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Understanding The Physiological, Biochemical, and Molecular Mechanisms of Salinity Tolerance in Strawberry Cultivars and in *Hv*TPK1-Overexpressed Barley

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Abstract

The research was carried out to investigate of main elements of salt stress response in two strawberry cultivars, Elsanta and Elsinore. Plants were grown under 0, 10, 20 and 40 mM NaCl for 80 days. Salinity dramatically affected growth in both cultivars, although Elsinore appeared to be more impaired than Elsanta. Moreover a significant reduction of leaf photosynthesis, evaporation, and stomatal conductance was recorded 24 hrs after the stress was applied in both cultivars, whereas physiological functions were differentially restored after acclimation. However, cv. Elsanta had more efficient leaf gas exchange and water status than cv. Elsinore. In general, Fruit yield reduced upon salinization, wheares fruit quality concerning fruit taste, aroma, appearance, total soluble solids and titratable acidity, did not change but rather was enhanced under moderate salinity. On the other hand fruit quality was impaired at severe salt stress. Fruit antioxidant content and antioxidant capacity were enhanced significantly by increasing salt concentration in both cultivars. The oxidative effects of the stress were defined by the measures of some enzymatic activities and lipid peroxidation. Consistently, an increase in superoxide dismutase (SOD), catalase (CAT), peroxide dismutase (POD) enzymes and higher content of proline and soluble proteins were observed in cv. Elsinore than in cv. Elsanta. The increase coincided with a decrease in lipid peroxidation. The research confirmed that although strawberry cultivars were sensitive to salinity, difference between cultivars exist; The experiment revealed that cv. Elsanta could stand severe salt stress, which was lethal to cv. Elsinore. The parameters measured in the previous experiment were proposed as early screening tools for the salt stress response in nine strawberry genotypes. The results showed that, wheares Elsanta and Elsinore cultivars had a lower dry weight reduction at 40 mM NaCl among cultivars, Naiad, Kamila, and Camarosa were the least salt-sensitive cultivars among the screened. In transgenic barley, the results showed that 13 lines were homozygous and resistance to hygromycin. Furthermore, the expression level of *Hv*TPK1 in transgenic lines was slightly increased compared to wild type and this resulted in different ion content and growth rate.

Keywords: salinity, strawberry, stomatal conductance, fruit quality, antioxidants systems, barley, *Hv*TPK1

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Dedication

To my parents:

Thanks for your love, precious support and sacrifice in my education. I hope that you can find here my deep gratitude and infinite reconnaissance

To my brothers and sisters:

Thanks for your encouragement, invaluable advices, financial and technical supports, and affection. My intense respect and esteem

To my wife Suha Alhomsi who supports and stands beside me during my study, I don't find words to express my deep love and appreciation…

To my daughter Rahaf:

To my angel who delights my life and brings happiness to my family

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Mohammad Alnayef

List of Abbreviations

A	Net photosynthesis
At	*Arabidopsis thaliana*
BCA	Bicinchoninic acid
bp	base pair
CAT	Catalase
cDNA	complementary DNA
CE	Catechin equivalent
C_i	Intercellular CO_2
cv.	cultivar
DAS	Day after salt treatments
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
E	Transpiration
EC	Electrical conductivity
FRAP	Ferric reducing antioxidant power
FV	Fast vascular channels
FW	Fresh weight
GAE	Galic acid equivalent
g_s	Stomatal conductance
Hv	Hordeum vulagre
LA	Leaf area
LOA	Leaf osmotic adjustment
LV	Lytic vacuoles
MDA	Malondialdhyde
MS salt	Murashige-Skoog salt
NTB	Nitro blue tetrazol
Os	Oryza sativa
P_a	External CO_2 partial pressure

PAR	Photosynthetically Active Radiation
PCR	Polymerase-chain-reaction
PEG	Polyethylene glycol
P_i	Intercellular CO_2 partial pressure
POD	Peroxidase
PSV	Protein storage vacuoles
PUFAs	Polyunsaturated fatty acids
PVPP	Polyvinylpyrrolidone
RGR	Relative growth rate
RH	Relative Humidity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse Transcriptase PCR
RWC	Relative water content
SOD	Superoxide dismutase
SV	Slow vacular channels
TA	Titratable acidity
TAC	Total anthocyanin content
TCA	Trichloro actic acid
TE	Trolox equivalent
TFC	Total flavonoids content
TPC	Total phenolics contents
TPK	Tow-pore K^+ channel
TSS	Total soluble solids
V/V	Volume by volume
VK	Vacular K^+ channel
w/v	Weight by volume
WUE	Water use efficiency
Ψ_p	Pressure potential
Ψ_t	Water potential
Ψ_π	Osmotic potential

Introduction

Abiotic stresses, such as drought and salinity, are serious threats to agriculture and natural status of the environment. These threats are indirectly catalyzed by global warming and population growth (Koyro et al., 2010). Global temperatures have increased by about 1 ºC over the course of the last century, and will likely rise even more rapidly in the coming decades. Increased drought and salinization of arable land are expected to have devastating global effects (Wing et al., 2003). The current amount of annual loss of arable area could double by the end of the century because of global warming (Evans, 2005). At the same time, rapid population growth increasingly generates pressure on existing cultivated land and other resources (Ericson et al., 1999). Therefore, adverse environmental conditions cause severe problems of poverty, social instability, and populations health threats (Moench, 2002). Salinity can be either natural or human induced by fertilizer or irrigation water. Excessive accumulation of salts in the rhizosphere can lead to growth inhibition, leaf necrosis, accelerated senescence, wilting and ultimate plant death. Different physiological mechanisms can also be involved. An osmotic mechanism may lead to the reduction of water potential and consequently the ability of plants to take up water decreases, which can seriously affect turgor potential and cell expansion. With time, salt may exert an additional effect on growth. If excessive amounts of Na^+ or Cl^- enter the plant, their concentration may rise to toxic levels in the older transpiring leaves. This injury, added to an already reduced leaf area, will then further limit the flow of carbon compounds to meristems and growing zones in leaves (Munns, 2010). The toxicity has also been related to increased generation of reactive oxygen species (ROS). Dual roles have been suggested for ROS in plants, as growth regulators, and potential signaling roles, as they may cause oxidatiove damage on vital cellular components at exccessive levels. It also has been reported that salt induced antioxidant enzymes may more or less successfully mitigate the potentially adverse effect of excessive ROS accumulation.

The salinity tolerance of plants is in most cases multigenic; it includes a wide range of morphological, physiological, and biochemical mechanisms on whole plant, tissue, and

cellular/ molecular levels (Wang et al., 2003). Only rarely is a single parameter of major importance for the ability to survive at high salinity. A comprehensive study with the analysis of at least a combination of several parameters is an essential to get a survey about the mechanisms which in the end leads to the salinity tolerance of individual species. These mechanisms are connected to the four major constraints of plant growth on saline conditions: water scarcity, leaf gas exchange, ion toxicity, and nutrient imbalance. It is therefore important to recognize and understand the processes that allow plants to adapt to water and salinity stress and allow an increase in biomass or plant yield for food production.

Accordingly, in this work we have tried to do a comprehensive study on the effect of salinity on strawberry and barley plants in order to understand the main mechanisms which allowed plants to mitigate the adverse effect of salinity. On the other hand, we tested the hypothesis that says that moderate salinity can improve strawberry fruit quality throughout enhancement of promoting health components and some biochemical attributes. As far as the Second Chapter is concerned, the objective of the study was to characterize morphological and physiological response of two strawberry cultivars namely (Elsanta and Elsinore) to different salt conditions. This study attempted to establish a functional link between morphological / physiological traits and on the one hand and stress tolerance on the other. In the Third Chapter: the objective of the study was to investigate the enzymatic antioxidant system and osmolytes accumulation, such as proline and soluble proteins and their roles in order to mitigate the adverse effect of salinity. In the Fourth Chapter, the objectives were to test the hypothesis which says that low-to-moderate levels of salinity are often used to improve fruit quality and to investigate as well the influence of adverse effect of salinity on fruit quality of both strawberry cultivars, especially on inner quality (nutrient value, antioxidant components, and biochemical attributes) and outer quality (appearance, aroma, and fruit size). In the Fifth Chapter; based on promising and valuable results of the first experiment, the second experiment was established by using nine cultivars of strawberry namely (Elsanta, Elsinore, Naiad, Siba, Kamila, Clery, Camarosa, Marmolada, and Madeleine) and the objective was to validate some screening tools of salt tolerance in order to differentiate between the less-sensitive-cultivars and sensitive-cultivars of strawberry for further breeding program. In the Sixth Chapter; this chapter is related to work on barley during my abroad training. In

this chapter, in my attempt to understand the molecular perspective of salinity tolerance, the objectives were to study overexpression *Hv*TPK1 in transgenic barley by leaf test, PCR, and RT-PCR and to characterize these transgenic plants with respect to growth and tolerance to a range of stresses, such as salinity stress and drought stress by measuring growth parameters and ion contents.

Low stomatal density and reduced transpiration facilitate strawberry adaptation to salinity

1 Introduction

Salinization of agricultural soils and irrigation water is one of the most critical environmental constraints limiting crop productivity and quality. Approximately 20% of irrigated land is affected by salinity (Rozema and Flowers, 2008). Although arid and semiarid regions of the world are more exposed to this phenomenon (Munns, 2005), salinization is increasingly expanding in less extreme environments since it is tightly associated with the practice of irrigation itself and, therefore, cannot be avoided (Rhoades et al., 1992; Flowers, 2004). Consequently, understanding the physiological and molecular basis underlying salt stress adaptation is pivotal to identify critical functions that should be potentiated to improve stress tolerance via traditional breeding or genetic engineering (Pardo, 2010). Key physiological processes and genetic determinants in salt stress adaptation have been identified in model systems and agricultural crops and have been shown to control mechanisms involved in ion/water homeostasis (Munns and Tester, 2008; Tavakkoli et al., 2011) and activation of multiple adaptation responses (Zhu, 2009; Klingler et al., 2010). Less explored, however, has been the role of metabolic components and physiological responses to saline stress with respect to morphological traits that may also facilitate stress adaptation (Maggio et al., 2001, 2007). Constitutively reduced transpiration fluxes, for example, may contribute to delay osmotic and ionic effects on shoot growth and may allow plants to adjust more effectively to unfavourable environments (Passioura and Munns, 2000). A deeper understanding of interlinks between metabolic, physiological and morphological determinants may also have a particular relevance when species-specific *salt tolerances* are framed in the context of different cultural/agricultural systems (Tavakkoli et al., 2010).

Strawberry (*Fragaria* x *ananassa* Duch.) is known as one of the most salt-sensitive crops with variable degrees of tolerance with respect to different cultivars and periods of exposure to high NaCl concentrations (Maas, 1990; Martinez Barroso and Alvarez, 1997; Turhan and Eris, 2005; Yilmaz and Kina, 2008). Salt stress generally impairs the

vegetative growth of strawberry and causes leaf necroses and premature senescence with consequent reduction of the photosynthetic leaf area (Keutgen and Pawelzik, 2009). As a result, the level of carbohydrates production and translocation to growing fruit is reduced (Saied et al., 2005). High NaCl levels in the root zone may also unbalance nutrients uptake, enhance competitions of Na^+ *vs.* K^+, Ca^{2+}, and Mg^{2+} (Khan et al., 2000) and impair assimilation of nitrogen (Alam, 1999; Mansour, 2000). Altogether these responses have a negative impact on yield and fruit quality (Awang and Atherton 1995a, 1995b).

Although evidence for a clear cause-effect relationship between physiological and/or molecular components and tolerance traits has somehow been hindered by the complex genetics of this species (Husaini and Abdin, 2008), correlative analyses have partially shed some light on critical tolerance mechanisms in strawberry (Turhan and Eris, 2005; Keutgen and Pawelzik, 2009). Ion redistribution and compartmentalization in different plant tissues and organs has been highlighted as an important mechanism to protect sensitive tissues from Na^+ accumulation and toxicity (Keutgen and Pawelzik, 2009). Transgenic analysis has also revealed that high constitutive osmolytes levels and reduced growth can both facilitate plant stress adaptation (Husaini and Abdin, 2008). Moreover, the activity of antioxidant enzymes and the control of the stomatal response to salinity have been indicated as physiological traits that may differentiate salt-tolerant *vs.* salt sensitive cultivars (Turhan et al., 2008).

Several studies have demonstrated that the stomatal control of transpiration-mediated ion fluxes to the shoot could delay the appearance of toxicity symptoms, while allowing plants to adapt to unfavorable conditions (Moya et al., 1999; Dalton et al., 2000; Maggio et al., 2007). Nevertheless, while adaptation via stomata closure may be advantageous under transitory stress (hours, days) to cope with osmotic stress and minimize water loss, it would negatively impact yield over a growth season since a reduced stomatal conductance would also restrict CO_2 uptake. That is why strategies to improve salinity stress tolerance via manipulation of stomatal conductance have rarely been successful (Thompson et al., 2007). It has been recently shown that a reduced stomatal density may partially compensate the trade-off between plant growth and adaptation (Ouyang et al., 2010) and therefore be advantageous under saline stress.

Using two strawberry cultivars with divergent responses to salinity, in this study we attempted to establish a functional link between morphological/physiological traits and

stress tolerance. Here we demonstrate that constitutive low transpiration fluxes associated to a reduced stomatal density may *uncouple* plant adaptation and yield reduction under saline stress in a specific agricultural context.

2 Materials and methods

Two experiments were carried out to characterize the response to salinity in the strawberry cultivars Elsanta and Elsinore, which had shown a diverse degree of stress tolerance in preliminary tests. Strawberry plantlets were kindly donated by a local nursery (Salvi vivai, Ferrara, Italy). The first experiment was conducted in a greenhouse in order to identify morphological and physiological traits that could be associated to salinity tolerance in the two cultivars under assessment. Subsequently, water relation parameters in response to salinity stress were analyzed in a growth chamber experiment under fully controlled environmental conditions.

2.1 Experiment 1

2.1.1 Plant material and growth conditions

The experiment was conducted in a glasshouse at the experimental station of the University of Bologna, located in *Ozzano dell'Emilia* (44º26"38 N, 11º26'18'' E, 98 m a.s.l.). Plantlets of similar height and diameter were transplanted into plastic pots of 5 l volume (1 plant/pot) filled with a mix of commercial growing media and pumice (2:1 v/v) on February 16th, 2010. Pots were placed over benches at a density of approximately 9 plants m^{-2}. Plants were irrigated automatically three times per day to ensure adequate substrate moisture. Fertigation was carried out once a week by adding to the irrigation water plant nutrients at the following concentrations: $N\text{-}NO_3$ = 6.0 mM; $N\text{-}NH_4$ = 1.0 mM; PO_4^{3-} = 3.0 mM; K^+ = 4.0 mM; SO_4^{2-} = 7.0 mM; Ca^{2+} = 5.0 mM; Mg^{2+} = 4.0 mM; microelements in traces, at a final EC = 1.75 dS m^{-1}. Before salt stress, plants stolons were removed to improve the vegetative growth and the quality of fruit. Inside the greenhouse temperatures ranged between12 and 38 °C, RH was 60-70% and Photosynthetically Active Radiation (PAR) during central hours of the day reached 700-1100 μmol m^{-2} s^{-1}.

2.1.2 Treatments and experimental design

Eight treatments, derived by the factorial combination of 2 cultivars (Elsanta and Elsinore) and 4 NaCl concentrations in the irrigation water (0, 10, 20 and 40 mM), were compared. The experimental design was a strip plot (*salt* assigned to the main plots and *cultivars* to the sub-plots) with 3 replications. Each plot included 8 plants. The salt stress treatment was initiated on April 1st (44 days after transplanting, when the plants had 6-7 leaves), by irrigating plants with a water solution of 0 mM NaCl (control, EC_w = 0.45 dS m^{-1}), 10 mM NaCl (EC_w = 0.97dS m^{-1}), 20 mM NaCl (EC_w = 1.95 dS m^{-1}) and 40 mM NaCl (EC_w = 3.90 dS m^{-1}). When the irrigation coincided with the fertigation (once a week), the EC_w were 1.75, 2.30, 4.30 and 6.50 dS m^{-1} for 0, 10, 20 and 40 mM NaCl treatments, respectively. This irrigation regime was maintained until the end of the experiment.

