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

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ABSTRACT

Salt stress is a serious environmental issue limiting crop growth and productivity worldwide. Lettuce salad is generally considered as a salt-sensitive species; however, different cultivars may exhibit different adaptive mechanisms. The application of biostimulants products has recently proved to be a strategic intervention to ameliorate plant response to abiotic stresses and foster resilience of plants during their cultivation. This study intended to explore the potential physiological mechanisms underlaying romaine lettuce plant responses to a period of salt stress when exogenously treated with glutamic acid. The glutamic acid treatment was applied as foliar spray the first time before the beginning of salt exposure, followed by further three applications during the stress. To understand the effect of salinity and glutamic acid treatment, physiological and molecular studies have been performed. High salinity induced a general stimulation of PSII and chlorophyll content in lettuce leaves, however, a reduction of yield (-26,5%) has been observed. Moreover, the concentration of proline has been stimulated under stressful condition whereas ABA levels decreased. The analyses of the genes encoding for ROS scavenging enzymes showed a general downregulation in response to salinity with the only exception of *LsSOD*.

Keywords: abiotic stress, amino acid, antioxidant, salinity, superoxide dismutase.

INTRODUCTION

Among environmental stressors, salinity is one of the most detrimental factors leading to severe losses in crops production, yield and product quality (Aslam et al., 2017; Grieve et al., 2011). According to FAO (FAO, 2015) more than 100 countries are affected by soils salinization and their extent is estimated at about 1 billion ha, worldwide. Despite the severity of salinization, no accurate and recent statistic is available about the global extent of the problem. Moreover, several researches report a constant increase in soil salinization due both to natural causes (such as climate change, increasing temperatures, rising seawater levels, intrusion of seawater and erosion of minerals), and to improper agricultural practices

(Adhikari et al., 2019; Annunziata et al., 2017; Aroca et al., 2013; Freitas et al., 2019; Molina-Montenegro et al., 2020). Soil salinity is widespread in different climates but it often occurs in irrigated areas, in arid and semiarid regions where precipitations are not enough to balance crop evapotranspiration and to ensure salt leaching (Connor et al., 2012; FAO, 2015; Pitman and Läuchli, n.d.). 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, horticultural sector is seriously jeopardised in Mediterranean areas, where more than 40% of soils is affected by salinity (Colla et al., 2010; Miceli et al., 2003; Nedjimi, 2014). Moreover, 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 these regions is usually higher than salt tolerance threshold level (Colla et al., 2010). Soils affected by salts include both those affected by salinity, where the electrical conductivity is higher than 4 dSm^{-1} and those affected by sodicity, where exchangeable sodium exceeds 6 % (FAO, 2015).

Salt stress can alter plant's physiological processes, disrupting photosynthesis and respiration, impairing protein biosynthesis, phytohormones regulation, inducing nutrient imbalance, and damaging cell organelles (Chaves et al., 2009; Munns, 2002; Nawaz et al., 2010; Yang and Guo, 2018; Zhu, 2000). The negative effects of salt stress can be divided in two phases: the osmotic phase and the ion toxicity phase (Isayenkov and Maathuis, 2019). The first one is characterised by a decreased ability of plants to uptake water from the soil. Soluble salts reduce the water potential of soil or substrates, and plants have to invest energy into water uptake with negative effects on cell metabolism and growth rate. This is the main reason for stunted growth under salt stress. The osmotic phase involves different processes and plant responses that are shared with drought stress. Thus, this phenomenon is also known as water-deficit effect of salinity and it usually occurs after minutes, hours and up to the first days of exposure to high salinity levels. During this time salts are not yet penetrated in plant tissues. On the contrary, the ion toxicity phase is caused by the excess of ions accumulated inside the plant and is also called salt-specific effect. Plant growth is limited by the ion toxicity inducing the reduction of nutrient uptake or transport. Ions accumulate into the vacuoles, but then they move to the cytoplasm, when the concentration is too high, threatening the metabolic activities and the normal functioning of the enzymes. Even though sodium chloride (NaCl) is the major compound present in salt affected soils, Ca^{2+}, Mg^{2+}, SO_4^{2-} and CO_3^{2-} may also contribute to soil salinization (Maas and R., 1999). High levels of Na^+ and Cl^- result in less absorption of other minerals such as calcium, manganese, and potassium. Moreover, high Na^+: K^+ ratio causes the inactivation of the enzymes mainly because K^+ is replaced by Na^+ in a series of biochemical reactions including protein formation, osmoregulation, photosynthesis, and maintenance of cell turgor pressure (Benito et al., 2014). Nutrient imbalances or deficiencies may also decrease the quality of fruits or other edible organs reducing the market value of many vegetables. Besides that, salt stress results in oxidative burst due to the overproduction and accumulation of reactive oxygen species (ROS) in cells, causing 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 leading to a 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 scavenge or detoxify the excess of ROS through 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 (AsA) acid 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 reaction, involving APX, MDHAR, DHAR, and GR (You and Chan, 2015).

