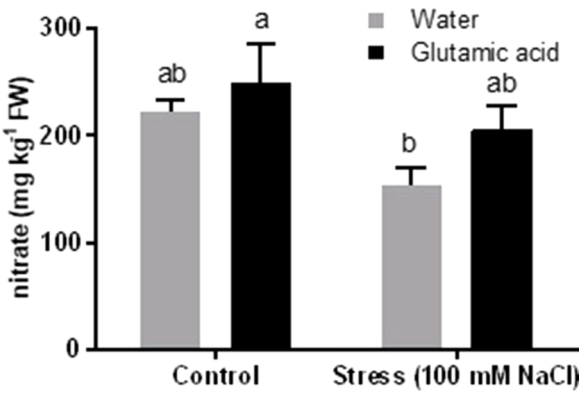

Fig. 3. Nitrate content measured in lettuce leaves under non stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Measures were taken at the end of the growing cycle. Values are means ± SE ($n = 6$). Data were subjected to two-way ANOVA and Tukey's multiple comparison test was used for evaluating the differences among means. Different letters, where present, represent significant differences ($P < 0.05$).

except between the control plants treated with the glutamic acid solution and the stressed plants treated with water. In general, nitrate concentration values ranged from 115 mg kg^{-1} FW and 409 mg kg^{-1} FW.

3.3. Proline, osmolytes, and abscisic acid

Proline levels were measured in order to assess its potential role in defining lettuce tolerance to NaCl in combination with glutamic acid treatment. Without salt, lettuce plants contained the same amount of proline in leaves, regardless the treatment (Table 1). Salt stress significantly ($p < 0.05$) affected the levels of proline and osmolytes in lettuce leaves (Table 1). In particular, the proline average value was 11.8 μg g^{-1} in plants grown under non-stressful condition and about 63.9 μg g^{-1} in stressed plants. A significant difference was observed between control and stressed plants treated with water. However, the high variability did not allow to see any significant effect of the glutamic acid treatment in stressed samples.

Salinity induced a significant increase (+ 52.2%) of osmolytes concentration in plants treated with glutamic acid, whereas no significant difference was observed in plants treated with water, as reported in Table 1.

Likewise, a significant ($p < 0.05$) effect of the salt stress resulted in the concentration of abscisic acid in lettuces leaves. ABA levels were generally low in plants grown under high salinity compared to those grown under control condition. In particular, salt stress induced a

Table 1
Proline, osmolytes and abscisic acid concentration in lettuce leaves under non stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Measures were taken at the end of the growing cycle. Values are means ± SE ($n = 6$). Data were subjected to two-way ANOVA and Tukey's multiple comparison test was used for evaluating the differences among means. Different letters, where present, represent significant differences ($P < 0.05$).

Stress	Treatment	Proline(μg g^{-1} FW)	Osmolytes(mOsm kg^{-1} g^{-1} FW)	Abscisic acid (ng g^{-1} FW)
CONTROL	WATER	12.5 ± 1.5 b	0.208 ± 0.021 ab	288.5 ± 117.8 ab
	GLUTAMIC ACID	11.1 ± 1.4 b	0.184 ± 0.025 b	417.3 ± 170.4 a
STRESS	WATER	44.8 ± 5.9 a	0.244 ± 0.019 ab	176.3 ± 72.3 b
	GLUTAMIC ACID	37.9 ± 8.0 a	0.280 ± 0.022 a	182.0 ± 74.3 b

significant decrease (- 56%) in plants treated with the glutamic acid solution while it had no significant effect on non-treated plants (Table 1). Similar to those observed in proline concentration, the high variability of the results reduced the statistical power.

3.4. Total thiols

The two-way ANOVA showed a significant ($p < 0.05$) effect of salinity on thiols concentration in lettuce leaves (Fig. 4). A slight decrease in their accumulation has been observed in plants treated with the glutamic acid solution and grown under non-stressful condition. On the contrary, the same treatment did not induce any modification in stressed plants since the thiols concentration in control plants treated with glutamic acid was already lower than that observed in plants treated with water and grown under the same condition. In particular, the average value of total thiols measured in plants exposed to salinity was about −43.7% if compared with control samples treated with water.

3.5. Expression analyses of LsSOD, LsCAT, LsAPX, LsMDHAR, LsDHAR, and LsGR genes

The changes in the expression of the genes involved in antioxidant defense system have been clustered into a heat map (Fig. 5). The figure shows that the expression levels of *LsCAT, LsAPX, LsMDHAR, and LsDHAR* were strongly downregulated by the salt stress, especially after 3 h. Different trends resulted in response to salt stress, treatments and during the time. Under control condition the expression levels of the genes were similar between plants treated with water and plants treated with the glutamic acid solution, both after 3 and 6 h. On the contrary, salt stress induced a general down-regulation of the genes, except for *LsSOD*, as shown by the color shades in the heat map. The expression values for each gene are reported in Fig. 6. A strong decrease was observed, especially in the transcripts level of *LsCAT, LsAPX, and LsMDHAR* after 3 h (Fig. 6B–D). At the same timepoint the expression of *LsSOD* increased in non-stressed plants, whereas the glutamic acid application did not induce any change if compared with the control. A threefold increase was measured in *LsSOD* transcripts of plants treated with glutamic acid only after 6 h (Fig. 6A).

4. Discussion

Salinity affects plant growth, development, and quality by altering several physiological and chemical processes. It represents a serious problem for crop productivity, especially in the Mediterranean regions.