2.1.3 Growth and yield assessment

Fruit-setting started on April 15th (14 days after stress treatment initiation, DAS). Fruit of all plants were harvested manually at full maturity on four dates: May 5th, 10th, 17th, and 27th (corresponding to 34, 39, 46 and 56 DAS, respectively). At the end of the experiment (June 7th, 67 DAS), three plants per each plot were randomly selected and harvested. Numbers of leaves, shoot fresh and dry weights were measured. The leaf area (LA) was determined by using a scanner and the image processor software *Image J* (Abramoff et al., 2004).

2.1.4 Stomatal size and density

Micromorphological observations were carried out at 15 DAS on three 1cm^2 portions per leaf (excised from areas between the main veins) using a bright-field light microscope (Meiji Techno Co., LTD, Japan). Stomata frequencies per surface unit (n mm^{-2}) were calculated on 3 representative fields of three leaves of similar age per plot.

2.1.5 Leaf gas exchanges

Leaf evaporation (E), stomatal conductance (g_s) and net photosynthesis (A) were measured at 2 and 58 DAS on the youngest fully expanded leaf of two plants per plot, using a CIRAS-2 infrared gas analyzer (PPSystem, Hitchin, UK) with a Parkinson's Automatic Universal Leaf Cuvette equipped with 2.5-cm^2 area cuvette inserts (environmental conditions inside the cuvette were set as follows: PAR=1000 μmol m^{-2} s^{-1}; leaf temperature=26 ºC, CO_2 =450 ppm, equal to the environmental conditions inside the glasshouse at the time of sampling).

2.1.6 Plant water relations

At 10 DAS, three pots per plot were sealed with a plastic film to prevent water loss from the soil surface, leaving the plant shoot protruding from the film. Before sealing the film at the crown, plants were re-watered to pot capacity with water (control), or water plus 10, 20 or 40 mM NaCl. Each plant was then placed on an electronic balance under glasshouse conditions and the weight loss was measured every hour for 24 hrs. Water loss values were normalized respect to whole plant dry weights or leaf areas taken at the end of the measurements.

2.1.7 K^+, Na^+ and Cl^- contents

Two plants per plot were collected at the beginning of fruit harvest, on May 7th (36 DAS), for ion determinations. Three hundred mg of dried and ground plant tissues were mixed with 30 ml of deionized water, shaken for 24 hours at 25 ºC and then centrifuged at 10.000 rpm for 5 min. Subsequently, the supernatant was collected and filtered through Whatman paper no. 1 (0.45μm Ø). A capillary electrophoresis system (Beckman P/ACE 5500, Pegasus scientific, Rockville, MD, USA) was used for the quantification of Cl^-, Na^+ and K^+ according to the method described in Dinelli et al. (1998), and Orsini et al., (2011). Ions were quantified using conventional 50cm long (from injection point to detector) untreated fused silica capillaries (75 μm internal diameter, Beckman, Rockville, MD, USA) at a constant temperature of 25 ºC. The indirect detection wavelength was 220 nm.

The applied voltage was -20 and 20 kV for anion and cation separation, respectively. The electrolyte buffer employed for the determination of the anions was 1.8 mM potassium dichromate, 34 mM boric acid, 14 mM sodium borate, and 1 mM diethylenetriamine. The electrolyte buffer for K^+ and Na^+ determination was 40 mM citric acid and 23 mM imidazole. Ion concentration was expressed as mg g^{-1} dry weight.

2.2 Experiment 2

2.2.1 Plant material and growth conditions

This experiment was conducted in a growth chamber with a photosynthetic photon flux of 500 μmol m^{-2} s^{-1} from cool-white fluorescent bulbs and a 16-h light/8-h dark photoperiod. Day and night temperatures were set at 22 ºC and 19 ºC, respectively. Plantlets were transplanted on September 10th, 2010 into plastic pots of 1.5 l volume (1 plant each pot) filled with a mix of perlite and pumice (2:1 v/ v). As in the first experiment, plants stolons were removed before salt stress application. Plants were automatically irrigated three times per day by using adequate amount of fertilized water (same composition as in exp.1). Plants were harvested at 90 days after transplanting, on December 9th, 2010. Biomass and leaf area determination were performed as in experiment 1.

2.2.2 Treatments and experimental design

Four treatments were compared, obtained from the factorial combination of 2 cultivars (Elsanta and Elsinore) and 2 NaCl concentrations in the nutrient solution (0 and 40 mM). The EC_w of the two nutrient solutions was 1.75 and 6.50 dS m^{-1}, respectively. The experimental design was a full randomization with plants being the elemental replicates (n = 24). Salt stress was applied starting 40 days after transplanting and was maintained until the end of the experiment.

2.2.3 Plant water relations

Total leaf water potentials (Ψ_t) were determined on six samples per plot at 20 DAS with a dew-point psychrometer (WP4, Decagon Devices, Washington, WA). The osmotic potential (Ψ_π) was estimated on frozen/thawed leaf samples and the pressure potential (Ψ_p) as the difference between Ψ_t and Ψ_π, assuming a matrix potential equal to 0. Leaf osmotic adjustment (*LOA*) was determined as follows: $LOA = \Psi_{\pi 0}\, V_0 - \Psi_\pi\, V$, where $\Psi_{\pi 0}\, V_0$ is the product of (osmotic potential) × (osmotic volume) of unstressed plants and $\Psi_\pi\, V$ is the product of (osmotic potential) × (osmotic volume) of leaves from salinized plants. For each measurement, the osmotic volume was approximated by the corresponding relative water content (*RWC*) value calculated as: *RWC* = (leaf fresh weight – leaf dry weight) / (leaf saturated weight – leaf dry weight). Leaf saturated weight was determined after leaf immersion in distilled water for 24 hrs (Orsini et al., 2010b).

3 Statistical analysis

Data were analyzed by ANOVA. Relationships between salt concentration in the irrigation water and plant dry weight, leaf area and yield were evaluated by regression analysis.

4 Results

4.1 Plant growth in response to salinity

Both cultivars were significantly affected by salinity. The regression analysis identified highly significant differences ($P \leq 0.001$) in both intercepts and slopes of the linear functions defined by LA and DW in response to salinity, with Elsanta being relatively more tolerant than Elsinore in terms of leaf area (LA) and dry biomass (DW) (Fig. 2-1). Elsinore plants had a 55% and 46% reduction of LA and DW respectively at 20 mM NaCl (Fig. 2-1). However, these plants did not tolerate the highest NaCl concentration (40 mM) tested in this experiment and virtually died at 60 DAS. In contrast, Elsanta plants presented only limited leaf damages at 40 mM NaCl, although they had

68% and 52% reductions in LA and DW, respectively. For both cultivars, the leaf number was not affected after 36 days of salinization (Table 2-1), whereas a general decrease in the number of leaves was observed at the end of the experiment (Fig. 2-2, Table 2-1). Elsinore presented higher yield respect to Elsanta in absence of stress (Fig. 2-3). Nevertheless, upon salinization, the yield was reduced by 32% and 16% in Elsinore and Elsanta, respectively.

A similar response was observed in the growth-chamber experiment (data not shown): at 40 mM NaCl, Elsinore plants were irreversibly damaged, whereas Elsanta presented only moderate damages with a 21% and 50% decrease in DW and LA, respectively.

4.2 Na^+, K^+ and Cl^- distribution

A different pattern of ion accumulation in plant organs was observed in the two cultivars. At increasing salinity, the concentration of K^+ was not significantly affected respect to control plants (ranging between 30 and 40 mg g^{-1} DW in leaves and 10-24 mg g^{-1} DW in the crown, Table 2-1), whereas Na^+ and Cl^- concentrations increased, reaching the highest values in Elsinore (Table 2-1; Fig. 2-4). Comparable concentrations of Na^+ and Cl^- were found in leaves and crowns of Elsanta. In contrast, the concentration of Na^+ in Elsinore was much higher in crowns, whereas Cl^- mainly accumulated in the leaves (Fig. 2-4). The tissue accumulation of Na^+ and the competition with K^+ altered the K^+: Na^+ ratio, which was reduced much more in both leaves and crowns of Elsanta than Elsinore at the highest salinity (Table 2-2). With the exception of an high value (4.4) for Elsinore at 40 mM NaCl, which was actually not statistically different from the value at 20 mM for the same cultivar (2.1), the K^+:Na^+ ratio was always higher in both leaves and crowns of Elsanta. Considering that no major differences were found in terms of K^+ accumulation, these results are consistent with the presence of more efficient root Na^+ exclusion mechanisms in Elsanta compared to Elsinore (Table 2-2).

4.3 Water relations and leaf gas exchanges

Salinity significantly reduced stomatal conductance (g_s), leaf transpiration (E) and net photosynthesis (A) (Table 2-1, Fig. 2-6). At 2 DAS, g_s was 20% lower than control

plants in Elsanta exposed to 10 and 20 mM NaCl (Fig. 2-5). For these plants, an additional 39% reduction was observed upon 40 mM NaCl stress. In contrast, g_s was affected in Elsinore only at 40 mM NaCl (46% decrease). After 58 DAS, g_s was reduced of -89 and -82% in Elsanta and Elsinore, respectively. However Elsanta plants were not further affected by salinity, whereas 49% and 88% reductions of g_s were observed in Elsinore at 10 and 20 mM NaCl, respectively. Plant transpiration (*E*) followed a similar pattern as g_s (data not shown).

The photosynthetic activity (*A)* decreased of 20% in Elsanta at 10 mM NaCl (at 2 DAS), yet it remained constant upon more severe salinization. The photosynthetic activity in Elsinore was not affected in the short term. Interestingly, at 58 DAS, no differences among the cultivars were observed for *A* at 0 and 10 mM NaCl. The photosynthetic activity was virtually absent in Elsinore at higher salinity, while only a 56% reduction was found in Elsanta. The transpiration efficiency (A/E) at 2 DAS was similar in Elsanta and Elsinore under control conditions (6.9 *vs.* 5.6 mM CO_2 mM H_2O) and at 40 mM NaCl (8.2 *vs.* 8.5 mM CO_2 mM H_2O). In contrast, at 58 DAS, A/E was higher in Elsanta respect to Elsinore under both control (10.5 *vs.* 4.4 mM CO_2 mM H_2O) and 40 mM NaCl (9.9 mM CO_2 mM H_2O in Elsanta; non-detactable in Elsinore).

These findings were substantiated when whole-plant water loss was monitored over 24 hours in salt-acclimated plants (Fig. 2-6). Under control conditions, the average water loss in Elsinore during the central hours of the day (between 12:00 PM and 3:00 PM) was approximately 1.5 fold higher than Elsanta. Upon salinization, water loss was moderately affected in Elsanta plants, whereas a substantial decrease was observed in Elsinore at all salinity levels tested. Consistently, the daily average of whole-plant water loss was higher in Elsinore as compared to Elsanta at 0, 10 and 20 mM NaCl and it declined sharply at increasing salinity. In contrast, the daily plant water loss was rather constant in Elsanta and was greater than Elsionre at 40 mM NaCl (Fig. 2-7).

The number of stomata per leaf area unit was 1.5-fold higher in Elsinore compared to Elsanta (Fig. 2-8). In addition, stomatal density was not affected by salinity in Elsinore, whereas in Elsanta the number of stomata per unit leaf area decreased by 25% and 21% at 10 and 20 mM NaCl, respectively.

Similar Ψ_t were found in the two cultivars under control conditions, while a greater reduction of Ψ_t was observed in Elsinore when salt was applied for 20 days (Fig. 2-9).

These results were in line with the greater water loss experienced by these plants upon salinization. In absence of stress, a moderately lower Ψ_π was observed in Elsinore compared to Elsanta, which could have actually facilitated adaptation to the oncoming stress in the former. However, the similar Ψ_π decay rate in response to salinity reflected the inability of Elsinore to effectively adjust to the hyperosmotic environment, as confirmed by the lower Ψ_t, and RWC values in Elsinore and the similar LOA between the two cultivars (Fig. 2-9).

5 Discussion

5.1 A functional leaf area is associated with yield improvement in salt stressed strawberry plants

A diverse degree of salt tolerance among strawberry cultivars has been documented (Yilmaz and Kina, 2008; Turhan et al., 2008; Keutgen and Pawelzik, 2009). However, the functional basis of such diversity has rarely been linked to physiological and genetic determinants (Turhan and Eris, 2005). Upon salt stress, yield was significantly reduced in the cultivar Elsinore, whose leaf area was much more affected than Elsanta at increasing salinity (Fig. 2-1). Specifically, salinity reduced the leaf number rather than the mean leaf area in a time-dependent manner. At 36 DAS the number of leaves was similar for the two cultivars and only later, at 67 DAS, a rapid decay of the number of leaves in Elsinore was observed (Fig. 2-2). Leaf area reduction is a common response in salt stressed plants, including strawberry (Saied et al., 2005; Yilmaz and Kina, 2008). On a plant basis, smaller leaf areas could be a consequence of stress induced inhibition of cell division (Verslues and Zhu, 2007) and photosynthetic activity (Yilmaz and Kina, 2008), both of which would affect the overall plant development (Keutgen and Pawelzik, 2009). However, leaf area reductions in salinized plants could also be determined by an anticipated leaf abscission as a consequence of hormone-mediated senescence and/or ion toxicity (Munns and Tester, 2008; Turhan and Eris, 2005). The patterns of Na^+ and Cl^- accumulation suggests that the observed leaf abscission was likely due to a faster and/or greater leaf ion accumulation in Elsinore which in turn manifested anticipated toxicity symptoms, including tissue necrosis and premature senescence (Fig. 2-4). Maintenance of a large leaf area upon saline stress may be critical to guarantee production, availability and

translocation of photosynthates to the fruit (Keutgen and Pawelzik, 2009) and it has been shown to benefit the final yield in strawberry. Saied et al. (2005) have demonstrated that upon exposure to 60 mM NaCl (EC_w=5.1 dS m^{-1}) the leaf area of the cv. Korona was reduced by 13% respect to the non-salinized control *vs.* 34% reduction of Elsanta, with the former being more tolerant, on a relative basis, in terms of fruit fresh weight per plant. Similarly, despite the lower yield of Elsanta compared to Elsinore in absence of stress (165 *vs.* 220 g $plant^{-1}$) the former was much less affected by salinity, as indicated by the slopes of the regression lines for yield *vs.* NaCl concentration (Fig. 2-3). Due to the constitutive (genetically determined) higher yield of Elsinore compared to Elsanta, the two cultivars had still comparable yield at high salinity tested; therefore these results must be interpreted in relative terms. Indeed, after a moderate yield reduction (-14%) at 10 mM NaCl, the fruit yield of Elsanta remained virtually unaffected up to 40 mM NaCl whereas it declined significantly in Elsinore. These results suggest that the maintenance of a functional leaf area under saline stress may beneficial for cultivars perscribed to be used exclusively in marginal environments in terms of salinity and/or drought. However, since a reduced stomatal density may partially compensate the trade-off between plant growth and adaptation (Husaini and Abdin, 2008; Ouyang et al., 2010), this could be an important stress tolerance trait that may have been lost in the selection of elite cultivars.