The effect of salinity on plants 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 even among varieties of a given crop (Machado and Serralheiro, 2017; Xu and Mou, 2015).

Lettuce (*Lactuca sativa* L.) is considered 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 major lettuce-producing countries 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 benefits 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 constituent of proteins and precursors of several metabolites involved in plant growth regulation and in responses to the external factors. Moreover, they are involved in the formation of pigments (Cho et al., 2009), vitamins (Asensi-Fabado and Munné-Bosch, 2010), secondary metabolites, and phytohormones (Westfall et al., 2013). They can act as osmolytes, regulate stomatal opening and ion transport (Rai, 2002). Among amino acids, glutamic acid has a key role in plant defence including cellular redox, 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 response of lettuce plants subjected to a period of salt stress 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 health status and quality of lettuce. The nitrate, proline, and osmolytes levels have been estimated since they are considered as biochemical indicators of plants responses to stress. Moreover, the expression of some of the key genes encoding for the enzymes responsible for ROS scavenging have been analysed to determine the responses induced by salt exposure and glutamic acid applications also at molecular level.

MATERIALS AND METHODS

Plant material, treatments and experimental plan

Two-week old Romaine lettuce (*Lactuca sativa* var. 'longifolia') plantlets were supplied from a local nursery and transplanted into 2.5 L plastic pots filled with a commercial peaty substrate. A total of 36 plants were grown in an

experimental greenhouse under controlled conditions (Temp. 24 ± 2 °C; R.U. 79 ± 12 %) at the Faculty of Agricultural and Food Science of Milan in 2018.

The experimental design was based on a combination of two factors: salt stress and glutamic acid (GA) treatment, each of them with two levels. 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 were applied by foliar spray 5 days after the transplant, every ten days for a total of four applications and each plant was treated with 10 mL of product. Treatments consisted of water and a glutamic acid solution (2 mM).

Lettuce plants were harvested at commercial maturity stage. 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 hours after the last treatment and stored at -80 °C until use for gene expression analyses.

Non-destructive analyses

Chlorophyll measurement *in vivo*

A rapid and direct estimation of chlorophyll in lettuce leaves has been 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 express as chlorophyll index (relative units).

Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence was measured *in vivo* using a hand-portable fluorometer (Handy-PEA, Hansatech Instruments, UK). Leaves were dark-adapted with leaf clips (4 mm diameter) for 30 minutes before the measurement. Afterwards, an array of three high-intensity light-emitting diodes produce a saturating light (3000 µmol m^{-2} s^{-1}) for 1 second that hits leaf tissues. JIP-test equations were applied to obtain derived parameters from the measured data. These parameters, provide information about the structural and functional status of photosynthetic apparatus (Table S1).

Destructive measurements

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.

144

145 *Nitrate*

146 Nitrate concentration was determined by the Cataldo method (Cataldo et al., 1975). Leaf samples were homogenized
147 (mortar and pestle) with 3 mL of distilled water per gram of fresh tissue. The homogenate was centrifuged at 4000 rpm
148 for 15 min at RT and the recovered supernatant was used for the colorimetric analysis. About 20 µL of the extract were
149 added to 80 mL of 5% (w/v) salicylic acid in concentrated H_2SO_4 (SA- H_2SO_4). Afterward, 3 mL of 1.5 N NaOH were added.
150 The samples were cooled to RT and absorbance was measured at 410 nm with a spectrophotometer. Nitrate content was
151 calculated referring to a KNO_3 standard calibration curve and expressed as mg of NO_3-N per kg of FW.