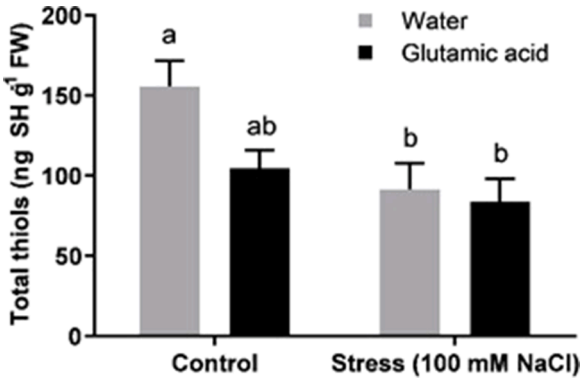

Fig. 4. Total thiols concentration in lettuce leaves grown under non stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Measures were taken at the end of the growing cycle. Values are means ± SE ($n = 12$). Data were subjected to two-way ANOVA and Tukey's multiple comparison test was used for evaluating the differences among means. Different letters, where present, represent significant differences ($P < 0.05$).

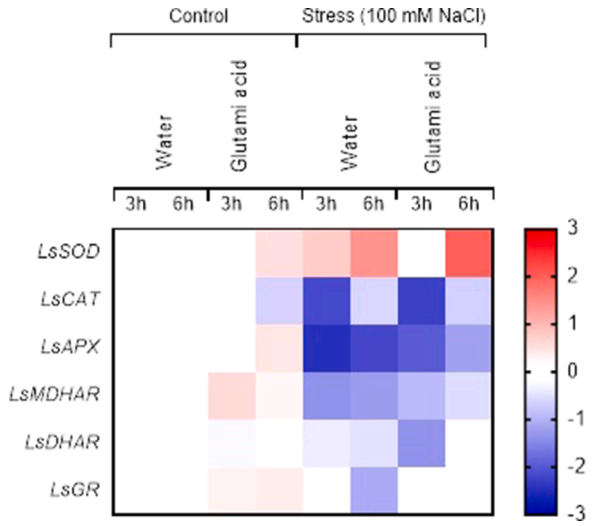

Fig. 5. Heatmap showing temporal expression of selected genes in lettuce plants grown under non stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Data represent the log2FC of the selected genes. The rows are the genes, and within each row the blue shaded areas indicate lower expression, whereas the red shaded areas indicate higher expression. No differences were visualized by white squares.

In these regions, the levels of EC in irrigation water, are often above the threshold tolerated by crops (Miceli et al., 2003; Xu and Mou, 2016). The severity of salinity stress is also exacerbated by high temperature and low water availability, especially, during summer.

Several approaches have been used to increase plant growth and productivity under abiotic stresses. An important strategy is represented by breeding programmes, aiming to improve crop stress tolerance. Unfortunately, the selection of new tolerant genotypes through genetic approaches, is a long-term process. Another approach is the induction of salt tolerance through the exogenous application of different bioactive molecules. The application of amino acids alone or in a combination with other molecules and products containing amino acids, has been considered as a strategy to face salt stress (Alfosea-Simón et al., 2020; SH Sadak et al., 2014). It has been observed that the application of a plant-derived protein hydrolyzate increased the fresh yield, dry biomass and plant performance, in lettuce grown under salinity conditions (NaCl 25 mM), probably due to the stimulation of root growth (Lucini et al., 2015). Interesting results have been observed after the application of glutamic acid in lentil seedlings and under salt stress (Fardus et al., 2021). The different effect observed by the authors, if compared to our experiment, might be related to the choice of the experimental plan and the plant species. The authors applied a higher concentration of glutamic acid (10 mM) to lentil seedling, while in this experiment the concentration applied to lettuce plants was 2 mM. We can thus hypothesize that, in this species, the glutamic acid treatment was not enough to induce a marked response due to both the concentration, and the phenological stage. Moreover, the lack of effect observed in response to the glutamic acid treatment could be due to the severity of the salt stress condition imposed in our experiment, where the NaCl concentration in the nutrient solution was 100 mM, much higher than the level tested in the paper mentioned before (Lucini et al., 2015).

In the present experiment the yield was significantly affected by the high salinity of the growing media. Lettuce yield response to the salt level of nutrient solution was in agreement with previous findings (Al-Maskri et al., 2010) and consistent with the stunted growth phenotype due to the reduced ability of plants exposed to high salinity levels to absorb water from the growing media. Moreover, the low yield of lettuce grown under salt stress conditions could be due to a decreased ability of stressed plants to uptake nutrients from the substrate.