5.2 Control of ion Na^+ and Cl^- fluxes and organ distribution

Strawberry can be considered a Na^+ excluder since it maintains low tissue Na^+ contents at increasing salinity, as demonstrated in several cultivars including Elsanta (Saied et al., 2005). The mechanism(s) through which Na^+ exclusion is achieved may include ion selectivity at root level and translocation/compartmentalization in non-photosynthesizing tissues (Tester and Davenport, 2003). Consistent with the existence of exclusion mechanisms, the leaf Na^+ concentration did not increase at advanced salinization in both cultivars (Fig. 2-4). In addition, the increased Na^+ concentration in the crown indicated that the protection of photosynthesizing tissues from Na^+ accumulation occurred via re-translocation and/or compartmentalization in this anatomical region (Munns and Tester, 2008). The mechanism of Na^+ exclusion from the shoot was more efficient in Elsanta, however, which had a relatively lower concentration of Na^+ in both leaves and

crown respect to Elsinore at increasing salinity (Fig. 2-4). This difference was consistent with a significantly higher K^+/Na^+ ratio observed in Elsanta (Table 2-2), which could be explained by exclusion/selective mechanisms at root level that may have restricted more efficiently Na^+ flux to the shoot in Elsanta (Hasegawa et al., 2000; Tester and Davenport, 2003). Moreover, the lower leaf and crown Na^+ levels of Elsanta could be the result of a reduced ion uptake by the roots and/or reduced transpiration and consequent restricted ion fluxes to the shoots in the former (Turhan and Eris, 2005). This hypothesis is substantiated by the pattern of Cl^- accumulation in the two cultivars (Fig. 2-4). In contrast to Na^+, which can be partially controlled at root level, leaf Cl^- concentrations increase progressively (Prior at el., 2007), most likely because Cl^- typically follows the transpiration flux (Lohaus et al., 2000; Maggio et al., 2007). Cl^- accumulation rates in Elsanta and Elsinore were consistent with their transpiration rates, which were lower in the former either in control or salinized plants (Figs. 2-5, 2-6 and 2-7). The relationship between plant growth and transpiration-driven ion flux to the shoot has been formally addressed by Dalton et al. (2000) who also proposed that a reduced transpiration rate would restrict the accumulation of Cl^- to the shoot and consequently delay the onset of a critical toxicity threshold (Moya et al., 1999; Dalton et al., 2000, 2001; Maggio et al 2002a). Although we cannot rule out the existence of different mechanisms for Na^+ and Cl^- detoxification at cellular and organ levels in both Elsanta and Elsinore, it is worth emphasizing that the transpiration-mediated restriction of Cl^- flux to the shoot (Gilliham and Tester, 2005) turned out to be an effective *escaping* strategy that delayed the shoot damages respect to the reproductive stage and allowed plants to maintain high photosynshtetic rates (Tavakkoli et al., 2011).

5.3 Low stomatal density and reduced transpiration facilitate strawberry adaptation to salinity

Most work on breeding and genetic engineering for salt tolerance has given minor attention to the functional link between transpirational flux and shoot ions accumulation as potential target to improve tolerance. This is because the beneficial effects due to a reduced transpiration under stress (e.g. re-establishment of tissue turgor; delayed ion accumulation) would also limit photosynthesis and consequently and consequently yield (Condon et al., 2002). Nevertheless, *field* and/or *soilless* salinization is a dynamic process

that evolves over a growth season, since it is mostly associated to irrigation (De Pascale et al., 2005) and as such it must be considered respect to the *species-specific* developmental pattern. In this respect, our data indicate that reduced gas exchanges early in the season may partially impair the photosynthetic activity (Fig. 2-5) but may also protect plants from a rapid dehydration (Fig. 2-9), which moreover would also activate a series of stress signals that would further affect plant growth (Maggio et al., 2002; Ruggiero et al., 2004). Over the entire crop cycle, a constitutive plant stress pre-adaptation status, associated with reduced levels of leaf gas exchanges, may have an important role in extending the functionality of the photosynthetic leaf surface in coincidence with fruit-set and fruit growth. Elsanta plants had a constitutive reduced daily transpiration compared to Elsinore (Fig. 2-7). This may have contributed to *pre-adapt* plants in the long run (growth season), as indicated by the reversed response of Elsanta and Elsinore in terms of daily water loss (Fig. 2-7) and photosynthesis (Fig. 2-5) at advanced salinization. The instantaneous measurements of stomatal conductance and leaf transpiration were consistent with a better hydration state of Elsanta compared to Elsinore and in general reflected the gravimetric measurements performed on a whole plant basis (Fig. 2-7) (i.e. the opposite response of Elsanta and Elsinore at 0 and 40 mM NaCl was confirmed). However, the apparent inconsistency between different water loss on a whole plant basis and similar g_s between the two cultivar in absence of salt (control), revealed some limitations of instantaneous gas exchange measurements that could be affected by many variables, including heterogeneity of stomatal opening (patchiness) and light conditions (Eisinger et al., 2000) and time of measurement. This is an important aspect to be considered either when gas exchange measurements are used for rapid screening of germplasm collections (Ashraf, 2004) or when these values are integrated into more complex models for predicting plant water use.

The control of transpiration water fluxes under drought or salinity stress has recently gained a renovated interest due to identification of genetic determinants regulating stomatal patterning, leaf gas exchanges and water use efficiency in stressed plants (Chartzoulakis and Klapaki, 2000; Sade et al., 2010). Low transpiration rates have been correlated to increased salt tolerance (Maggio et al., 2006) and water deficit tolerance as a result of 25% reduction of abaxial stomatal density (Yoo et al., 2010). In line with these findings, the reduced water loss of Elsanta was also correlated with a 27% reduction

of stomatal density (Fig. 2-8). It is worth emphasizing that the constitutive pre-adaptation state, determined by a reduced stomatal density, seemed to benefit plant growth and yield under saline stress more effectively than a rapid salt-induced stomatal closure. Turhan et al. (2008) have demonstrated that exposure to NaCl caused a prompt g_s reduction in the more sensitive cultivar Camarosa, whereas salinity up to 34.0 mM NaCl did not have any influence on g_s of the more tolerant Tioga and Chandler, further indicating that stomatal closure may act as signal to activate multiple stress responses, including plant growth inhibition, which may be deleterious in terms of yield (Awang and Atherton, 1995a; Maggio et al., 2002b; Ruggiero et al., 2004).

6 Conclusion

The results of this study indicated that low stomatal density, and the consequent constitutively reduced transpirational flux, is a critical stress tolerance determinant that may allow plants to adapt more effectively to salinity. The reduced transpiration rate of the cultivar Elsanta was functional *to delay* the accumulation of Cl^- ions to the shoot (and their effects on leaf senescence) and *to ameliorate* plant water status under stress. At low/moderate salinity, Na^+ exclusion systems may have also contributed to sustain salt stress adaptation in Elsanta.

Acknowledgements

We wish to thank Salvi vivai for providing the plant material. We acknowledge the support of Prof. Giovanni Dinelli and Dr. Mattia Accorsi for the ion analyses.

7 Tables

Table 2-1: Summary ANOVA table for the parameters under assessment in two strawberry cultivars, Elsanta and Elsinore, in response to four salinity treatments (0, 10, 20 and 40 mM NaCl). DAS = days after stress treatment initiation; g_s = stomatal conductance; E = leaf transpiration; A = net photosynthesis; ns = non significant differences; * = significant differences at P≤0.05; ** = significant differences at P≤0.01; *** = significant differences at P≤0.001.

Variable			DAS	Block (Bl)	Salt	Cultivar (C)	S x C
Leaf number			36	ns	ns	ns	ns
			67	ns	ns	***	*
Ion concentration	K^+	Leaf	36	ns	ns	ns	ns
		Crown	36	ns	ns	ns	ns
	Na^+	Leaf	36	ns	***	**	**
		Crown	36	ns	***	***	**
	Cl^-	Leaf	36	ns	***	**	ns
		Crown	36	ns	***	ns	ns
Leaf gas exchanges	g_s		2	ns	**	**	ns
			58	ns	**	**	**
	E		2	ns	**	*	ns
			58	ns	**	**	**
	A		2	ns	**	**	*
			58	ns	**	*	**
Water loss	Per plant		10	***	***	*	***
	Per unit DW		10	***	***	***	***
Stomata	Length		15	ns	***	***	ns
	Density		15	ns	***	***	***
Water relations	Ψ_t		20	ns	**	***	***
	Ψ_π		20	ns	***	***	ns
	RWC		20	ns	ns	***	**
	LOA		20	ns	nd	ns	nd

Table 2-2: Effect of salt stress (0, 10, 20 and 40 mM NaCl) K^+/Na^+ ratio in leaf and crown of two strawberry cultivars (Elsanta and Elsinore).

NaCl (mM)	**Elsanta**		**Elsinore**	
	Leaf	Crown	Leaf	Crown
0	14.6	12.9	8.8	6.8
10	3.7	3.0	2.4	0.9
20	5.5	2.4	2.1	0.8
40	2.8	0.8	4.4	0.3

8 Figures

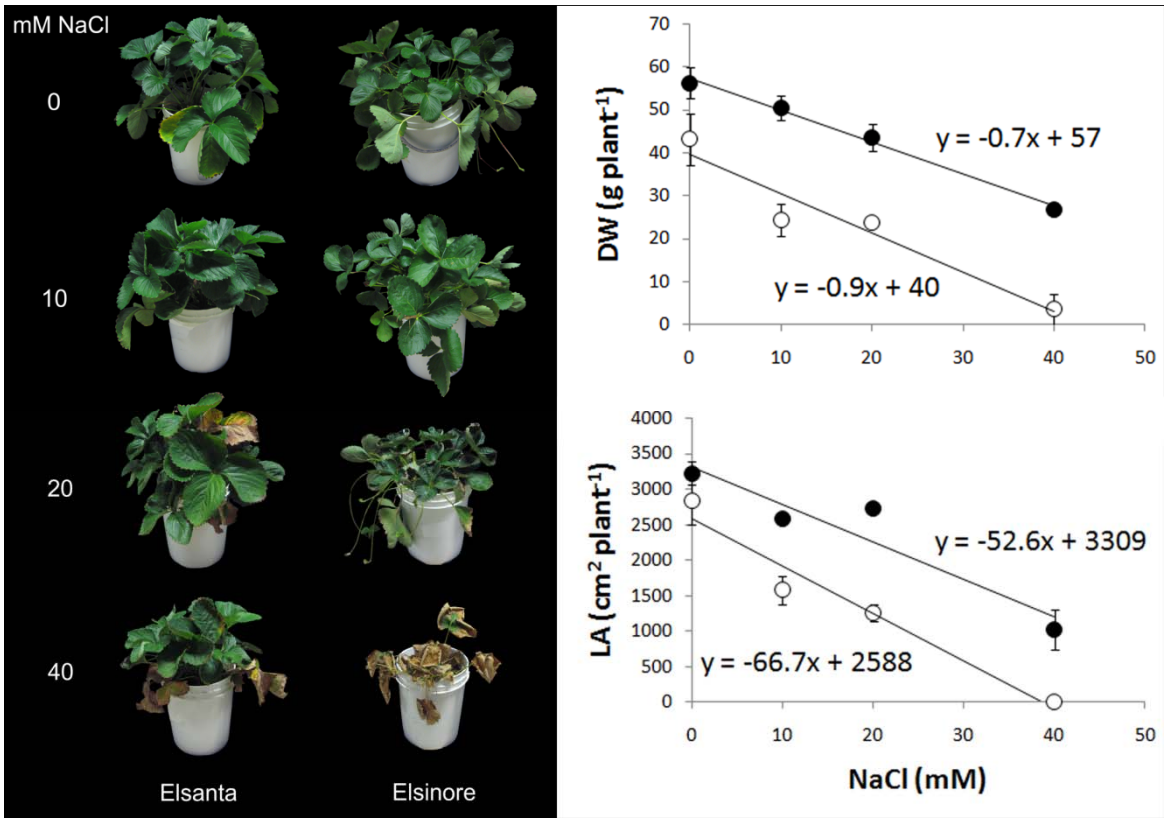

Figure 2-1: Effect of saline treatments (0, 10, 20 and 40 mM NaCl) on dry weight and leaf area in two strawberry cultivars, Elsanta (closed circles) and Elsinore (open circles). At the time of measurements plants were 111 days old. Equations indicate regression lines. Mean values ± SE (n = 9).

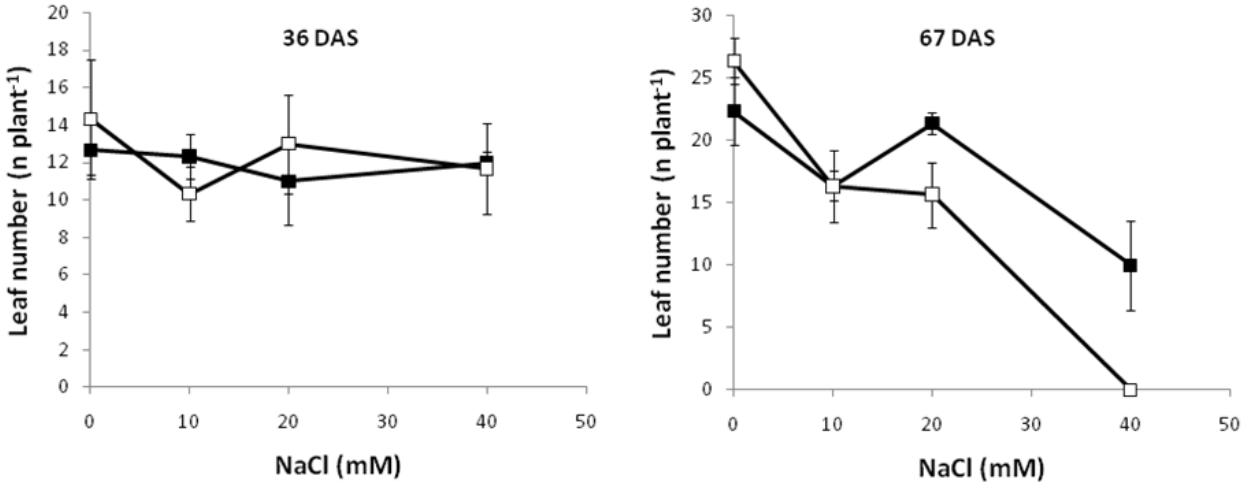

Figure 2-2: Leaf number of two strawberry cultivars, Elsanta (closed circles) and Elsinore (open circles), after 36 and 67 days of growth at different NaCl concentrations (0, 10, 20 and 40 mM) in the nutrient solution. Mean values ± SE (n = 9).