152

153 *Osmolytes*

154 Fresh leaf tissues were homogenized (mortar and pestle) in distilled water (1 g fresh tissue per 3 mL water). The
155 homogenate was centrifuged at 4000 rpm for 15 min at RT and the recovered supernatant was analysed. The osmolarity
156 was measured using an automatic freezing point depression osmometer (Digital Osmometer, Roebling, Berlin, Germany)
157 calibrated with sodium chloride solutions.

158

159 *Proline*

160 Proline concentration was determined by the ninhydrin-based colorimetric assay improved by Bates (Bates et al., 1973).
161 Approximately 1 g of leaf tissue was grinded (mortar and pestle) with 10 mL of 3% sulfosalicylic acid. Samples were
162 centrifugated at 4000 rpm for 5 min at RT. Afterwards, 100 µL of supernatant was added to a reaction mixture prepared
163 with 3% sulfosalicylic acid (100 µL), glacial acetic acid (200 µL) and acidic ninhydrin (200 µL). The tubes were mixed, each
164 lid was punctured with a needle to avoid high pressure and the tubes were incubated at 96 °C for 60 min. The reaction
165 was terminated putting the tubes on ice. The extraction was made adding 1 mL toluene to the reaction mixture. The tubes
166 were vortexed and leaved on the bench for 5 min to allow the separation between the organic and water phases. The
167 chromophore phase containing toluene was collected and the absorbance at 520 nm was red using toluene as reference.
168 Proline concentration was calculated referring a standard calibration curve and expressed as µg per g FW.

169

170 *Total thiols*

171 The concentration of total thiols in lettuce leaves was determined by Leão method (Leão et al., 2014). About 0.5 g of leaf
172 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
173 EDTA, and 1% ascorbic acid. Samples were centrifugated at 4000 rpm for 10 min at 4 °C. the supernatant was collected
174 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
175 added. After 15 min of reaction at 37 °C, the absorbance at 412 nm was determined. Total thiols concentration was
176 calculated using a molar extinction coefficient of 13,100 M^{-1} cm^{-1}.

177

Total RNA isolation and analysis of gene expression

Frozen leaves of lettuce were thoroughly ground with liquid N using cold mortar and pestle. Approximately 100 mg was transferred to a cryotube and stored at −80 °C. The isolation of total RNA was performed using the Spectrum Plant Total RNA Kit with on-column DNase-treatment (Sigma-Aldrich, Italy) following the steps of protocol A with a few modifications. The concentration and the purity of RNA were evaluated by measuring the absorbance at 230 nm, 260 nm and 280 nm using a NanoDrop N-1000 spectrophotometer (NanoDrop technologies). A ratio of absorbance at 260 and 280 ≈ 2.0 is generally accepted as pure for RNA and expected 260/230 values are commonly in the range of 2.0-2.2, usually higher than the respective 260/280 value. About 3 µg of RNA were reversely transcribed to cDNA using the SuperScript IV cDNA Synthesis Kit according to the manufacturer's instruction (Invitrogen, Italy). The SYBR® Green PCR Master Mix (Applied Biosystems) was used 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). The gene expression levels were analysed with the AB software program and results were calculated using the 2^{-ddct} method described by Livak and Schmittgen (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 reference gene (housekeeping) due to the highest stability in its expression levels, whereas the non-stressed and non-treated sample after 3 hours was chosen as internal calibrator.

Statistical analyses

Data were subjected to ANOVA and differences among means were determined by Tuckey post-test ($P < 0.05$). Statistics 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's legend.

RESULTS

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. Thesomministration/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 (Figure 1 A).

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 (Figure 1 B).