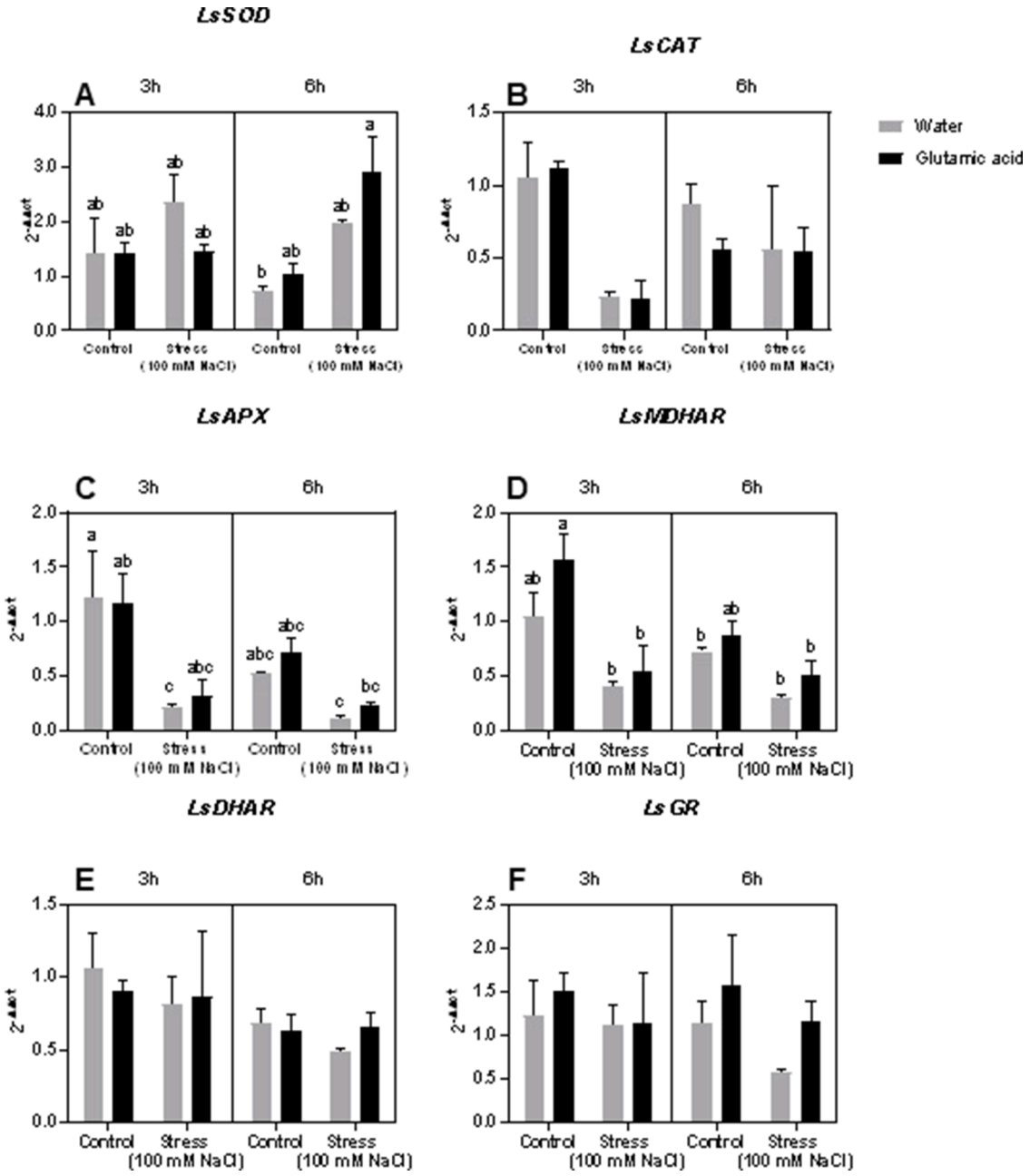

Fig. 6. Expression levels of *LsSOD* (A), *LsCAT* (B), *LsAPX* (C), *LsMDHAR* (D), *LsDHAR* (E), *LsGR* (F) in lettuce leaves grown under non stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Measures were taken at harvest. Values are means ± SE ($n = 6$). Data were subjected to three-way ANOVA and Tukey's multiple comparison post-test was used for evaluating the differences among means. Different letters, where present, represent significant differences ($P < 0.05$).

Chlorophyll fluorescence can be used as a non-invasive indicator of the physiological status of the plant photosynthetic function. The level of chlorophyll measured *in vivo* and the PI increased in lettuce plants grown under high salinity condition. The PI is an indicator of the sample vitality and the increase observed in lettuce leaves is probably linked to the increase of the amount of the photosynthetic reaction centres (RC/ABS) measured in the same samples. Moreover, the Fv/Fm ratio was not significantly affected by high salt treatment, in accordance with the observation of Xu and Mou (2015) and Adhikari et al. (2019). Since the decrease of Fv/Fm usually suggests damages of PSII blocking the electron transport, the stressful condition imposed by this study did not inhibit the electron flow of PSII (Shu et al., 2013). Additionally, an increase in the electron transport flux (ETo/CS), in the Area, and a decrease in the energy dissipation (DIo/RC) was observed. Similar results were reported in *Cucumis* sp., *Salvinia auriculate, Dunaliella salina,* and rice subjected to different levels of salt stress (Asch et al., 2000; Gomes et al., 2017; Kuşvuran et al., 2008; Sedjati et al., 2019). Likewise, the increase of the PI in response to salinity stress due to an increase of the efficiency of primary photochemistry and photochemical efficiency of photosynthetic electron transport associated with a decreased DIo/RC was observed in one hybrid of *Brassica napus* (Bacarin et al., 2011). The measurement of chlorophyll *in vivo* correlates the green color of the leaves with the content of chlorophyll. It is well established that chlorophyll *a* represents the main pigment involved in the photosynthetic activity, whereas chlorophyll *b* act as accessory pigment. Moreover, chlorophyll *a* absorbs energy wavelengths of blue-violet and orange-red light and it is responsible for the green color of the leaves while chlorophyll *b* absorbs energy wavelengths of green light. An increase in

chlorophyll *a* and a decrease in chlorophyll *b* content in response to salt stress was observed (Gomes et al., 2017). This is in line with other studies reporting that salt stress affects more chlorophyll *b* than chlorophyll *a* (Houimli et al., 2010). Moreover, since the first step in the degradation of chlorophyll *b* is its conversion in chlorophyll *a* (Fang et al., 1998), this might explain the high levels of greenness measured in lettuce leaves in our experimental conditions.