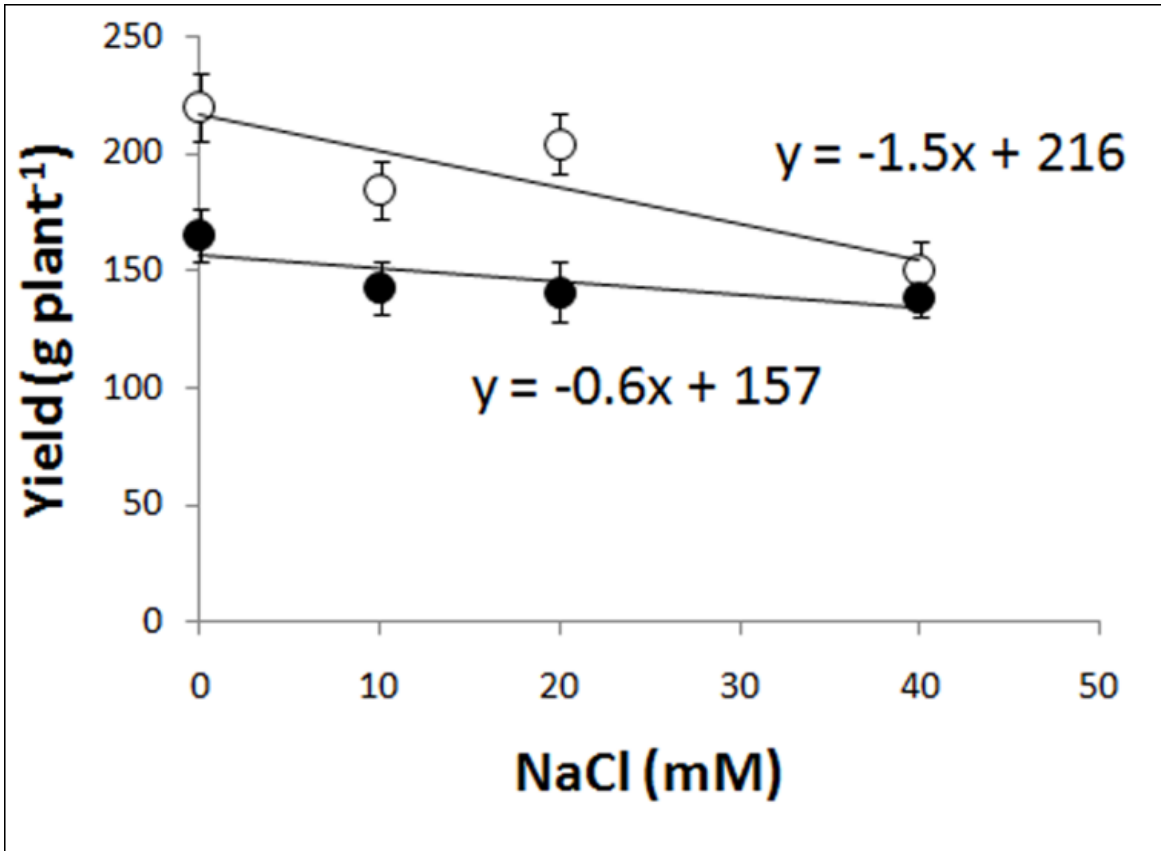

Figure 2-3: Fruit yield of two strawberry cultivars, Elsanta (closed circles) and Elsinore (open circles), grown at different NaCl concentrations (0, 10, 20 and 40 mM) in the nutrient solution. At the time of measurements plants were 111 days old. Equations indicate regression lines. Mean values ± SE (n = 18).

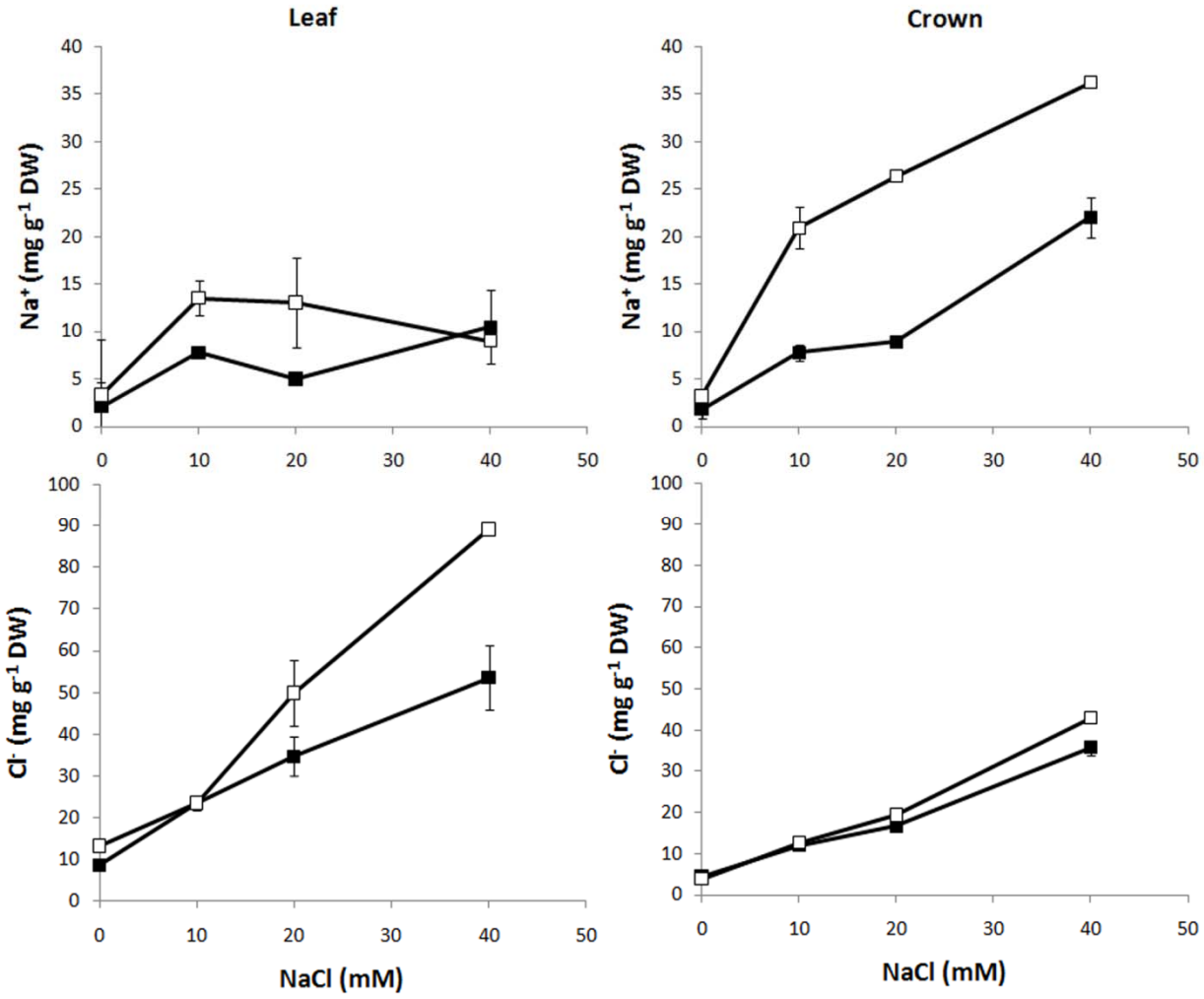

Figure 2-4: Ion contents in leaves and crowns of two strawberry cultivars, Elsanta (closed squares) and Elsinore (open squares), grown at different NaCl concentrations (0, 10, 20 and 40 mM) in the nutrient solution for 36 days. At the time of measurements plants were 80 days old. Mean values ± SE. (n = 6).

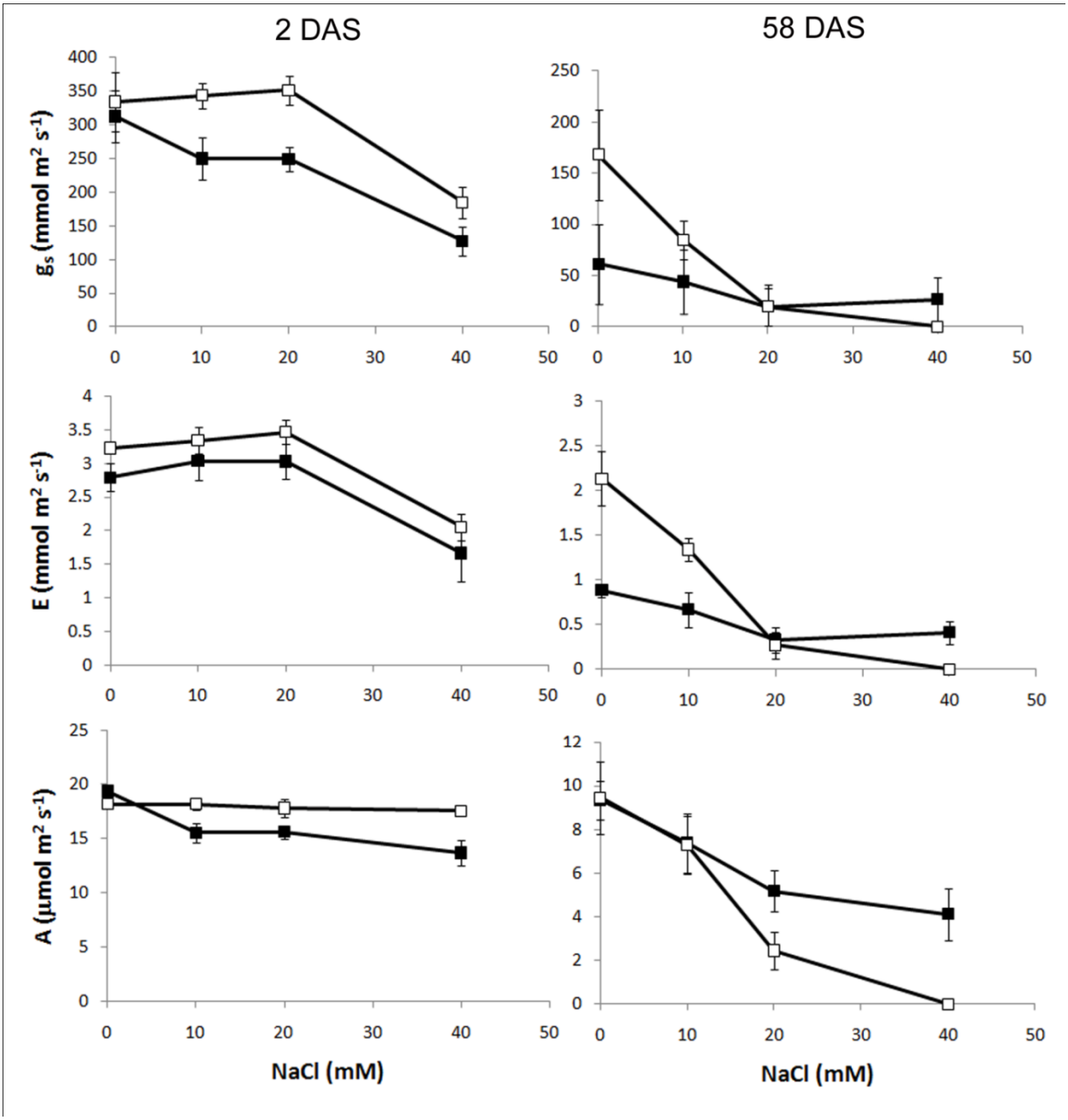

Figure 2-5: Leaf gas exchanges in two strawberry cultivars, Elsanta (closed squares) and Elsinore (open squares), exposed to saline irrigation (0, 10, 20 and 40 mM NaCl). DAS = days after salt treatment initiation; g_s = stomatal conductance; E = leaf transpiration; A = net photosynthesis. Mean values ± SE (n = 6).

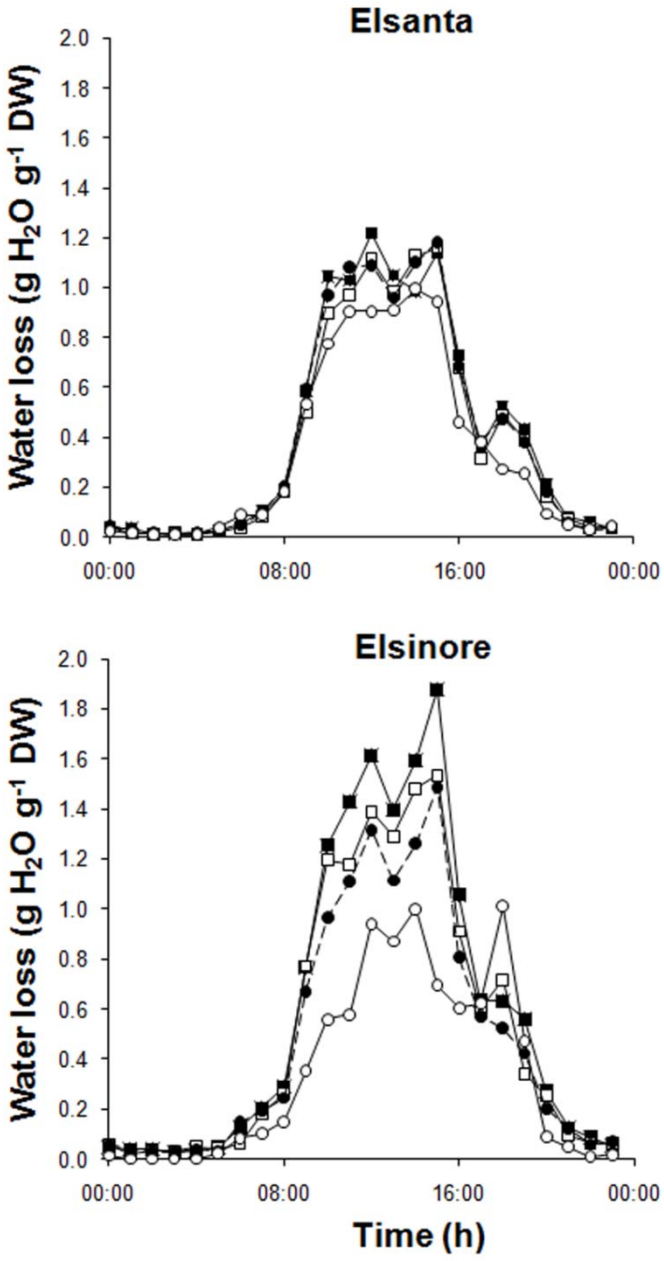

Figure 2-6: Daily pattern of water loss in two strawberry cultivars, Elsanta and Elsinore, irrigated with 0 (closed squares), 10 (open squares), 20 (closed circles) and 40 (open circles) mM NaCl solutions. At the time of measurements plants were 54 days old. Measures were carried out after ten days of irrigation with different NaCl solutions. Plants were grown singularly in 5 l pots, which were sealed in plastic wrap and placed on electronic balances. Water loss was determined every 60 min for 1 day. Mean values ± SE (n = 9).

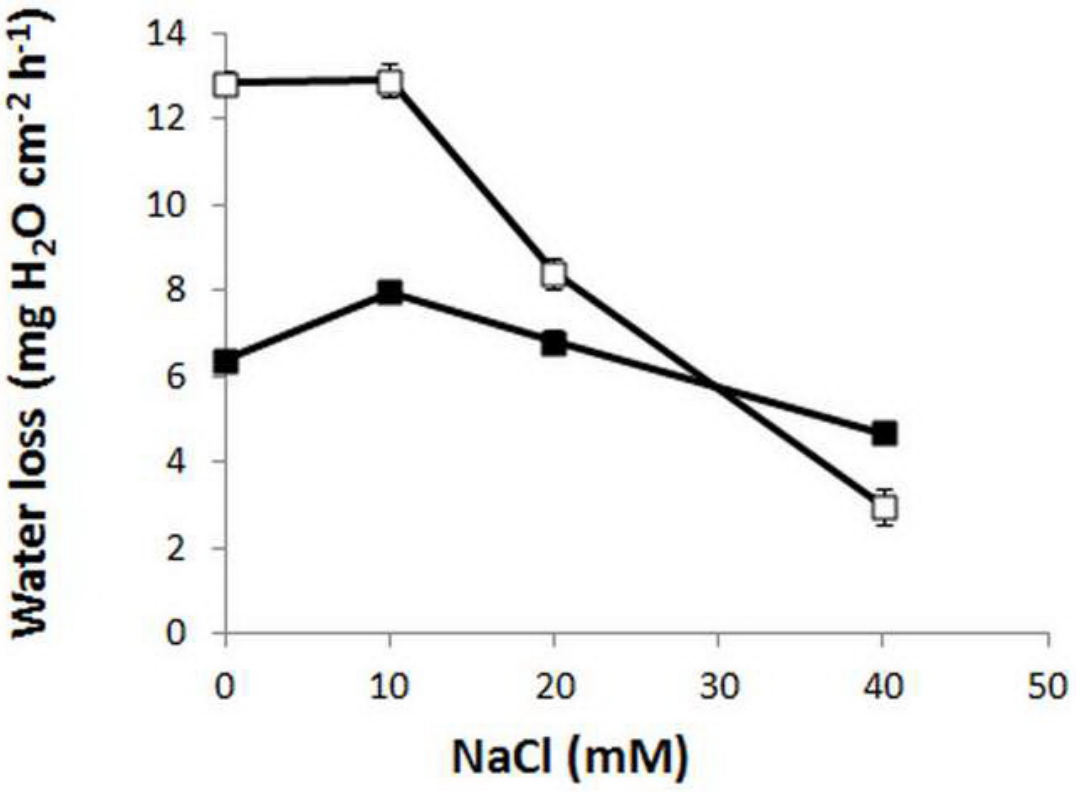

Figure 2-7: Daily average whole-plant water loss in two strawberry cultivars, Elsanta (closed squares) and Elsinore (open squares), after ten days of saline irrigation (0, 10, 20 and 40 mM NaCl). At the time of measurements plants were 54 days old. Plants were grown singularly in 5 l pots, which were sealed in plastic wrap and placed on electronic balances. Water loss was determined every 60 min for 1 day. Mean values over the 24-hours are shown (n = 9).