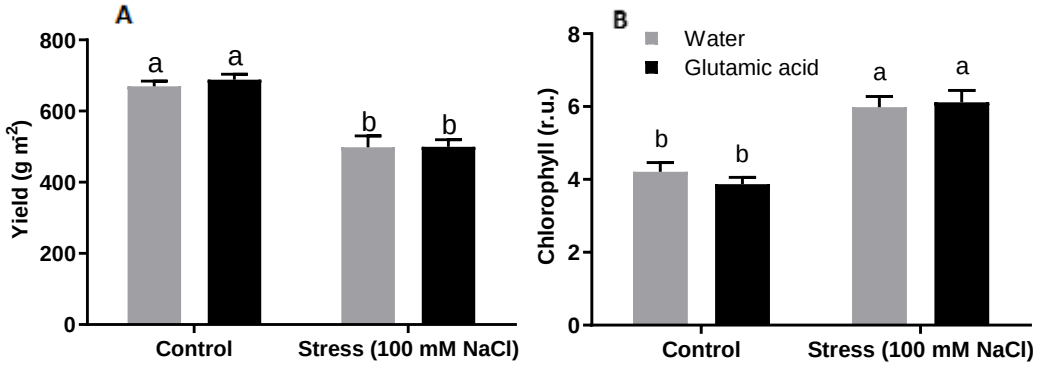

Figure 1. Yield (**A**) and chlorophyll content (**B**) of lettuce plants grown under non-stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Measures were taken at the end of the growing cycle. Values are means ± SE (yield: n = 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 ($p < 0.05$).

A general response of photosynthesis to salt stress and glutamic acid treatment is presented in the multiparametric graph of chlorophyll *a* fluorescence parameters (2). In this chart, all the parameters' values are normalized to 0 -grey 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 grey 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, minimal fluorescence (Fo), maximal fluorescence (Fm), variable fluorescence (Fv) and maximum quantum efficiency of PSII (Fv/Fm) were not significantly affected by the stress and by the treatment. The Fv/Fm values in both growing conditions were about 0.86.

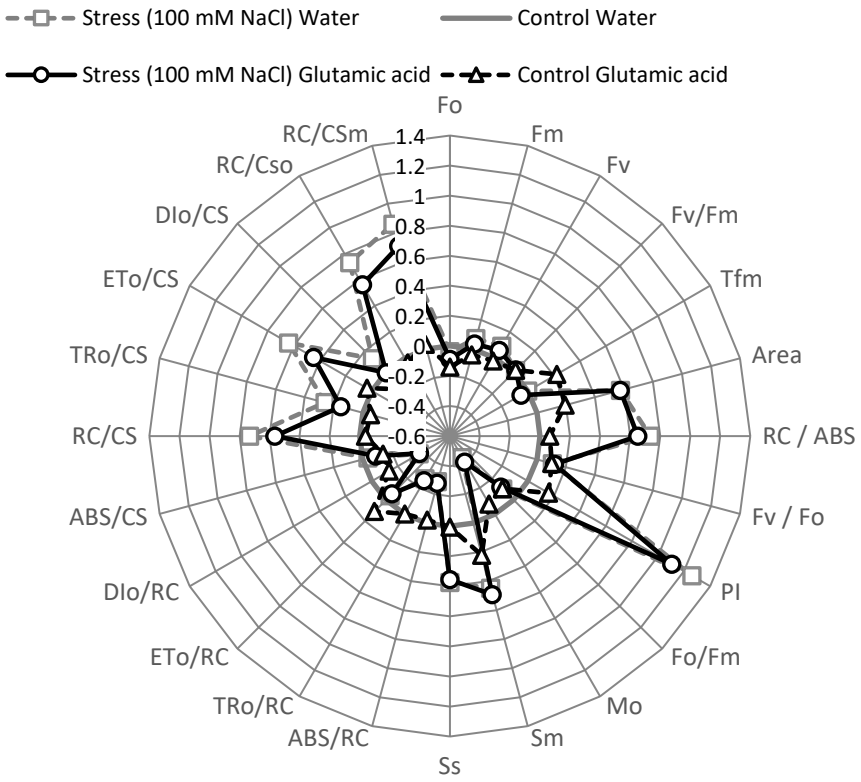

Figure 2. Chlorophyll a fluorescence parameters of lettuce plants grown under non stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Measures were taken at the end of the growing cycle. Values are means ± SE (n =6). Data plotted are fluorescence parameters normalized by formulae: (Ft – Fcw)/Fcw, where "Ft" and "Fcw" represent the parameter values of the treated plants and control plants treated with water, respectively. Values of "Fcw" plants were normalized to 0 (control plants treated with water, grey circle = 0).