Nitrate concentration is an indicator of leafy vegetables quality and the maximum level permitted for the commercialization is determined by the EC regulation 1258/2011. The concentration of nitrate in lettuce leaves significantly decreased in plants grown under high salinity. This effect has been reported also by other authors and it may be due to the inhibition of nitrate absorption, and to a reduction in the nitrate reductase activity (Meloni et al., 2004; Scuderi et al., 2009; Shimomachi et al., 2008). The reduction of nitrate uptake in plants growing under salt stress conditions could also be related to the decrease of water absorption or to the high level of chloride, which could lead to a reduced nitrate accumulation (Abdelgadir et al., 2005; Miceli et al., 2003). The competitive and dynamic interaction between nitrate and chloride is related to their comparable properties at physical and osmotic levels. Moreover, the two anions share ion transport mechanisms with dubious selectivity (Rosales et al., 2020). This antagonism might explain the reduced content of nitrate in plants, as observed in the present work.

The NaCl stress induced a significant increase of proline levels in lettuce leaves. This is a common response of plants subjected to salt stress, as reported by several studies (Agarwal and Pandey, 2004; Eraslan et al., 2007; Jimenez-Bremont et al., 2006; Karabal et al., 2003; Santander et al., 2020). It is known that soil salinization leads to a decrease of water uptake causing ion imbalance, ion toxicity, and osmotic stress. The accumulation of compatible solutes in the cytosol such as proline is a common plants response to withstand salt stress. High levels of proline are usually linked to a higher tolerance of plants to a stressful condition. Moreover, proline is accumulated, especially in leaves where it is involved in the protection of photosynthetic activity, maintaining the chlorophyll level and cell turgor (Silva-Ortega et al., 2008). In the present experiment, the highest levels of proline observed in lettuce plants subjected to salt stress might have contributed to the health status of the photosynthetic apparatus, as shown by the chlorophyll *a* fluorescence parameters. However, unlike proline trend, the osmolytes levels increased only in stressed plants treated with the glutamic acid solution. This could mean an involvement of proline in different mechanisms other than osmoregulation. Moreover, the glutamic acid is a common substrate in the biosynthesis of several amino acids and its application might have been stimulated the production/accumulation of amino acids, which in turn act as compatible osmolytes in plants (Forde and Lea, 2007). However, it has been reported that a high concentration of osmolytes is not always associated with a tolerance toward stress and it seems to be specific to a species or a particular growth condition or stage (Forni et al., 2017).

Abscisic acid plays a central role in plant responses to stress, both in the regulation of several gene expression and in the mechanism of stress signal transduction, and it usually increases in response to salt stress (Fricke et al., 2004; Sah et al., 2016; Zhang et al., 2006). In this experiment ABA content did not change in water-treated plants in response to salt stress while it decreased in plants treated with glutamic acid and subjected to high salinity. ABA levels measured in non-stressed and non-treated plants were in line to other experiments in lettuce leaves (Aroca et al., 2008). Lettuce plants may have been activated ABA-independent signaling responses to salt stress, for example the osmotic adjustment in order to restore the cellular homeostasis, as observed in the increase in osmolytes level in the same plants. So far there is no report of a direct link between glutamic acid and abscisic acid in plants under normal or stressful conditions.

Thiols are a group of molecules involved in plant responses to almost all stress factors, protecting the cell from oxidative stress and preventing the damage caused by ROS. Thiols take part in the non-enzymatic antioxidant defense system, protecting the plant cells from oxidative damages (Pivato et al., 2014). In most studies different thiols compounds increase in response to stressful conditions and it has been associated with stress tolerance (Zagorchev et al., 2013). The decreased concentration of total thiols observed in salt-stressed lettuce plants might be due to their conversion into other compounds or might indicate a toxic effect of salt stress on thiols metabolism.

The expression of the genes involved in the antioxidant defense system decreased under high salinity. The only exception was the expression of *LsSOD*. The SOD enzyme acts as a first line of defense to cope with ROS production, catalysing the reaction, transforming the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2). *LsSOD* gene expression was induced by salinity, especially after 6 h of stress and the treatment with glutamic acid amplified this response, as shown in Fig. 6A. These results are similar with other previously published (Kalhor et al., 2018; Santander et al., 2020) in lettuce plants. H_2O_2 has a versatile role in plants, acting as a signaling molecule at normal levels, or inducing oxidative damage at toxic concentrations. The enzymes APX and CAT are able to scavenge H_2O_2 through different mechanisms. In the present experiment, salinity induced a decrease in the *LsCAT* and *LsAPX* gene expression. This indicates that the H_2O_2 produced might not have reached toxic levels to induce *LsAPX* or *LsCAT* overexpression. Moreover, CAT is one of the major ROS scavenging enzymes in plants and considering a cause/effect relationship between CAT production and ROS concentration, it can be said that lower levels of CAT indicate lower levels of ROS, meaning a less oxidative stress and *vice versa* (Milne et al., 2012). An inhibition of CAT activity under stress condition has been reported also in other plants (Khedr, 2003; Kohler et al., 2009). At the same time, the increase of the activity of these enzymes in response to high salinity conditions has been observed (Shams et al., 2016). The expression of *LsMDHAR* showed the same trend of *LsAPX*. Both these enzymes are involved in the conversion and restore of AsA into monodehydroascorbate and *vice versa*. A general decrease in the expression of these genes was observed over time, probably due to a circadian regulation of their expression. Moreover, in this experiment, salinity caused a further decrease right after 3 h of stress. These observations, together with the lack of changes in the expression of *LsDHAR* and *LsGR* might indicate a minor involvement of ascorbate-glutathione cycle in plant response to stress and it is reasonable to hypothesize the involvement of detoxification mechanisms different from antioxidant enzymes.