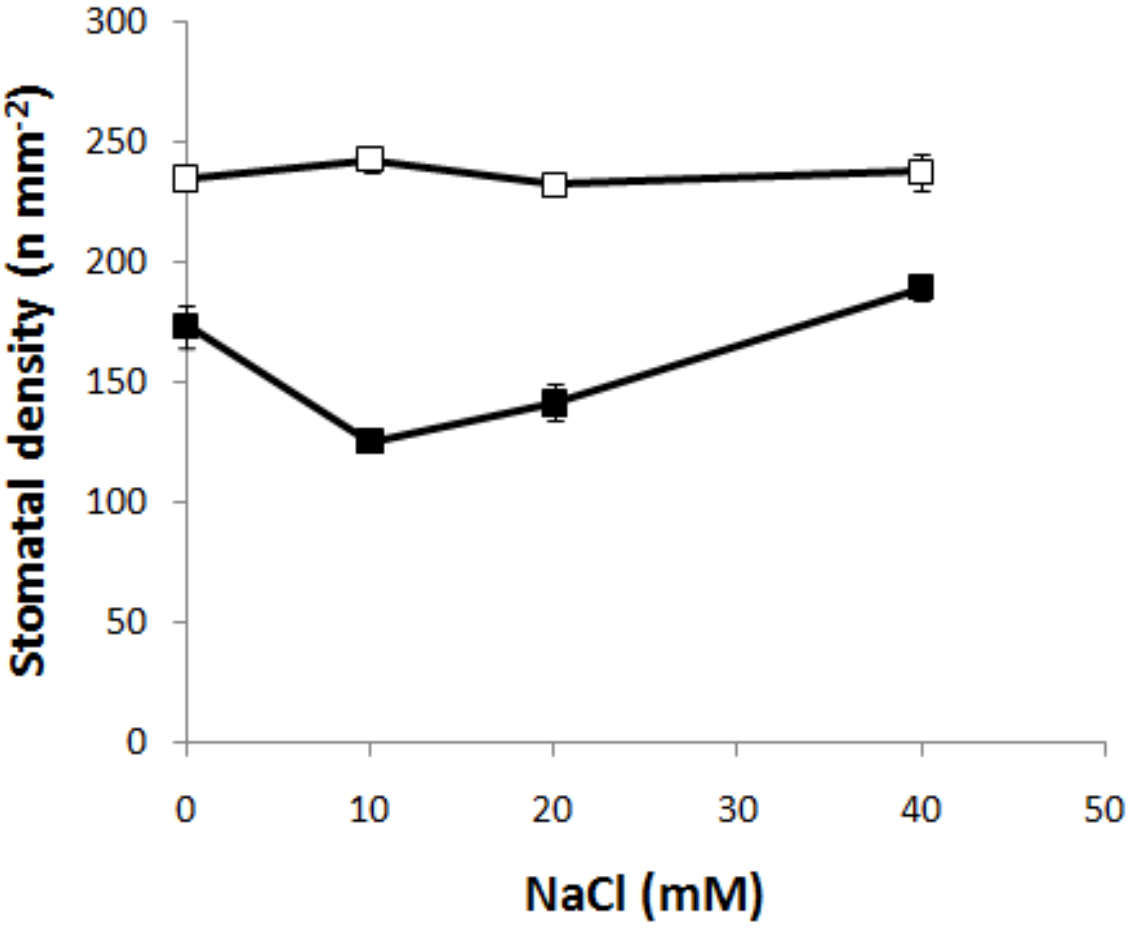

Figure 2-8: Stomatal density in two strawberry cultivars, Elsanta (closed squares) and Elsinore (open squares), exposed to saline irrigation (0, 10, 20 and 40 mM NaCl) for 15 days. At the time of measurements plants were 59 days old. Mean values ± SE (n = 9).

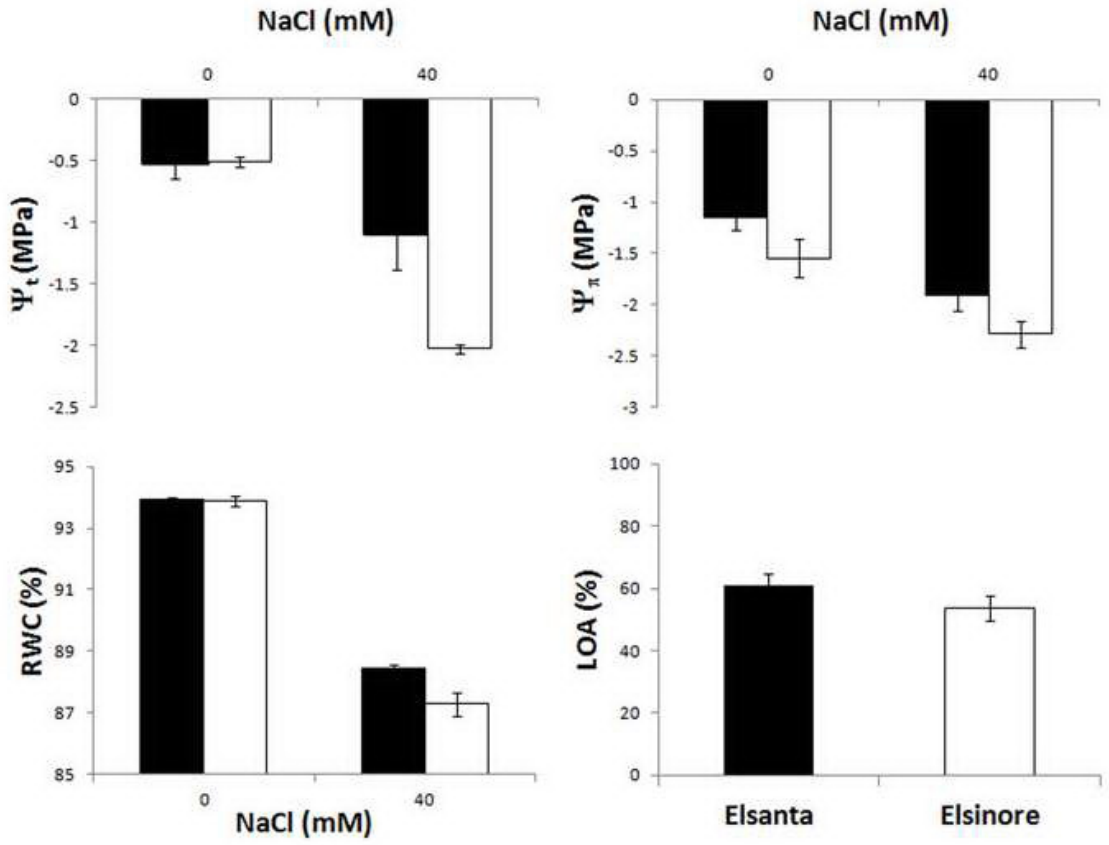

Figure 2-9: Water potential (Ψ_t), osmotic potential (Ψ_π), relative water content (RWC) and leaf osmotic adjustment (LOA) in two strawberry cultivars, Elsanta (closed symbols) and Elsinore (open symbols), grown in a growth chamber under cool-white fluorescent light and irrigated with saline water (0 and 40 mM NaCl) for 20 days. At the time of measurements plants were 60 days old. Mean values ± SE (n = 6).

Response of endogenous proline, total soluble proteins, lipid peroxidation, and antioxidative enzymes in leaves of two strawberry cultivars (Elsanta and Elsinore) to long- terms of salt stress

1 Introduction

In plants, salt stress leads to an enhanced generation of reactive oxygen species (ROS), such as superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2). Double functions for ROS in plants have been proposed, as they serve as key regulators of growth, development, and defense pathways, as well as at excessive levels they cause oxidative damage to fundamental cellular components, such as membranes, proteins and nucleic acids (Miller et al., 2010; Mittler et al., 2004). The detoxification of excess ROS is achieved by an efficient antioxidative system comprising of the non enzymatic system, such as ascorbate, phenolics, proline and soluble proteins and the enzymatic system, such as superoxide dismutase (SOD), Catalase (CAT) and peroxidase (POD) (Gill and Tuteja., 2010). Change in the activities of antioxidant enzymes and the levels of some nonenzymatic antioxidants were assessed for their use as markers of salt tolerance in different plant species (Sharma et al., 2011). The maintenance of a high antioxidant capacity to scavenge the toxic reactive oxygen species (ROS) has been linked to plants tolerance increase of abiotic stress (Agarwal and Shaheen., 2007; Zaefyzadehet et al., 2009).

In strawberry, moderate salinity induced an increase of antioxidant activity in cv. Korona and cv. Elsanta (Keutgen and Pawelzik., 2007). The effect of salinity on enzymatic antioxidant systems has been studied on different strawberry organs. On fruit, on the one hand, an increment of superoxide dismutase (SOD) activity in the fruit of cv. Korona under salt condition was observed (Keutgen and Pawelzik., 2007). On leaf, on the other hand, SOD activity did not change in cv. Selva while POD activity was enhanced under salt condition (Tanou et al., 2009). CAT activity decreased in response to salt condition in cv. Tioga, while in cv. Camarosa and cv. Chandler no modifications were observed (Turhan and Eris., 2008). The level of lipid peroxidation has been widely used as an indicator of free radical mediated damage to cell membranes under stressful conditions

(Sharma et al., 2011). Many investigations showed that the content of malondialdehyde (MDA), one of the end-products of lipid peroxidation, increased in response to salinity in fruit as well as in leaves of strawberry cultivars (Keutgen and Pawelzik., 2008; Tanou et al., 2009). One of the most common mechanisms enabling plants to cope with salt and drought stress is the accumulation of intercellular solutes, such as free amino acids and sugars (Heuer., 2011). Proline has been proposed to contribute to osmotic adjustment, the detoxification of ROS, buffer cytosolic pH, and the protection of membrane integrity. In addition, this amino acid acts as a carbon and nitrogen storage (Heuer., 2011; Gill and Tuteja., 2010). It has also been suggested to consider proline as a selection criterion for breeding programs (Heuer., 2011). In strawberry cultivars, a dramatic accumulation of proline following salt stress was observed (Tohoma and Esitken., 2011; Rahimi and Biglarifard., 2011; Keutgen and Pawelzik., 2008). Proteins might function as compatible cytoplasmic solutes in osmotic adjustment in order to equalize the osmotic potential of the cytoplasm with the vacuoles in adverse conditions of salinity (Greenway and Munns., 1980; Dubey and Rani., 1989). The level of proteins differs in salt-tolerance and salt-sensitive genotypes when they are subjected to salinity stress (Sharma and Dubey., 2011; Mahmoodzadeh., 2009; Tada and Kashimura., 2009). However the overall mechanism of how these proteins could provide adaptation is not clearly understood. (Sharma and Dubey., 2011).

Enzymatic activities and osmolytes accumulation in two cultivars of strawberry leaves (Elsanta and Elsinore) under long-term salt stress were addressed in this study. Therefore the present study is mainly aimed to differentiate cultivars response to different levels of salinity as a function of enzymes activities and solutes accumulation, such as proline and soluble proteins.

2 Materials and methods

2.1 Experimental design and growth conditions

(Please see chapter 2)

2.2 Plant materials

Plant material for determinations was collected from three plants of each cultivars and treatments with exception of cv. Elsinore undergoing 40 mM NaCl condirtion, since all plants died at the end of the experiment. Adequate amounts of leaf samples were weighed and ground in liquid nitrogen by pre-cooled mortar and pestle and then stored at – 80 °C to be used for enzymes analysis.

2.3 Enzymatic activities

2.3.1 Catalase

Catalase activity (CAT) was measured according to Havir and McHale (1987) assay. About 0.5 g of frozen leaf powder was suspended in 5 ml of extraction buffer (NaKPi 100 mM, pH 7.0 – 4 °C). Then 1% of polyvinylpyrrolidone (PVPP) was added. For enzyme extraction, the mixture was incubated in ice for 30 minutes, and then centrifuged at 10000 x g for 30 minutes at 4 °C. The supernatant was desalted by elution buffer (NaKPi 50 mM, pH 7.0 – 4 °C) on a Sephadex G-25 gel column (NAP-25, Amersham Biosciences). Ten µl of the desalted sample was added to 0.95 ml of 10 mM H_2O_2 in elution buffer, and the kinetics was followed at λ=240 nm during the 2nd minute. The activity was calculated on the maximum slope, assuming an extinction coefficient of 0.036 $mM^{-1}cm^{-1}$. One catalase unit is defined as the amount of enzyme to decompose 1 µmol of H_2O_2 to water and oxygen per minute. The analysis was done in triplicate and the results were expressed as unit/g FW.

2.3.2 Superoxide dismutase

The superoxide dismutase (SOD) assay relies on the ability of SOD to inhibit the photochemical reduction of colourless NTB (nitro blue tetrazol) to blue formazan by flavins under illumination (Masia., 1998). Enzyme extraction was carried out as described

for catalase description. Glass tubes containing 10, 20, 40, 60, 80 and 500 µl of desalted extract were prepared, then the reaction mixture (2 µM riboflavin 10 mM methionine, 50 µM nitroblue tetrazolium, 20 µM KCN, 6.6 mM Na_2EDTA, final concentrations) and buffer (65 mM NaPi, pH 7.8) were added to a 3 ml final volume. The reaction was started by illumination under 4 fluorescent lamps for 30 minutes. Absorbance was measured at λ=560 nm. One superoxide dismutase unit is defined as the amount of enzyme required for 50% inhibition of the NTB reduction in producing blue formazan. The analysis was done in triplicate and the results were expressed as unit/g FW.

2.3.3 Peroxidase

Peroxidase (POD) was assayed as described by Ushimaru et al (1997), using pyrogallol as electron donor. The plant material was extracted in cold buffer (200 mM NaPi, 5 mM Na_2EDTA, 1% PVPP, pH 7.0) and the supernatant was used for the assay. The reaction mixture (2.5 ml) included 50 mM phosphate buffer, pH 7.0, 0.1 mM H_2O_2, 50 mM pyrogallol, and 100 µl raw extract; H_2O_2 and pyrogallol were prepared fresh just before use. Absorbance (λ=430 nm) was taken after 5 minutes incubation at room temperature and referred to a blank with no extract added. One unit of guaiacol peroxidase is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of pyrogallol min-1 under the described conditions. An absorbance coefficient of 2.47 mM^{-1} cm^{-1} was assumed for calculations.

2.4 Lipid peroxidation

Lipid peroxidation was determined by MDA assay. Malondialdhyde (MDA), the final product of lipids peroxidation particularly of polyunsaturated fatty acids (PUFAs), was spectrophotometerly determined according to the method proposed by Heath and Packer (1968) with slight modifications. Frozen powder of leaf sample was homogenized in 5 ml of 0.1% trichloroacetic acid (TCA) for 10 minutes at room temperature. The mixture was shaken occasionally and centrifuged at 13000 x g for 10 minutes. 1 ml of supernatant was mixed with 4ml of reacting solution (Thiobarbituric acid 0.5% in TCA 20%), and then incubated at 100°C for 30 minutes. The samples were cooled down by tap water to stop reaction and centrifuged at 10000 x g for 5 minutes. The absorbance for each sample was read at λ=532 and λ=600 nm with a Cary. The concentration of MDA was

calculated according to the difference of the two wavelengths, based on standard curve of MDA bisdimthylactale diluted in (0-15 µM) HCl at different concentrations. The results were expressed as µmol MDA/g FW.