241 *Nitrate*

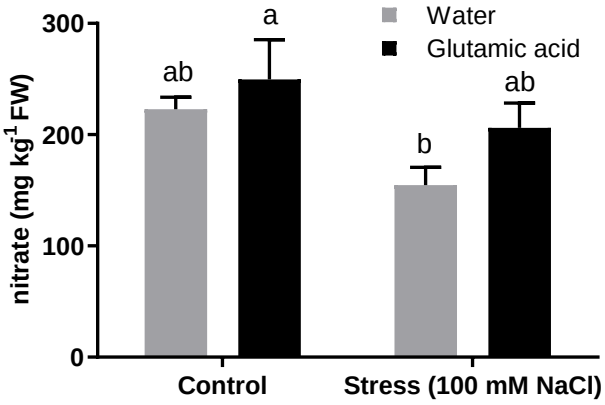

Figure 3. Nitrate content measured in lettuce leaves under non stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Measures were taken at the end of the growing cycle. Values are means ± SE (n =6). Data were subjected to two-way 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).

242

243 Nitrate concentration in lettuce leaves was significantly ($p < 0.05$) affected by the salt stress. In particular, the lower level
244 was measured in untreated plants grown under high salinity (Figure 3). However, no significant differences emerged
245 comparing the treatments, except between the control plants treated with the glutamic acid solution and the stressed
246 plants treated with water. In general, nitrate concentration values ranged from 115 mg kg^{-1} FW and 409 mg kg^{-1} FW.

247

248 *Proline, osmolytes, and abscisic acid*

249 Proline levels were measured in order to assess its potential role in defining lettuce tolerance to NaCl in combination with
250 glutamic acid treatment. Without salt, lettuce plants contained the same amount of proline in leaves, regardless the
251 treatment (Table 2). Salt stress significantly ($p < 0.05$) affected the levels of proline and osmolytes in lettuce leaves (Table
252). In particular, the proline average value was about 11.8 µg g^{-1} in plants grown under non-stressful condition and about
253 63.9 µg g^{-1} in stressed plants. A significant difference was observed between control and stressed plants treated with
254 water. However, the high variability did not allow to see any significant effect of the glutamic acid treatment in stressed
255 samples.
256 Salinity induced a significant increase (+52.2%) of osmolytes concentration in plants treated with glutamic acid, whereas
257 no significant difference was observed in plants treated with water, as reported in Table 1.
258 Likewise, a significant ($p < 0.05$) effect of the salt stress resulted in the concentration of abscisic acid in lettuces leaves. ABA
259 levels were generally low in plants grown under high salinity compared to those grown under control condition. In
260 particular, salt stress induced a significant decrease (- 56%) in plants treated with the glutamic acid solution while it had no
261 significant effect on non-treated plants (Table 2). Probably, similarly to those observed in proline concentration, the high
262 variability of the results reduced the statistical power.

263

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

Total thiols

The two-way ANOVA showed a significant ($p<0.05$) effect of salinity on thiols concentration in lettuce leaves (Figure 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.

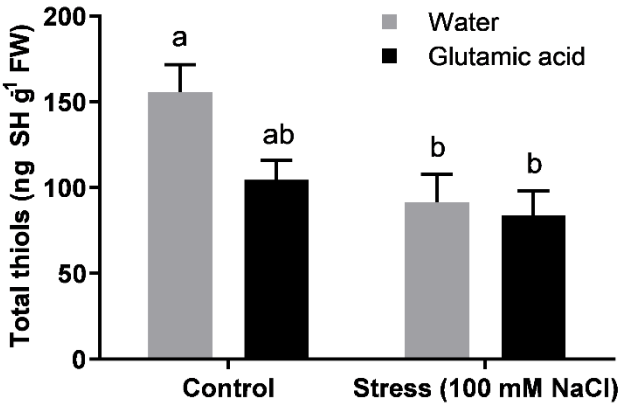

Figure 4. Total thiols concentration in lettuce leaves grown under non stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Measures were taken at the end of the growing cycle. Values are means ± SE (n =12). Data were subjected 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).