5. Conclusion

Taken together, the results obtained in this experiment confirm that Romaine lettuce is a crop moderately tolerant to salt stress (De Pascale and Barbieri, 1995), in fact few stress responses were observed, both at physiological and molecular levels. However, it is important to consider that the sensitivity of lettuce to salinity may differ among cultivars. For example, Romaine lettuce was found more tolerant to NaCl than another variety by different authors (Nasri et al., 2011; Pasternak et al., 1986). The application of the glutamic acid did not show a strong effect on lettuce plants, neither under optimal nor under stressful condition. We might suppose that the lack of a clear response is related to the tolerance of this cultivar to the stressful condition tested in our experiment. Interestingly, the induction of *LsSOD* expression in response to salt stress and to the treatment with this amino acid solution might indicate a link between glutamic acid and this enzyme. A similar result, suggesting a connection between the glutamic acid and *LsSOD* was also observed in a previous work where glutamic acid was applied on lettuce plants subjected to a period of water deprivation (Franzoni et al., 2021). Further experiments aiming to clarify this aspect are necessary.

CRediT authorship contribution statement

Giulia Franzoni: Investigation, Formal analysis, Writing – original

draft. **Giacomo Cocetta:** Investigation, Writing – review & editing. **Alice Trivellini:** Investigation. **Christian Garabello:** Conceptualization. **Valeria Contartese:** Conceptualization. **Antonio Ferrante:** Conceptualization, Supervision, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2022.111027.

References

Abdelgadir, E.M., Oka, M., Fujiyama, H., 2005. Characteristics of nitrate uptake by plants under salinity. J. Plant Nutr. 28, 33–46. https://doi.org/10.1081/PLN-200042156.

Adhikari, N.D., Simko, I., Mou, B., 2019. Phenomic and physiological analysis of salinity effects on lettuce. Sensors 19. https://doi.org/10.3390/s19214814 (Switzerland).

Agarwal, S., Pandey, V., 2004. Antioxidant enzyme responses to NaCl stress in cassia angustifolia. Biol. Plant. 48, 555–560. https://doi.org/10.1023/B:BIOP.0000047152.07878.e7.

Alfosea-Simón, M., Zavala-Gonzalez, E.A., Camara-Zapata, J.M., Martínez-Nicolás, J.J., Simón, I., Simón-Grao, S., García-Sánchez, F., 2020. Effect of foliar application of amino acids on the salinity tolerance of tomato plants cultivated under hydroponic system. Sci. Hortic. 272, 109509 https://doi.org/10.1016/j.scienta.2020.109509.

Al-Maskri, A., Al-Kharusi, L., Al-Miqbali, H., Khan, M.M., 2010. Effects of salinity stress on growth of lettuce (Lactuca sativa) under closed-recycle nutrient film technique. Int. J. Agric. Biol. 12, 377–380.

Annunziata, M.G., Ciarmiello, L.F., Woodrow, P., Maximova, E., Fuggi, A., Carillo, P., 2017. Durum wheat roots adapt to salinity remodeling the cellular content of nitrogen metabolites and sucrose. Front. Plant Sci. 7, 1–16. https://doi.org/10.3389/fpls.2016.02035.

Arif, Y., Singh, P., Siddiqui, H., Bajguz, A., Hayat, S., 2020. Salinity induced physiological and biochemical changes in plants: an omic approach towards salt stress tolerance. Plant Physiol. Biochem. 156, 64–77. https://doi.org/10.1016/j.plaphy.2020.08.042.

Aroca, R., Ruiz-Lozano, J.M., Zamarreño, Á.M., Paz, J.A., García-Mina, J.M., Pozo, M.J., López-Ráez, J.A., 2013. Arbuscular mycorrhizal symbiosis influences strigolactone production under salinity and alleviates salt stress in lettuce plants. J. Plant Physiol. 170, 47–55. https://doi.org/10.1016/j.jplph.2012.08.020.

Aroca, R., Vernieri, P., Ruiz-Lozano, J.M., 2008. Mycorrhizal and non-mycorrhizal Lactuca sativa plants exhibit contrasting responses to exogenous ABA during drought stress and recovery. J. Exp. Bot. 59, 2029–2041. https://doi.org/10.1093/jxb/ern057.

Asch, F., Dingkuhn, M., Dorffling, K., 2000. Salinity increases CO_2 assimilation but reduces growth in field-grown, irrigated rice. Plant Soil 218. https://doi.org/10.1023/A:1014953504021.

Asensi-Fabado, M.A., Munné-Bosch, S., 2010. Vitamins in plants: occurrence, biosynthesis and antioxidant function. Trends Plant Sci. 15, 582–592. https://doi.org/10.1016/j.tplants.2010.07.003.

Aslam, M., Ahmad, K., Akhtar, M.A., Maqbool, M.A., 2017. Salinity stress in crop plants: effects of stress, tolerance mechanisms and breeding strategies for improvement. J. Agric. Basic Sci. 2, 70–85.

Bacarin, M.A., Deuner, S., da Silva, F.S.P., Cassol, D., Silva, D.M., 2011. Chlorophyll a fluorescence as indicative of the salt stress on Brassica napus L. Braz. J. Plant Physiol. 23, 245–253. https://doi.org/10.1590/S1677-04202011000400001.

Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39, 205–207. https://doi.org/10.1007/BF00018060.

Botta, A., 2012. Enhancing plant tolerance to temperature stress with amino acids: an approach to their mode of action. Acta Hortic. 1009, 29–36.

Cataldo, D.A., Maroon, M., Schrader, L.E., Youngs, V.L., 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun. Soil Sci. Plant Anal. 6, 71–80. https://doi.org/10.1080/00103627509366547.

Chen, H., Jiang, J.G., 2010. Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. Environ. Rev. 18, 309–319. https://doi.org/10.1139/A10-014.