2.5 Proline content

Free proline content was determined according to the method proposed by Bates et al (1973) with slight modifications. 0.2- 0.4 g of leaf tissue was ground by pre-cooled pestle and mortar in liquid nitrogen and suspended in 2 ml of distilled water. Tubes were incubated at 100 °C for 20 minutes, cooled down by tap water, and centrifuged at 10000 x g for 5 minutes at room temperature. Supernatant samples and blanks (water) were mixed with 2 ml ninhydrin reagent, incubated at 100 °C for 1 hour and cooled down. Six ml of toluene were added and mixed vigorously; samples were kept in the dark to separate the two phases. Toluene fraction was read twice at λ=520 nm against the blank. Analysis was done in triplicate and the results were expressed as µg proline/g FW.

2.6 Total soluble proteins

The soluble proteins concentration was measured by the method based on bicinchoninic acid (BCA) for colorimetric detection and quantitation of total protein, according to the manufacturer instructions of the Pierce® BCA Protein Assay kit (Thermo Scientific, Rockford, IL).

3 Results

3.1 Lipid peroxidation (MDA)

MDA content in leaf tissues subjected to different salt treatments decreased significantly ($p<0.05$) in both cultivars (Fig. 3-1A). MDA content stayed firmly constant under mild stress up to 20 mM NaCl then decreased dramatically by 33% at 40 mM NaCl in cv. Elsanta while MDA content stepwise decreased by 34% at 20 mM NaCl in cv. Elsinore. A significant difference ($p<0.05$) was detected among cultivars.

3.2 Enzymes activities

Compared to control plants, there was a significant reduction of CAT and SOD activities upon exposure to mild stress in both cultivars (Table 3-1). CAT and SOD activities were 17% and 58% less in cv. Elsanta, where the reduction was 65% and 67% in cv. Elsinore at mild stress (10mM NaCl) (Fig. 3-1B and 3-1C). Concerning CAT activity, dynamic response was detected in cv. Elsinore rather than in cv. Elsanta by increasing the salt concentration, where CAT activity increased 1-fold in cv. Elsinore at 20 mM NaCl and 1-fold in cv. Elsanta at 40 mM NaCl compared to 10mM NaCl for both cultivars (Fig. 3-1B). SOD activity under high salt concentration increased 0.5-fold at 20 mM NaCl in cv. Elsinore whereas it increased 1-fold and 2-fold at 20 and 40 mM NaCl respectively in cv. Elsanta compared to 10 mM NaCl (Fig. 3-1C). POD activity did not change much under mild salinity in cv. Elsinore, whereas various responses were detected in cv. Elsanta. Higher salt treatments significantly increased ($P<0.01$) POD activity by 73% and 14% at 40 mM NaCl and 20 mM NaCl in cv. Elsanta and cv. Elsinore respectively compared to control plants (Fig. 3-1D). However, strawberry genotype revealed significant effect CAT, SOD, and POD activities towards salinity (Table 3-1). Furthermore, interaction of salt treatments and varieties was significant except in the case of SOD activity.

3.3 Proline content

Proline content was significantly affected ($p<0.001$) by salinity treatments (Table 3-1), where proline content decreased sharply by 65% in cv. Elsanta while it decreased by 25% in cv. Elsinore at 10mM NaCl compared to control plants (Fig. 3-2A). Furthermore, cv. Elsinore exhibited greater response to higher salt concentration when proline content increased 3-fold at 20 mM NaCl while increased gradually up to 0.7-fold at 40mM NaCl in cv. Elsanta compared to 10 mM NaCl (Fig. 3-2A). The data indicated that cultivars and their interaction with salt treatments had a significant effect ($P<0.001$) on proline content.

3.4 Total soluble protein

A significant accumulation ($P<0.001$) of soluble protein was detected in both cultivars under salt stress (Table 3-1). Soluble protein content did not change much up to

20mM NaCl but then increased dramatically by 11-fold at 40 mM NaCl compared to control plants in cv. Elsanta, whereas soluble protein sharply accumulated 10-fold at 20 mM NaCl compared to control plants in cv. Elsinore (Fig. 3-2B). The strawberry genotype had significant effect ($p<0.05$) on total soluble protein.

4 Discussion

4.1 Enzymes activity

Salinity and drought stresses disturb the equilibrium of the production and the scavenging of ROS, which may lead to impulsive increase in intracellular levels of ROS that are highly reactive and toxic causing either protective and signaling factors or damage to protein, lipids, and DNA. Eventually programmed cell death depends on the equilibrium at the appropriate site and time (Miller et al., 2008; Gill and Tuteja., 2010; Demiral et al., 2011). Stress-induced ROS accumulation is countered by both enzymatic antioxidant systems, such as SOD, POD and CAT and non-enzymatic systems, such as phenolics, flavonoids, carotenoids and tocopherols. Superoxide dismutase is ubiquitous in all aerobic organisms and provides the first line of defense against the toxic effects of high levels of ROS under biotic and abiotic stresses. The SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (Gill and Tuteja., 2010). In our experimental condition, data showed that plants acted in two different steps in response to salt stress. At the first step, SOD activity decreased dramatically in both cultivars under mild stress, whereas the second one was characterized by an increase of the SOD activity in response to high salt concentration compared to mild stress. Furthermore, a significant difference was detected among cultivars in control condition while similar trend of SOD activity was remarkable in both cultivars under salt treatments. Tanou et al (2009) reported that SOD activity was not affected by the salt stress compared to control plants in cv. Selva of strawberry. Many studies revealed various responses in SOD activity towards salt stress. On cotton, Meloni et al (2003) found that salinity increased SOD activity in cv. Pora while salinity had no significant effect on SOD activity in cv. Guazunch. Furthermore, Gosset et al (1994) suggested that salinity increases SOD activity in salt-tolerant cultivars and decreases it in salt-sensitive cultivars of cotton. On tomato, Shalata

and Tal (1998) reported that SOD activity increased in wild salt-tolerance relative species and decreased in hybrid M82 in salt conditions. On potato, high salt level (200 mM NaCl) increased SOD activity, while 100 mM NaCl did not significantly influence on the SOD activity (Fidalgo et al., 2004). Detoxification of hydrogen peroxide (H_2O_2) in plants is crucial for cell protection and signaling (Apel and Hirt., 2004).

Catalase provides very efficient tools for the gross removal and control of high H_2O_2 levels. CAT activity had similar trend of POD activity where its activity decreased at 10 mM NaCl and then increased upon salt exposure in both cultivars, taking into consideration that CAT activity differed significantly among both cultivars in control and it was higher in cv. Elsinore compared to cv. Elsanta at 20 mM NaCl. Tanou et al (2009) found that CAT activity decreased significantly in strawberry leaves exposed to NaCl. In addition, he proposed that the higher hydrogen peroxide accumulation concurrent with reduction of CAT activity in response to salinity presumed that peroxisomes are sensitive to H_2O_2 accumulated under salt stress. CAT activity in three strawberry cultivars had different trends in response to salt treatments, where CAT activity decreased in Tioga while its activity continued steadily or increased slightly in Camarosa and Chandler (Turhan et al., 2008). In response to salinity, a significant reduction of CAT activity in salt-sensitive genotype of maize and an insignificant effect on CAT activity in salt-tolerant genotype have been reported by (Azevedo Neto et al., 2005).

The coordinated function of different hydrogen peroxide scavenging enzyme, such as CAT and preoxidase with the scavenging enzyme superoxide dismutase is vital to prevent the formation of the highly toxic hydroxyl radical (Mittler and Poulos., 2005). Our data showed that the POD activity remained around the control level in both cultivars at mild stress whereas increased slightly in cv. Elsinore and vigorously in cv. Elsanta at 20 and 40 mM NaCl respectively. POD activity elevated by 66% compared to control in strawberry leaves exposed to salt stress. This high POD activity might help to scavenge the H_2O_2 or may contribute to prevent the generation of $^{\bullet}OH$ (Tanou et al., 2009). In response to salinity, POD activity in leaves of two genotypes of mulberry elevated significantly with higher elevation in the salt-tolerant genotype compared to the salt-sensitive one (Sudhakar et al., 2001). Similar increase in the POD activity has been reported on cotton cultivars subjected to salt stress, where Pora cultivar, characterized by

higher POD activity compared to Guazuncho cultivar consequently, had a higher capability to scavenge H_2O_2 produced by SOD (Meloni et al., 2003).

4.2 Lipid peroxidation (MDA)

MDA is formed as a result of PUFA peroxidation and considered as a parameter to estimate the level of lipid devastation in environmental stresses (Gill and Tuteja., 2010). In the present study, in response to salt treatments, a higher reduction of MDA content was observed in cv. Elsinore rather than in cv. Elsanta compared to control plants (Fig. 3-1A). The reduction of MDA content in both cultivars coincided with an initial decline of SOD activity followed by an increase and an increase in POD activity, which may scavenge H_2O_2 free radical and thus reducing lipid peroxidation. Initial reduction of MDA content was also detected in cv. Gloria of strawberry under low level of salt followed by an increase under a high level (1500 mg/l NaCl) of salinity (Yilmaz and Kina., 2008). However, a significant increase in lipid peroxidation was observed in leaves of cv. Selva in response to salt conditions (Tanou et al., 2009).

4.3 Proline content

Proline accumulation is a common metabolic response to adverse effect of drought and salinity stresses. Proline is considered as a non enzymatic antioxidant that protects membrane and proteins against the adverse effect of ionic toxicity derived from salinity and may function as hydroxyle radical scavenger (Gill and Tuteja, 2010; Smirnoff and Cumbes., 1989).

In the present study, an initial decrease of proline content was detected in both cultivars in response to mild stress, and a higher decrease of proline was detected in cv. Elsanta compared to cv. Elsinore under higher NaCl concentration. The proline content accumulation was higher in cv. Elsinore compared to cv. Elsanta (Fig. 3-2A). Our results have shown that activities of SOD, POD and CAT enzymes coincided with proline tendency. Similar results have been obtained by Hoque et al (2007), where salinity decreased activities of SOD, POD and CAT in Tobacco (BY-2) and the exogenous application of proline mitigated the reduction of CAT and POD activity. However, Tohoma and Esitken (2011); Rahimi and Biglarifard (2011) reported that proline

accumulated significantly in cv. Camarosa of strawberry under salt treatments and may have functioned as osomatic adjustment. Furthermore, Keutgen and Pawelzik (2008) proposed that in their experimental condition, proline accumulation was not a substantial factor against salinity in strawberry fruit. In addition, although a higher proline accumulation rate was found in susceptible cv. Elsanta compared to control, that did not provide vital protection against the adverse effects of salinity.

4.4 Total Soluble proteins

A soluble protein is considered as an important parameter to estimate the physiological state of plant. It has been reported by Singh et al (1987) that protein accumulation in response to salinity may supply a storage form of nitrogen which may be re-utilized later and may function as osmotic adjustment (Parvaiz and Satyawati., 2008). In our experimental condition, enormous accumulation of protein content was observed in response to salt treatments in both cultivars with distinct response in cv. Elsinore compared to cv. Elsanta (Fig. 3-2B); an appropriate elucidation that protein may be synthesized *de novo* in response to salt stress (Keutgen and Pawelzik., 2007; Pareek-Singla and Grover., 1997). Similar results have been reported by Keutgen and Pawelzik (2008), where the fruit of cv. Elsanta accumulated higher protein content compared to fruit of cv. Korona in response to salt stress. Furthermore, Rahimi and Biglarifard (2011) reported that protein content was enhanced in cv. Camarosa of strawberry at 30 and 60 mM NaCl, while Tohoma and Esitken (2011) reported that protein content decreased in cv. Camarosa at 60 mM NaCl.

Acknowledgements

We would like to thank Prof. Andrea Masia and Dr. Antonio Cellini for their collaboration in order to achieve these analyses.

5 Tables

Table 3-1: Summary of two-ways ANOVA table for the enzymatic and non enzymatic antioxidant of leaves of two strawberry cultivars, Elsanta and Elsinore, in response to salinity treatments (0, 10, 20 and 40 mM NaCl). ns = non significant differences; * = significant differences at P≤0.05; ** = significant differences at P≤0.01; *** = significant differences at P≤0.001.

Variables	**Block**	**Salt stress** (S)	**Cultivar** (C)	**S x C**
CAT	ns	**	**	*
SOD	ns	***	*	ns
POD	ns	**	*	*
MDA	ns	**	*	ns
Proline	ns	***	***	***
Soluble proteins	ns	***	**	ns

6 Figures

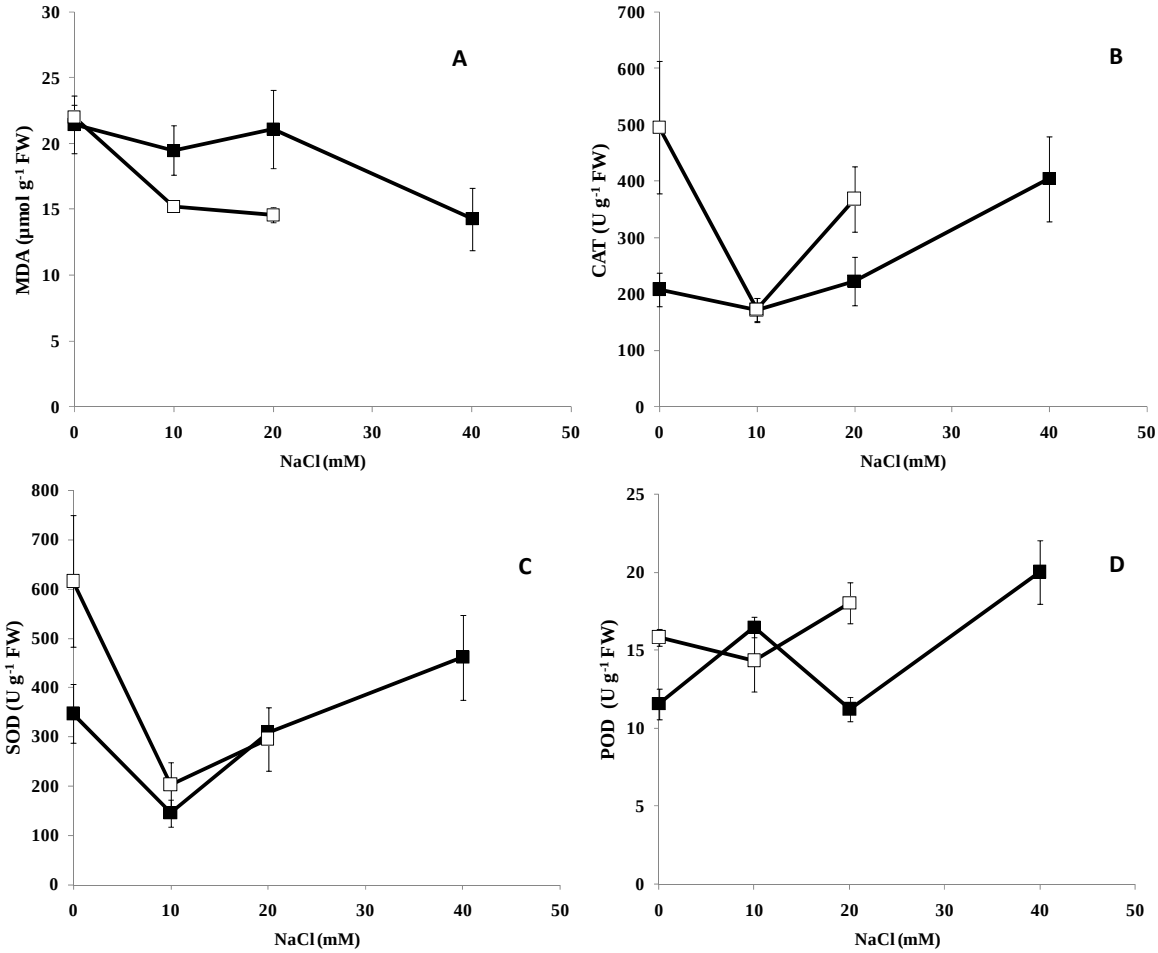

Figure 3-1: A) MDA content, B) catalase enzyme activity, C) superoxide dismutase enzyme activity and D) peroxidase enzyme activity in the leaves of two strawberry cultivars, Elsanta (closed square) and Elsinore (open square). Mean values ± SD (n = 3) under salinity treatments of 0, 10, 20 and 40 mM NaCl.