Expression analyses of LsSOD, LsCAT, LsAPX, LSMDHAR, LsDHAR, and LsGR genes

The changes in the expression of the genes involved in antioxidant defence system have been clustered into a heatmap (**Error! Reference source not found.**). Moreover, a graph representing the expression analysis of each gene is presented (Figure 6**Error! Reference source not found.**). Different trends resulted in response to salt stress, treatments and during 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 hours. On the contrary, salt stress induced a general down-regulation of the genes, except for *LsSOD*, as shown by the colour shades in the heatmap. A strong decrease was observed especially in the transcripts of *LsCAT*, *LsAPX, and LsMDHAR* after 3 h (Figure 6 B, C, and D). At the same timepoint the expression of *LsSOD* increased in plants treated with water whereas the glutamic acid treatment did not induce any change if compared with the control. A three-fold increase was measured in *LsSOD* transcripts of plants treated with glutamic acid only after 6 hours (Figure 6 A).

The expression levels of *LsCAT, LsAPX, LsMDHAR, and LsDHAR* were strongly downregulated by the salt stress especially after 3h (Figure 5).

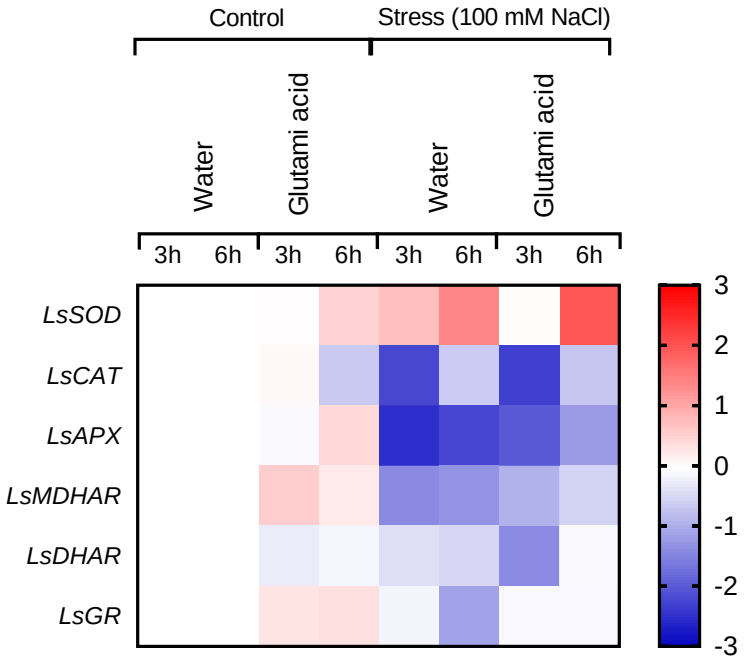

Figure 5. Heatmap showing temporal expression of selected genes in lettuce plants grown under non stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Data represent the log2FC of the selected genes. The rows are the genes, and 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.