Cho, J., Lee, E.J., Yoo, K.S., Lee, S.K., Patil, B.S., 2009. Identification of candidate amino acids involved in the formation of blue pigments in crushed garlic cloves (Allium sativum L.). J. Food Sci. 74, C11–C16. https://doi.org/10.1111/j.1750-3841.2008.00986.x.

Colla, G., Rouphael, Y., Leonardi, C., Bie, Z., 2010. Role of grafting in vegetable crops grown under saline conditions. Sci. Hortic. 127, 147–155. https://doi.org/10.1016/j.scienta.2010.08.004.

Das, K., Roychoudhury, A., 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front. Environ. Sci. 2, 1–13. https://doi.org/10.3389/fenvs.2014.00053.

De Pascale, S., Barbieri, G., 1995. Effects of soil salinity from long-term irrigation with saline-sodic water on yield and quality of winter vegetable crops. Sci. Hortic. 64, 145–157. https://doi.org/10.1016/0304-4238(95)00823-3.

Eraslan, F., Inal, A., Savasturk, O., Gunes, A., 2007. Changes in antioxidative system and membrane damage of lettuce in response to salinity and boron toxicity. Sci. Hortic. 114, 5–10. https://doi.org/10.1016/j.scienta.2007.05.002.

Fang, Z., Bouwkamp, J.C., Solomos, T., 1998. Chlorophyllase activities and chlorophyll degradation during leaf senescence in non-yellowing mutant and wild type of Phaseolus vulgaris L. J. Exp. Bot. 49, 503–510. https://doi.org/10.1093/jxb/49.320.503.

FAO, 2015. Intergovernmental Technical Panel on Soils. Status of the World's Soil Resources. Intergovernmental Technical Panel on Soils.

Fardus, J., Hossain, M.S., Fujita, M., 2021. Modulation of the antioxidant defense system by exogenous l-glutamic acid application enhances salt tolerance in lentil (Lens culinaris Medik.). Biomolecules 11, 587. https://doi.org/10.3390/biom11040587.

Ferrante, A., Trivellini, A., Malorgio, F., Carmassi, G., Vernieri, P., Serra, G., 2011. Effect of seawater aerosol on leaves of six plant species potentially useful for ornamental purposes in coastal areas. Sci. Hortic. 128, 332–341. https://doi.org/10.1016/j.scienta.2011.01.008.

Forde, B.G., Lea, P.J., 2007. Glutamate in plants: metabolism, regulation, and signalling. J. Exp. Bot. 58, 2339–2358. https://doi.org/10.1093/jxb/erm121.

Forni, C., Duca, D., Glick, B.R., 2017. Mechanisms of plant response to salt and drought stress and their alteration by rhizobacteria. Plant Soil 410, 335–356. https://doi.org/10.1007/s11104-016-3007-x.

Franzoni, G., Cocetta, G., Ferrante, A., 2021. Effect of glutamic acid foliar applications on lettuce under water stress. Physiol. Mol. Biol. Plants 27, 1059–1072. https://doi.org/10.1007/s12298-021-00984-6.

Freitas, W.E.D.S., De Oliveira, A.B., Mesquita, R.O., de Carvalho, H.H., Prisco, J.T., Gomes-Filho, E., 2019. Sulfur-induced salinity tolerance in lettuce is due to a better P and K uptake, lower Na/K ratio and an efficient antioxidative defense system. Sci. Hortic. 257, 108764 https://doi.org/10.1016/j.scienta.2019.108764.

Fricke, W., Akhiyarova, G., Veselov, D., Kudoyarova, G., 2004. Rapid and tissue-specific changes in ABA and in growth rate in response to salinity in barley leaves. J. Exp. Bot. 55, 1115–1123. https://doi.org/10.1093/jxb/erh117.

Gomes, M.A.D.C., Pestana, I.A., Santa-Catarina, C., Hauser-Davis, R.A., Suzuki, M.S., 2017. Salinity effects on photosynthetic pigments, proline, biomass and nitric oxide in Salvinia auriculata Aubl. Acta Limnol. Bras. 29 https://doi.org/10.1590/s2179-975X4716.

Grieve, C.M., Grattan, S.R., Maas, E.V., 2011. Plant Salt Tolerance, 2nd ed. Agricultural Salinity Assessment and Management. https://doi.org/10.1061/9780784411698.ch13.

Houimli, S.I.M., Denden, M., Mouhandes, B.D., 2010. Effects of 24-epibrassinolide on growth, chlorophyll, electrolyte leakage and proline by pepper plants under NaCl-stress. Eurasian J. Biosci. 96–104. https://doi.org/10.5053/ejobios.2010.4.0.12.

Isayenkov, S.V., Maathuis, F.J.M., 2019. Plant salinity stress: many unanswered questions remain. Front. Plant Sci. 10 https://doi.org/10.3389/fpls.2019.00080.

Jimenez-Bremont, J.F., Becerra-Flora, A., Hernandez-Lucero, E., Rodriguez-Kessler, M., Acosta-Gallegos, J.A., Ramirez-Pimentel, J.G., 2006. Proline accumulation in two bean cultivars under salt stress and the effect of polyamines and ornithine. Biol. Plant. 50, 763–766. https://doi.org/10.1007/s10535-006-0126-x.

Kalhor, M.S., Aliniaeifard, S., Seif, M., Asayesh, E.J., Bernard, F., Hassani, B., Li, T., 2018. Enhanced salt tolerance and photosynthetic performance: implication of ϒ-amino butyric acid application in salt-exposed lettuce (Lactuca sativa L.) plants. Plant Physiol. Biochem. 130, 157–172. https://doi.org/10.1016/j.plaphy.2018.07.003.

Karabal, E., Yücel, M., Öktem, H.A., 2003. Antioxidant responses of tolerant and sensitive barley cultivars to boron toxicity. Plant Sci. 164, 925–933. https://doi.org/10.1016/S0168-9452(03)00067-0.