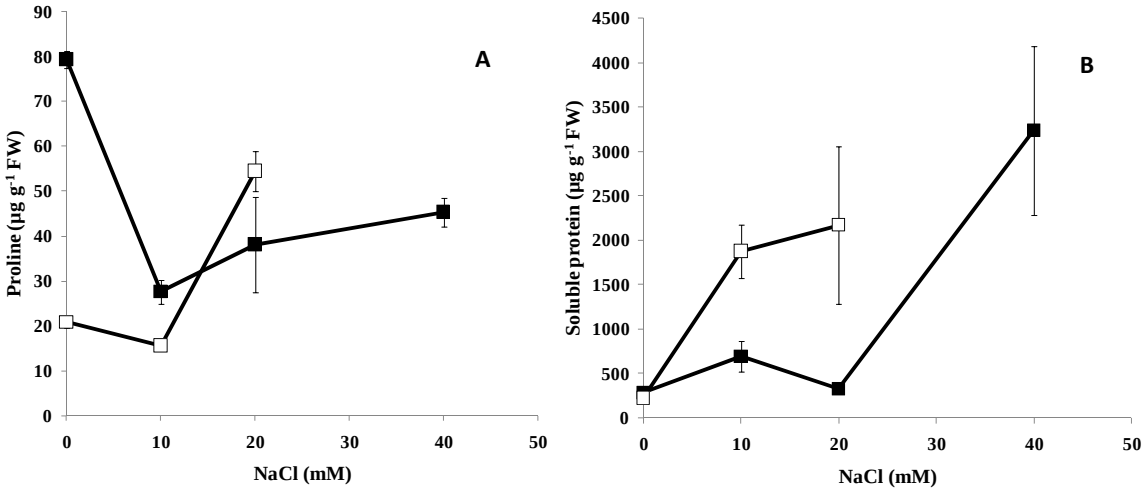

Figure 3-2: A) proline content and B) total soluble proteins content in the leaves of two strawberry cultivars, Elsanta (closed square) and Elsinore (open square). Mean values ± SD (n = 3) under salinity treatments of 0, 10, 20 and 40 mM NaCl.

Quality and bioactive compounds of strawberry fruit under long-term salt stress

1 Introduction

Salinity and drought stress lead to progression of morphological, physiological, biochemical, and molecular changes that unfavorably affect plant growth and productivity (Wang et al., 2001). Strawberry fruit is commonly considered to be a valuable nutritional resource of vitamins, minerals and various photochemical compounds that concern consumer satisfaction and health. Genotype and the environment are considered important factors that may modify the organoleptic attributes of strawberry fruit and these modifications cause alteration of fruit quality. Strawberry is considered as salt-sensitive plant but response to salinity may vary among cultivars (Martinez Barroso and Alvarez, 1997; Saied et al., 2005; Yilmaz and Kina, 2008; Turhan et al., 2008; Keutgen and Pawelzik, 2009). Low to moderate levels of NaCl have been used in order to improve fruit quality Keutgen and Pawelzik, 2007a). The application of controlled salinity may be adopted in order to improve nutritional value of fruit with minimum unfavorable effect on yield (Grieve, 2011). Modifications of fruit quality, in response to salt conditions were studied over the last few decades in strawberry cultivars differing in their sensitivity to salinity. Ehlig and Bernstein (1958) reported that the improvement of fruit quality was observed when Lassen and Shasta cultivars were exposed to 2-3 dS m^{-1} of salinity under open field conditions, although total yield was reduced by 50% under 2.3 dS m^{-1} and 2.6 dS m^{-1} for Shasta and Lassen, respectively. Saied et al (2005) reported that aroma, taste, texture, total soluble solids (TSS), titratable acidity (TA) and TSS/TA ratio decreased in both cv. Korona and cv. Elsanta under salinity, although cv-related differences could be observed. Moreover, Keutgen and Pawelzik (2007a) showed that in response to salinity, fruit quality was more impaired in cv. Elsanta due to reduction of fruit weight, size and TSS/TA ratio as compared to cv. Korona.

Concerning human health, phytochemical compounds, such as vitamin C, phenolics compounds and flavonoids are associated with a reduced risk of diseases mediated by oxidative stress and inflammation, such as atherosclerosis and neurodegenerative diseases (Seeram, 2007). Among 21 common fruit and vegetables consumed in the United State, strawberry ranked the fourth in total phenolic content after cranberry, apple and red grape (Sun et al., 2002; Chu et al., 2002). Accumulation of health promoting components, such as phenolics compound has been linked to the adverse effect of salinity on many plant species. In addition, these properties have been used as markers to improve plant salt tolerance by both conventional agricultural practices or through genetic engineering programs (Ashraf, 2009; Juan et al., 2005), although genetically modified food has to face adverse feeling from consumers (Martinez-Ballesta et al., 2008). Consistently, the application of an abiotic stress, such as salinity, can serve to enhance concentrations of desirable phytochemicals in fruit and vegetables. High quality crops produced by a strictly agronomic management practice may be more acceptable to consumers (Grieve, 2011). However, Keutgen and Pawelzik (2007b) addressed the increase of antioxidant capacity under moderate salinity in both salt-tolerant cultivar Korona and the relatively salt-sensitive one Elsanta and they also proposed that relative salt-tolerance strawberry cultivars could be grown under moderate salinity in order to improve fruit quality.

The quality of strawberry fruit results as function of their chemical composition and organoleptic attributes under long-term salt stress is our area of study. Understanding those attributes of fruit quality and their interactions with salinity is important for both screening or improving new strawberry cultivars and developing diverse kind of food with high nutritional value. Therefore, the present study addressed the identification of main biochemical response to salinity in two strawberry cultivars (namely Elsanta and Elsinore), with the final aim of defining how moderate salinity may improve the fruit nutritional value with no significant effects on its organoleptic attributes and plant production.

2 Materials and methods

2.1 Plant material and growth conditions

The experiment was conducted in a glasshouse at the experimental farm of the University of Bologna, located in *Ozzano dell'Emilia* (44º26'38 N, 11º26'18'' E, 98 m a.s.l.). Plantlets of similar height and diameter were transplanted into plastic pots of 5 l volume (1 plant/pot) filled with a mix of commercial growing media and pumice (2:1 v/v) on February 16th, 2010. Pots were placed over benches at a density of approximately 9 plants m^{-2}. Plants were irrigated automatically three times per day to ensure adequate substrate moisture. Fertigation was carried out once a week by adding to the irrigation water plant nutrients at the following concentrations: N-NO_3 = 6.0 mM; N-NH^4 = 1.0 mM; PO_4^{3-} = 3.0 mM; K^+ = 4.0 mM; SO_4^{2-} = 7.0 mM; Ca^{2+} = 5.0 mM; Mg^{2+} = 4.0 mM; microelements in traces, at a final EC = 1.75 dS m^{-1}. Before salt stress, plants stolons were removed to improve vegetative growth.

2.2 Treatments and experimental design

Eight treatments, derived by the factorial combination of 2 cultivars (Elsanta and Elsinore) and 4 NaCl concentrations in the irrigation water (0, 10, 20 and 40 mM), were compared. The experimental design was a strip plot (*salt* assigned to the main plots and *cultivars* to the sub-plots) with 3 replications. Each plot included 8 plants. The salt stress treatment was initiated on April 1st (44 days after transplanting, when the plants had 6–7 leaves), by irrigating plants with a water solution of 0 mM NaCl (control, EC_w = 0.45 dS m^{-1}), 10 mM NaCl (EC_w = 0.97 dS m^{-1}), 20 mM NaCl (EC_w = 1.95 dS m^{-1}) and 40 mM NaCl (EC_w = 3.90 dS m^{-1}). When the irrigation coincided with the fertigation (once a week), the EC_w were 1.75, 2.30, 4.30 and 6.50 dS m^{-1} for 0, 10, 20 and 40 mM NaCl treatments, respectively. This irrigation regime was maintained until the end of the experiment.

Fruit-setting started on April 15th (14 days after stress treatment initiation, DAS). Fruit of all plants were harvested manually at full maturity on four dates: May 5th, 10th, 17th, and 27th (corresponding to 34, 39, 46 and 56 DAS, respectively). Number and weight of fruit were determined at the end of the experiment.

2.3 Titratable acid, pH, Brix

TA was calculated by potentiometric acidity titration, (Giusti and Wrolstad, 2001). Briefly; 100-200 g of fresh fruit were blended at 100 rpm for 1 minute then the supernatant collected after carefully centrifuge for 5 minutes at 5000 rpm at room temperature, 5 ml sample pipetted into 250 ml beaker and carefully titrated with 0.1 N NaOH solution to the end point of pH 8.2 under stirring with a magnetic-stirrer. TA was calculated in term of citric acid as standard acid using the following equation

$$\text{TA (g/100 ml)} = [(V)\,(N)\,(\text{meq. wt.})\,(100)] / [(1000)\,(v)] \qquad (1)$$

Where (V): Volume of sodium hydroxide solution used for titration (ml); (N): Normality of sodium hydroxide solution; (meq. wt): Milliequivalent weight of the standard (Citric acid =64.04); and (v): sample Volume (ml).

In order to measure pH, 5 ml of supernatant of blended strawberry fruit was pipetted into cleaned beaker, stirred slowly and pH value was recorded by pH-meter (GLP 22, CRISON). The total soluble solids TSS content was determined using a refractometer (PCE, Italia).

2.4 Sensory panel test

Strawberry fruit were harvested at the ripening stage and offered immediately for sensory evaluation. The sensory panel test was conducted by 10 panellists. Encoded strawberry fruit boxes were randomly posted on tables under well-controlled condition with adequate distance between boxes in order to prevent aroma interference. The panellists were asked to rate the following sensory attributes: appearance, aroma, taste. In addition, the panellists were given water as a neutralizing beverage between samples testing (Azodanlou et al., 2003). Evaluations of fruit appearance, aroma, and taste were based on model of Quality Assessment of Strawberries which was developed by Azodaluo et al (2003), the model (scale 1–9) consist of three quality levels and the respective average for “medium” value was 4.5 (range 4–5), “good” value was 6 (range 5–7) and “very good” was 8.5 (range 8–9).

2.5 Determinations of phenolics and anti-oxidant activities

The frozen samples were thawed at room temperature and 10g of each sample was homogenized with 50 ml (1:5 w/v) of methanol/H_2O/acetone. (60+30+10; v/v/v) (Hartmann et al., 2008), then, the mixture was centrifuged (ALLEGRA™ 25R Centrifuge, BECKMAN, USA) at 10000 rpm for 10 minutes and the supernatant was collected. The extraction was repeated one time and the final extraction (100 ml) was used for the determination of total phenolic, flavonoids, anthocyanin, and antioxidative capacity.

2.5.1 Total phenolic content

Total phenolic content (TPC) was determined according to Folin-Ciocalteau colorimetry methods (Giusti and Wrolstad, 2002). Briefly, 20 µL of sample extraction was mixed with 1.6 mL of distilled water in 2 ml plastic cuvette followed by 100 µL of Folin-Ciocalteau phenolic reagent and incubated for 8 minutes at room temperature after mixing well, (300 µL) of sodium carbonate was added and mixed thoroughly then incubated for (2 hrs) at room temperature. The sample absorbance was measured at 765 nm by spectrophotometer (DU530® life science UV/VIS spectrophotometer, BECKMAN, USA). All samples were measured in duplicate and the total phenolic content was expressed as galic acid equivalent in milligram per 100 g of fresh weight of strawberry's fruit (mg of GAE/100 g FW).

2.5.2 Total flavonoids content

Total flavonoids (TFC) content was determined by aluminium chloride colorimetric assay (Zhishen et al., 1999). Briefly, 250 µL of sample extraction was mixed with 1.25 mL of distilled water, and 75 µL of 5% $NaNO_2$ was added in a 2 mL plastic cuvette. After 6 minutes, 150 µL of 10% $ALCl_3$ was added, followed by 500 µL of 1 M NaOH after 5 minutes. Then the sample absorbance was measured at 510 nm by spectrophotometer and the calibration was carried out by a standard curve of catechin (5, 10, 20, 25, and 50 ppm). The results were expressed as mg of catechin equivalents (CE)/100 g of fruit fresh weight. Samples were measured in duplicate.

2.5.3 Total anthocyanin content

The total anthocyanin content (TAC) was determined by the PH-differential method (Giusti and Wrolstad, 2001). Briefly; two dilutions of the sample extract were prepared in 2 ml cuvettes (1:20 v/v), one with potassium chloride buffer, (pH 1.0) and the other with sodium acetate buffer, (pH 4.5). Then, these dilutions were equilibrated for 15 minutes. The absorbance of each dilution was measured at 500 nm and 700 nm by spectrophotometer, against a blank cuvette filled with distilled water. The total anthocyanin content was calculated by the following equation and the results were expressed in mg pelargonidin-3-glucoside per 100 g of fruit fresh weight:

$$pg-3-glu\ mg/Kg\ FW = \frac{\{(A_{500}-A_{700})_{\mathrm{pH1.0}}-(A_{500}-A_{700})_{\mathrm{pH4.5}}\}\times MW\times DF\times V\times 10^{6}}{\varepsilon\times W\mathrm{x}\ 10} \quad (2)$$

Where A is absorption; MW is molecular weight of Pg-3-glu (433.2 g/mol); DF is dilution factor; V is volume of the extract in L; molar absorption coefficient ε of Pg-3-glu 15600 L/ (mol × cm); and W: sample weight in g (Hartmman et al., 2008). The samples were measured in duplicate.

2.6 Antioxidant activity

2.6.1 FRAP assay

Total antioxidant activity was measured by *Ferric reducing antioxidant power (FRAP) assay* according to Benzie and Strain (1999) and modified by Aaby et al (2007).
The composition of FRAP reagents was:

1. Acetate buffer 300 mM pH 3.6: weigh 3.1 g sodium acetate trihydrate and add 16 ml of glacial acetic acid and make the volume to 1 l with distilled water.
2. TPTZ (2, 4, 6-tripyridyl-s-triazine) (MW 312.34) 10 mM in 40 mM HCl (MW 36.46) (250 ml)
3. $FeCl_3.6H_2O$ (MW 270.30) 20 mM (250 ml)

The final FRAP reagent was prepared by mixing 1, 2 and 3 in the ratio of 10:1:1 at the time of use and covered with aluminium. Briefly, freshly prepared FRAP reagent (2.4 mL) was mixed with 80 µL of sample (0.1 g/ml) in duplicate. The mixture was equilibrated for 1 hour at room temperature before absorbance was measured at 593 nm.

Aqueous solutions of Fe-(II) ($FeSO_4.6H_2O$) in the concentration range of 125–1250 µmol/L were used for calibration of the FRAP assay. FRAP values were expressed as mmol of Fe (II) per 100 g of sample (mmol of Fe (II) / 100 g of FW).