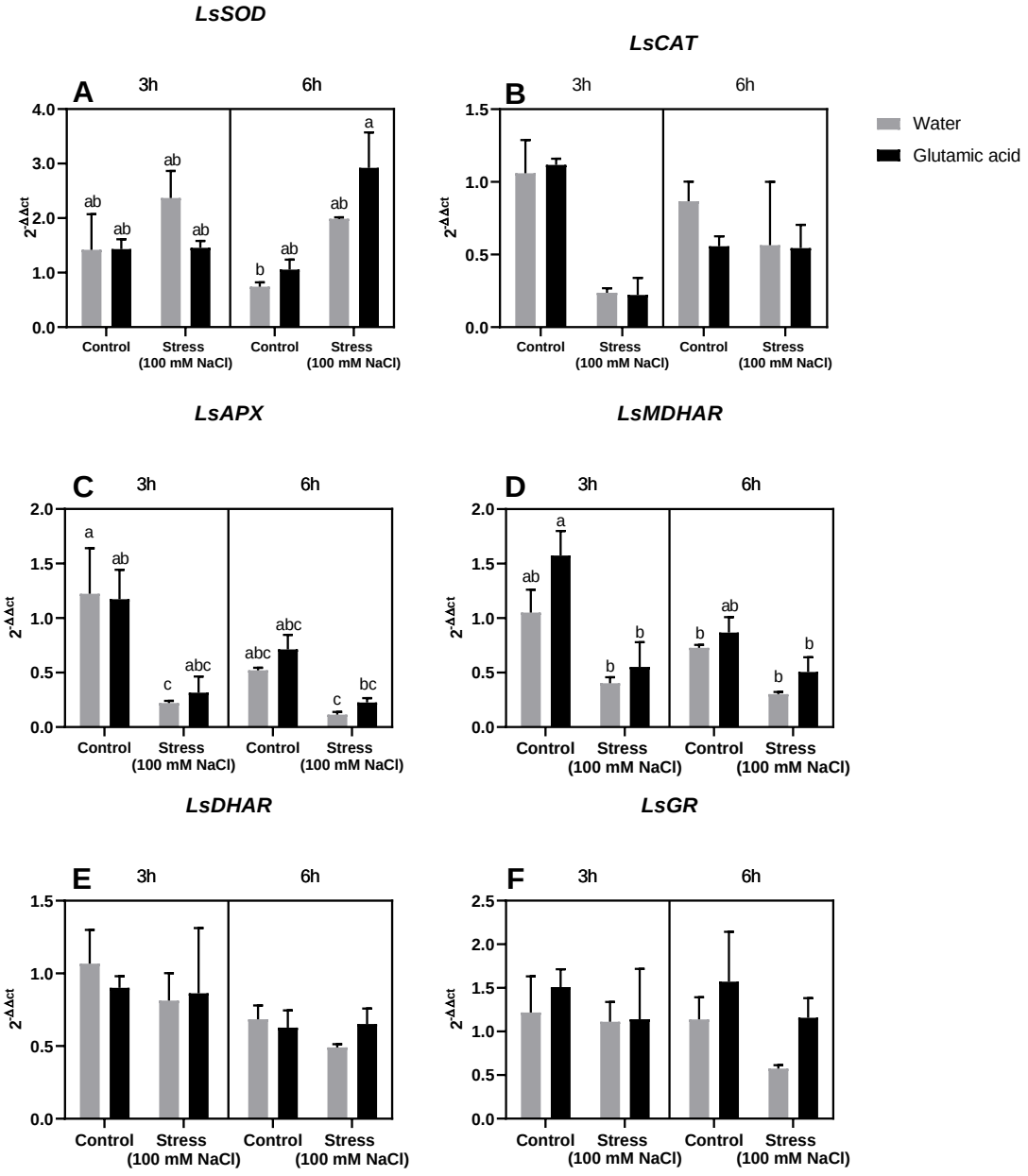

Figure 6. Changes in the expression of *LsSOD* (A), *LsCAT* (B), *LsAPX* (C), *LsMDHAR* (D), *LsDHAR* (E), *LsGR* (F) in lettuce leaves grown under non stressful (Control) and salt stress condition (Stress 100 mM NaCl) and treated with water or a glutamic acid solution (2 mM). Measures were taken at the end of the growing cycle. 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 ($P < 0.05$).

DISCUSSION

Salt stress severely affects plant growth, development, and quality by altering physiological and chemical processes. It represents a serious problem for commercial horticulture, especially in Mediterranean regions where the use of water

from the ground wells causes seawater intrusion. Here, the high levels of EC in water used for irrigation easily overcome the threshold tolerated by most of the species (Miceli et al., 2003; Xu and Mou, 2016). The severity of salinity stress is also enhanced by the high temperature and lower water availability in summer season. Moreover, the reduction of water in soil increases the concentration of soluble salts and the stress intensity.