Khedr, A.H.A., 2003. Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of Pancratium maritimum L. to salt-stress. J. Exp. Bot. 54, 2553–2562. https://doi.org/10.1093/jxb/erg277.

Kohler, J., Hernández, J.A., Caravaca, F., Roldán, A., 2009. Induction of antioxidant enzymes is involved in the greater effectiveness of a PGPR *versus* AM fungi with respect to increasing the tolerance of lettuce to severe salt stress. Environ. Exp. Bot. 65, 245–252. https://doi.org/10.1016/j.envexpbot.2008.09.008.

Kuşvuran, Ş., Yaşar, F., Abak, K., Ellialtıoğlu, Ş., 2008. Changes occur in lipid peroxidation, chlorophyll and ion contents of some salt tolerant and sensitive Cucumis sp. genotypes grown under salinity stress. J. Agric. Sci. 18, 13–20.

Leão, G.A., de Oliveira, J.A., Felipe, R.T.A., Farnese, F.S., Gusman, G.S., 2014. Anthocyanins, thiols, and antioxidant scavenging enzymes are involved in Lemna gibba tolerance to arsenic. J. Plant Interact. 9, 143–151. https://doi.org/10.1080/17429145.2013.784815.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Lucini, L., Rouphael, Y., Cardarelli, M., Canaguier, R., Kumar, P., Colla, G., 2015. The effect of a plant-derived biostimulant on metabolic profiling and crop performance of lettuce grown under saline conditions. Sci. Hortic. 182, 124–133. https://doi.org/10.1016/j.scienta.2014.11.022.

Machado, R., Serralheiro, R., 2017. Soil salinity: effect on vegetable crop growth. management practices to prevent and mitigate soil salinization. Horticulturae 3, 30. https://doi.org/10.3390/horticulturae3020030.

Matysiak, K., Kierzek, R., Siatkowski, I., Kowalska, J., Krawczyk, R., Miziniak, W., 2020. Effect of exogenous application of amino acids L-arginine and glycine on maize under temperature stress. Agronomy 10, 769. https://doi.org/10.3390/agronomy10060769.

Mehta, P., Jajoo, A., Mathur, S., Bharti, S., 2010. Chlorophyll a fluorescence study revealing effects of high salt stress on photosystem II in wheat leaves. Plant Physiol. Biochem. 48, 16–20. https://doi.org/10.1016/j.plaphy.2009.10.006.

Meloni, D.A., Gulotta, M.R., Martínez, C.A., Oliva, M.A., 2004. The effects of salt stress on growth, nitrate reduction and proline and glycinebetaine accumulation in Prosopis alba. Braz. J. Plant Physiol. 16, 39–46.

Miceli, A., Moncada, A., D'Anna, F., 2003. Effect of salt stress in lettuce cultivation. Acta Hortic. 609, 371–375. https://doi.org/10.17660/ActaHortic.2003.609.56.

Milne, C.J., Laubscher, C.P., Ndakidemi, P.A., Marnewick, J.L., Rautenbach, F., 2012. Salinity induced changes in oxidative stress and antioxidant status as affected by applications of silicon in lettuce (Lactuca sativa). Int. J. Agric. Biol. 14, 763–768.

Molina-Montenegro, M.A., Acuña-Rodríguez, I.S., Torres-Díaz, C., Gundel, P.E., Dreyer, I., 2020. Antarctic root endophytes improve physiological performance and yield in crops under salt stress by enhanced energy production and Na+ sequestration. Sci. Rep. 10, 1–10. https://doi.org/10.1038/s41598-020-62544-4.

Nasri, N., Kaddour, R., Rabhi, M., Plassard, C., Lachaal, M., 2011. Effect of salinity on germination, phytase activity and phytate content in lettuce seedling. Acta Physiol. Plant. 33, 935–942. https://doi.org/10.1007/s11738-010-0625-4.

Nedjimi, B., 2014. Effects of salinity on growth, membrane permeability and root hydraulic conductivity in three saltbush species. Biochem. Syst. Ecol. 52, 4–13. https://doi.org/10.1016/j.bse.2013.10.007.

Pasternak, D., De Malach, Y., Borovic, I., Shram, M., Aviram, C., 1986. Irrigation with brackish water under desert conditions IV. Salt tolerance studies with lettuce (Lactuca sativa L.). Agric. Water Manag. 11, 303–311. https://doi.org/10.1016/0378-3774(86)90046-6.

Pivato, M., Fabrega-Prats, M., Masi, A., 2014. Low-molecular-weight thiols in plants: functional and analytical implications. Arch. Biochem. Biophys. 560, 83–99. https://doi.org/10.1016/j.abb.2014.07.018.

Rai, V.K., 2002. Role of amino acids in plant responese to stresses. Biol. Plant. 45, 481–487.

Rosales, M.A., Franco-Navarro, J.D., Peinado-Torrubia, P., Díaz-Rueda, P., Álvarez, R., Colmenero-Flores, J.M., 2020. Chloride improves nitrate utilization and NUE in plants. Front. Plant Sci. 11 https://doi.org/10.3389/fpls.2020.00442.

Sah, S.K., Reddy, K.R., Li, J., 2016. Abscisic acid and abiotic stress tolerance in crop plants. Front. Plant Sci. 7, 1–26. https://doi.org/10.3389/fpls.2016.00571.

Santander, C., Ruiz, A., García, S., Aroca, R., Cumming, J., Cornejo, P., 2020. Efficiency of two arbuscular mycorrhizal fungal inocula to improve saline stress tolerance in lettuce plants by changes of antioxidant defense mechanisms. J. Sci. Food Agric. 100, 1577–1587. https://doi.org/10.1002/jsfa.10166.

Schön, A., Krupp, G., Gough, S., Berry-Lowe, S., Kannangara, C.G., Söll, D., 1986. The RNA required in the first step of chlorophyll *b*iosynthesis is a chloroplast glutamate tRNA. Nature 322, 281–284. https://doi.org/10.1038/322281a0.

Scuderi, D., Giuffrida, F., Noto, G., 2009. Effects of salinity and plant density on quality of lettuce grown in floating system for fresh-cut. Acta Hortic. 219–226. https://doi.org/10.17660/ActaHortic.2009.843.28.

Sedjati, S., Santosa, G., Yudiati, E., Supriyantini, E., Ridlo, A., Kimberly, F., 2019. Chlorophyll and carotenoid content of dunaliella salina at various salinity stress and harvesting time. In: Proceedings of the IOP Conference Series: Earth and Environmental Science, 246, 012025. https://doi.org/10.1088/1755-1315/246/1/012025.

SH Sadak, M., Abdelhamid, M.T., Schmidhalter, U., 2014. Effect of foliar application of amino acids on plant yield and physiological parameters in bean plants irrigated with seawater. Acta Biológ. Colomb. 20, 140–152. https://doi.org/10.15446/abc.v20n1.42865.

Shahbaz, M., Ashraf, M., Al-Qurainy, F., Harris, P.J.C., 2012. Salt tolerance in selected vegetable crops. CRC Crit. Rev. Plant Sci. 31, 303–320. https://doi.org/10.1080/07352689.2012.656496.

Shahid, M.A., Sarkhosh, A., Khan, N., Balal, R.M., Ali, S., Rossi, L., Gómez, C., Mattson, N., Nasim, W., Garcia-Sanchez, F., 2020. Insights into the physiological and biochemical impacts of salt stress on plant growth and development. Agronomy. https://doi.org/10.3390/agronomy10070938.

Shams, M., Yildirim, E., Ekinci, M., Turan, M., Dursun, A., Parlakova, F., Kul, R., 2016. Exogenously applied glycine betaine regulates some chemical characteristics and antioxidative defence systems in lettuce under salt stress. Hortic. Environ. Biotechnol. 57, 225–231. https://doi.org/10.1007/s13580-016-0021-0.

Shannon, M.C., Grieve, C.M., 1998. Tolerance of vegetable crops to salinity. Sci. Hortic. 78, 5–38. https://doi.org/10.1016/S0304-4238(98)00189-7.

Shimomachi, T., Kawahara, Y., Kobashigawa, C., Omoda, E., Hamabe, K., Tamaya, K., 2008. Effect of residual salinity on spinach growth and nutrient contents in polder soil. Acta Hortic. 419–424. https://doi.org/10.17660/ActaHortic.2008.797.60.

Shu, S., Yuan, L.Y., Guo, S.R., Sun, J., Yuan, Y.H., 2013. Effects of exogenous spermine on chlorophyll fluorescence, antioxidant system and ultrastructure of chloroplasts in Cucumis sativus L. under salt stress. Plant Physiol. Biochem. 63, 209–216. https://doi.org/10.1016/j.plaphy.2012.11.028.

Silva-Ortega, C.O., Ochoa-Alfaro, A.E., Reyes-Agüero, J.A., Aguado-Santacruz, G.A., Jiménez-Bremont, J.F., 2008. Salt stress increases the expression of p5cs gene and induces proline accumulation in cactus pear. Plant Physiol. Biochem. 46, 82–92. https://doi.org/10.1016/j.plaphy.2007.10.011.

Vernieri, P., Perata, P., Armellini, D., Bugnoli, M., Presentini, R., Lorenzi, R., Ceccarelli, N., Alpi, A., Tognoni, F., 1989. Solid phase radioimmunoassay for the quantitation of abscisic acid in plant crude extracts using a new monoclonal antibody. J. Plant Physiol. 134, 441–446. https://doi.org/10.1016/S0176-1617(89)80007-0.

Westfall, C.S., Muehler, A.M., Jez, J.M., 2013. Enzyme action in the regulation of plant hormone responses. J. Biol. Chem. 288, 19304–19311. https://doi.org/10.1074/jbc.R113.475160.

Xu, C., Mou, B., 2016. Responses of spinach to salinity and nutrient deficiency in growth, physiology, and nutritional value. J. Am. Soc. Hortic. Sci. 141, 12–21. https://doi.org/10.21273/JASHS.141.1.12.

Xu, C., Mou, B., 2015. Evaluation of lettuce genotypes for salinity tolerance. HortScience 50, 1441–1446. https://doi.org/10.21273/HORTSCI.50.10.1441.

You, J., Chan, Z., 2015. ROS regulation during abiotic stress responses in crop plants. Front. Plant Sci. 6, 1–15. https://doi.org/10.3389/fpls.2015.01092.

Yu, Z., Duan, X., Luo, L., Dai, S., Ding, Z., Xia, G., 2020. How plant hormones mediate salt stress responses. Trends Plant Sci. 25, 1117–1130. https://doi.org/10.1016/j.tplants.2020.06.008.

Zagorchev, L., Seal, C.E., Kranner, I., Odjakova, M., 2013. A central role for thiols in plant tolerance to abiotic stress. Int. J. Mol. Sci. 14, 7405–7432. https://doi.org/10.3390/ijms14047405.

Zhang, J., Jia, W., Yang, J., Ismail, A.M., 2006. Role of ABA in integrating plant responses to drought and salt stresses. Field Crops Res. 97, 111–119. https://doi.org/10.1016/j.fcr.2005.08.018.