Several approaches have been used to increase plant growth and productivity under abiotic stresses. An important strategy is breeding for stress tolerance, however, developing tolerant plants through genetic 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 mixture and products containing amino acids as a strategy to face the negative effect of salt stress has been widely evaluated (Alfosea-Simón et al., 2020; SH SADAK et al., 2014). Authors observed that the application of a plant-derived protein hydrolysate on lettuce salad increased the fresh yield, dry biomass and plant performance under salinity conditions (NaCl 25 mM) if compared to untreated plants, probably due to a more extensive roots apparatus (Lucini et al., 2015).

In our 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 the findings of All-Maskri (Al-Maskri et al., 2010) and coherent with the stunted growth phenotype due to the reduce ability of plants exposed to high salinity levels to absorb water from the growing media. Indeed, the first phase of salt stress is represented by the osmotic stress and it similar to those caused by drought (Machado and Serralheiro, 2017). Moreover, the low yield of lettuce plants grown under salt stress conditions could be attributed to a decrease in the nutrient uptake. 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).

Chlorophyll fluorescence can be used as non-invasive indicator of the physiological status of 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 in high salt treatment, in accordance with the observation of Xu and Mou (Xu and Mou, 2015) and Adhikari (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 colour 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 from wavelengths of blue-violet and orange-red light and it is responsible for the green colour of the leaves while

chlorophyll *b* absorbs energy from 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 affect 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 nutritional quality of leafy vegetable and its maximum level for commercialization is limited by the EC regulation 1258/2011. The concentration of nitrate in lettuce leaves was 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 be related to the decrease of water absorption or to the high level of chloride reducing nitrate accumulation (Abdelgadir et al., 2005; Miceli et al., 2003).

The NaCl stress induced a significant increase of proline levels in lettuce leaves. This is a common response of plants upon 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 ions imbalance, ions 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 our experiment, the high levels of proline observed in lettuce plants subjected to salt stress might have contributed to the health status of photosynthetic apparatus, as shown by the chlorophyll 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 our 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 signalling 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.

Lettuce plants grown subjected to high salinity had lower levels (-25%) of total thiols if compared to non-stressed and non-treated plants, regardless the application of the glutamic acid solution. 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 reactive oxygen species. They take part in the non-enzymatic antioxidant defence system working in plants to control and protect 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 stressed plants might be due to their conversion on other compounds or might indicate a toxic effect of salt stress on thiols metabolism.

The expression of the genes involved in antioxidant defence system decreased in plants subjected to high salinity. The only exception was the expression of *LsSOD* which acts as first line of defence to cope with ROS production, catalysing the reaction transforming the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2). The expression of *LsSOD* was induced by salinity, especially after 6 hours of stress and the treatment with glutamic acid amplified this response, as shown in Figure 6A. Our results are similar with other observations (Kalhor et al., 2018; Santander et al., 2020) in lettuce plants. H_2O_2 is a versatile molecule in plants, it acts as signal at normal levels, whereas it induces oxidative damages at toxic concentrations. The enzymes APX and with less affinity CAT are able to detoxify H_2O_2 through different mechanisms. In our experiment, 100 mM of NaCl in the nutrient solution induced a decrease in the *LsCAT* and *LsAPX* gene expression. This indicate that the H_2O_2 produced might not have reached toxic levels to induce *LsAPX* or *LsCAT* overexpression and the H_2O_2 eventually produced by *LsSOD* is involved in different biological processes. 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, different studies report the increase of the activity of these enzymes in response to high salinity conditions (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 their expression was observed over time, probably due to a circadian regulation of these genes. Moreover, the salt condition imposed in our experiment caused a further decrease right after 3 hours of stress. These observations, together with the unchanged expression of *LsDHAR* and *LsGR* might indicate a minor involvement of ascorbate-glutathione cycle in plant response to stress and it is reasonable hypothesize the involvement of detoxification mechanisms different from antioxidant enzymes.

Conclusion

Collectively, the results obtained in this experiment confirm that Romaine lettuce variety is moderately tolerant to salt stress (De Pascale and Barbieri, 1995) based on the less severe stress responses activated both at physiological and molecular levels. However, it is important to remember that 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 didn't show a strong effect on lettuce plants neither under optimal nor in 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). Moreover, further experiments aimed to clarify this aspect are necessary.

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.

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