2.6.2 DPPH assay

2, 2-Diphenyl-1-picrylhydrazyl DPPH assay was done according to Alamanni and Cosu (2004) and modified by (Hartmman et al., 2008). The stock solution was prepared by dissolving DPPH in methanol (0.1 mmol/l), covered with aluminium and then stored at -20 °C until the use. 10 ml of 0.1 mmol/l fresh DPPH solution in methanol were mixed with 0.1 mL sample extraction. After 30 minutes of incubation at room temperture, the absorbance was measured at 517 nm by spectrophotometer. Trolox in the concentration range between between 25 and 1000 µM was used for calibration, and the antioxidant capacity was expressed in µmol/L of trolox equivalents TE per 100 FW.

3 Statistical analysis

Statistical analyses were performed by using SPSS statistical program. The significance of differences between treatments and between cultivars, and of the interaction between these two factors, was determined using two-way ANOVA. Where significant effects were found, ANOVA was followed by LSD test.

4 Results

Salinity treatments had a significant effect fruit weight of both Elsanta and Elsinore cultivars compared to control plants (Table 4-1). Fresh weight reductions were 21% and 26% for both Elsanta and Elsinore cultivars respectively at 40 mM NaCl (Table 4-2). Nevertheless cv. Elsanta presented significantly bigger fruit (+30%) as compared to cv. Elsinore under control conditions (Table 4-2). On the other hand, number of fruit per plant was not significantly affected by increasing salinity (Table 4-2). However, a significant difference was observed among the cultivars (Table 4-1), with cv. Elsinore having 58% fruit than cv. Elsanta.

4.1 pH, Brix, TA, Brix/TA

Salinity did not significantly affect the pH, titratable acidity and TSS/TA ratio (Table 4-1), where pH remained stable in both cultivars and varied from 3.51 to 3.73 (Table 4-3). Regarding TSS, cultivars and salinity did not significantly affected this attribute. Nevertheless, TSS decreased by 24% and 8% in cv. Elsinore and cv. Elsanta respectively under 40 mM NaCl as compared to control plants (Table 4-3). In term of TA, a significant difference was observed between both cultivars, where cv. Elsanta had 28% higher TA content compared to Elsinore (Table 4-3). Subsequently, TSS/TA ratio significantly differed at both levels of cultivars and salinity x cultivars interaction (Table 4-1).

4.2 Appearance, Aroma, Taste

According the assessment model, appearance for both cultivars at all treatments levels was generally considered as "good" (5.2–6.9) with exceptions in fruit of cv. Elsinore considered being "medium" at 20 and 40 mM NaCl (Table 4-4). Statistically, the appearance of both cultivars was significantly deteriorated ($P \leq 0.001$) under salinity (Table 4-1), where the value 18% and 29% less in cv. Elsanta and cv. Elsinore respectively compared to control plants.

For what concern the aroma, results showed that fruit of cv. Elsanta under control condition had the highest value and were considered as "very good" according the model of assessment, while fruit of cv. Elsanta under 10, and 20 mM NaCl and cv. Elsinore under control, 10, and 20 mM NaCl were considered as "good". On the other hand, fruit of both cultivars under 40 mM NaCl had the lowest value and were considered as "medium" (Table 4-4). Statistically, aroma was significantly ($P \leq 0.001$) impaired by increasing salt concentration in the growing media (Table 4-1) with a 39% and 30% decrease in cv. Elsanta and cv. Elsinore respectively at 40 mM NaCl as compared to control plants.

The taste of fruit of both cultivars was considered to be "good" with the exception of fruit of cv. Elsanta, which was "medium" (Table 4-4) under 40 mM NaCl. Based on statistical analysis, no significant effect of salinity was found *taste* (Table 4-1). No significant differences were also observed between both levels of cultivars and cultivars x salinity interaction over all attributes.

Above all, salinity significantly affected both cultivars, although with distinct effect cv. Elsinore rather than cv. Elsanta. Therefore, cv. Elsanta had a higher value compared to cv. Elsinore. Subsequently, cv. Elsanta met the satisfaction requirement of the consumer more than cv. Elsinore, especially at control and mild NaCl concentration.

4.3 Antioxidants content

A high significant genotypic difference was detected ($P \leq 0.001$) between both cultivars (Table 4-1), where cv. Elsanta showed 23% higher TPC compared to cv. Elsinore in control fruit. Beside the genotypic difference, salt treatments had a significant effect ($P \leq 0.001$) TPC in both cultivars, where TPC values stepwise increased up to the highest values at 29% and 43% in cv. Elsanta and cv. Elsinore respectively by increasing salinity up to 40 mM NaCl compared to control fruit (Table 4-5).

Anthocyanin content was significantly improved ($P \leq 0.001$) under the effect of salt treatments in both cultivars (Table 4-1), where anthocyanin content increased at 23% and 6% in cv. Elsanta and cv. Elsinore respectively at 40 mM NaCl compared to control fruit (Table 4-5). Nevertheless, significant differences for both cultivars ($P \leq 0.001$) were observed in control plants, where cv. Elsanta had 19% lower anthocyanin content compared to cv. Elsinore. Cultivars x salt treatments interaction ($P \leq 0.05$) were statistically significant.

Flavonoid contents had a similar tendency of TPC and anthocyanin contents. Salinity had high significant influence flavonoid content in both cultivars ($P \leq 0.001$). Flavonoid content increased at 9% and 14% in cv. Elsanta and cv. Elsinore respectively at 40 mM NaCl compared to control fruit (Table 4-5). Additionally, variation among both cultivars was highly significant, where cv. Elsanta had 12% higher content compared to cv. Elsinore. Also, interaction cultivars x salt treatments were significant ($P \leq 0.01$).

Antioxidant capacity was measured by DPPH and FRAP assay and both methods showed that salt treatments significantly enhanced antioxidants capacity in both cultivars. A significant correlation (Table 4-6) between DPPH value and TPC, AC and TFC in cv. Elsanta and TPC in cv. Elsinore was observed. Regarding FRAP assay, a significant correlation between FRAP value and TPC, AC and TFC in cv. Elsanta was observed

(Table 4-6), whereas analyzed data did not show any correlation FRAP value and TPC, AC and TFC content in cv. Elsinore.

5 Discussion

Fruit weight may affect the fruit quality, which may subsequently affect the consumer's preference. Furthermore, Crespo (2010) has reported that fruit weight may affect a production cost through harvest speed. Under our experimental condition, salinity caused a significant reduction in fruit weight for both cultivars with distinct effect cv. Elsinore compared to cv. Elsanta. A similar response was found by Awing and Atherton (1995), where yield reduction was a result of reduction in fruit weight rather than fruit number. On the other hand, Saied et al (2005) reported that yield reduction was due to decrease in fruit number while fruit size was not significantly affected by salinity. Furthermore, Sakamoto et al (1999) and Sato et al (2006) have proposed that salinity impair fruit weight by decreasing plant water potential. According to Keutgen and Pawelzik (2008) a significant increase of Na^+ and Cl^- in fruit impaired fruit weight.

High acidity helps stabilize color; pH values ranging between 3.27–3.86 are favored for strawberry as reported by the Oregon Strawberry Commission (2011); and Roudeillac and Trajkowski (2004). On the other hand, salinity did not affect the pH and the values for both cultivars were within the favored range, as demonstrated by (Keutgen and Pawelzik (2007a) and Saied et al (2005). High sweetness and relatively high acidity content have been considered as substantial parameters for flavor, which satisfies consumers (Wang and Millner, 2009; Keutgen and Pawelzik, 2007a; Wang et al., 2002). In the present study, TSS contents were within a very good range of 8–9 according to the model of assessment of strawberry quality created by Azodaluo et al (2003) and an optimum range of 9–10% according to Roudeillac and Trajkowski (2004). Also, a range of 7% minimum TSS content for acceptable quality of strawberry fruit was recommended by Mitchan et al (2000) and Kader (1999). Moreover, TSS content varied between 7% and 12% depending on genotype (Crespo, 2010; Galletta et al., 1995). The coordinated relation of organic and phenolic acids in berries is responsible for the titratable acidity of the fruit and it is widely measured as an indicator of fruit quality (Talcott, 2007). Mitchan et al (2000) reported that TA values over 0.8% are considered as too acidic for acceptable

flavor. In the present study, Elsanta had significantly higher TA content compared to Elsinore (Fig. 4-1) and (Table 4-2); therefore, TA may have affected the fruit taste and consequently the consumer's preference. This result in accordance with the reported results of Awang and Atherton (1995a), where TA significantly increased in fruit of cv. Rapella plants treated by salinity. Keutgen and Pawelzik (2007a) reported that TSS and TA decreased significantly by salinity in both cultivars Elsanta and Korona. A similar effect of salinity on inner fruit attributes were reported by Saied et al (2005); Kepenk and Koyuncu (2002) and Kaya et al (2001). A good balance of TSS/TA ratio at 8.5 to 13.79 in strawberry fruit is preferable to meet consumer requirement (Oregon Strawberry Commission, 2011). TSS/TA ratio was significantly higher in cv. Elsinore compared to cv. Elsanta. On the other hand, salinity had no effect TSS/TA ratio, while salinity x cultivar interaction was significantly detected. Keutgen and Pawelzik (2007a) studies showed that TSS/TA ratio was not influenced by salt stress, while Saied et al (2005) reported that TSS/TA ratio significantly decreased in response to salt treatments for both cultivars, Elsanta and Korona. Fruit appearance, brightness, and shape are essential attributes that influence marketing and consumers' preferences (Crespo, 2010; Resende, 2008). under our experimental conditions, sensory evaluation of fresh fruit reflected a significant negative response of both cultivars to salinity at 40 mM NaCl. Furthermore, a significant ($p< 0.05$) correlation was observed between fruit weight and appearance, and aroma of strawberry fruit regardless to cultivars (0.51) and (0.48) respectively. Accordingly, we can draw the conclusion that high salinity might have impaired fruit appearance and aroma through its effect fruit weight for both cultivars, in addition to the fact that there was a genotypic difference between both cultivars in fruit weight. On the other hand, under moderate salinity (10 mM NaCl), the total scores of sensory evaluation were similar to control plants and that gave a considerable indicator of strawberry fruit quality. Aroma and TSS / TA ratio substantially contribute to the taste attribute (Resende, 2008; Wozniak et al., 1997). Salinity impaired the taste attribute in cv. Elsanta, whereas it did not affect the taste attribute in cv. Elsinore at 40 mM NaCl compared to control plants. Keutgen and Pawelzik (2007a); Saied et al (2005) investigation showed that salinity negatively influenced sensory attributes (appearance, aroma, taste) in cv. Elsanta at 80 mM NaCl and the fruit of cv. Elsanta was completely rejected by the panelists.

Total phenolic content and total anthocyanin in strawberries have been reported over studies to range from 43 to 273 mg 100 g^{-1} FW and 6 to 102 mg 100 g^{-1} FW respectively (Zhao, 2007). Irrespectively to salinity effect and cultivars, our result confirmed those studies, where total phenolic content and total anthocyanin in strawberry fruit ranged from 170 mg GA 100g^{-1} FW to 269 mg GA 100 g^{-1} FW, and 23 pg-3-glu E 100 g^{-1} FW to 29 pg-3-glu E 100 g^{-1} FW respectively. Total flavonoids ranged between 15 CE 100 g^{-1} FW and 20 CE g^{-1} FW, This content was lower as compared to the results presented by Marinova et al (2005), where TFC was reported at 69.7 CE 100 g^{-1} FW in strawberry (*Fragaria vesca*) fruit. Nevertheless, a significant higher TPC and TFC rates were detected in cv. Elsanta compared to cv. Elsinore. TAC rate was, however, higher in cv. Elsinore compared to cv. Elsanta (Fig. 4-1). In addition to the variation of TPC, TAC, and TFC between both cultivars, a variation in response to salt treatments was also detected. As a result, salinity improved phytochemical contents of strawberry in both cultivars, where TPC increased 11% and 16% under moderate salinity and 29% and 43% high salinity in cv. Elsanta and cv. Elsinore respectively. Consequently, the antioxidant activity increased in fruit of both cultivars by increasing the salt concentration in the growing medium (Fig. 4-2). It should be noted that the antioxidant capacity of phenolics components in strawberry fruit vary upon the oxidation assay applied (Giusti and Jing (2007). Linear correlation significantly was observed between TPC, TAC and TFC and antioxidant capacity measured by DPPH and FRAP in cv. Elsanta, while significant correlation was recorded between TPC and DPPH in cv. Elsinore. In previous studies, results showed that salinity increased anthocyanin and total phenolic contents in both Elsanta and Korona cultivars, where the highest increase of 94% occurred in cv. Elsanta at 40 mM NaCl. Thus, salinity enhanced antioxidant activity measured by FRAP assay up to 64% (Keutgen and Pawelzik, 2008; Keutgen and Pawelzik, 2007a). Also, a higher concentration of total phenolics and antioxidant activity was detected in fruit under deficit irrigation treatments (Terry et al., 2008). However, results of the past studies showed that strawberry fruit are always characterized by a high antioxidant activity and this activity is influenced by various factors, such as planting date and the growing environment (Anttonen et al., 2006), the cultural systems (Wang and Millner, 2009), and cultivars (Tulipani et al., 2009; Hernanz, 2007; Cordenunsi et al., 2003 and Scalzo et al., 2003).

In conclusion, the results of this study showed that under moderate salinity (10 mM NaCl) fruit quality improved including organoleptic attributes and phytochemical compositions. These improvements may to economically compensate the reduction 14–18% of fruit yield, while sensory panel evaluation showed that the panelists considerably appreciated fruit size and relatively organoleptic attributes while health promoting compositions were not given attention. However, cv. Elsanta was characterized by bigger fruit size, higher panel evaluation scores and higher phytochemical contents compared to cv. Elsinore. Although, the health promoting compounds significantly enhanced under severe salt stress 40 mM NaCl in both cultivars, other organoleptic attributes and fruit yield significantly decreased and the fruit were given the lower scores by panelists (Fig. 4-2). This kind of fruit might be useful for developing value-added strawberry fruit products rich with health promoting compounds especially for elder people, and may be not as fresh products.

6 Tables

Table 4-1: Summary of two-ways ANOVA table for the morphological and chemical fruit parameters of two strawberry cultivars, Elsanta and Elsinore, in response to salinity treatments (0, 10, 20 and 40 mM NaCl). ns = non significant differences; * = significant differences at P≤0.05; ** = significant differences at P≤0.01; *** = significant differences at P≤0.001.

Variables	Block	Salt stress (S)	Cultivar (C)	S x C
Fruit weight	ns	***	***	ns
Fruit number	ns	ns	***	ns
Yield	ns	*	***	ns
pH	ns	ns	ns	ns
Brix	**	ns	ns	ns
TA	ns	ns	***	ns
Brix/TA	*	ns	***	*
Appearance	ns	***	ns	ns
Aroma	**	***	ns	ns
Taste	ns	ns	ns	ns
Polyphenols	ns	***	***	ns
Flavonoids	ns	***	***	**
Anthocyanin	**	***	***	*
DPPH	ns	***	ns	*
FRAP	*	**	ns	ns

Table 4-2: Influence of salinity treatments (0, 10, 20 and 40 mM NaCl) on fruit weight, fruit number, and yield per plant.

Cultivar	NaCl (mM)	Fruit weight g fruit^{-1}	Fruit number N plant^{-1}
Elsanta	0	18.26 ± 0.49 a	10 ± 0.18 b
	10	15.70 ± 0.56 b	10 ± 0.84 b
	20	15.37 ± 0.84 b	10 ± 0.37 b
	40	14.45 ± 1.02 bc	10 ± 0.67 b
Elsinore	0	14.08 ± 1.71 bc	17 ± 0.38 a
	10	13.27 ± 1.51 cd	15 ± 0.16 a
	20	12.05 ± 0.84 de	17 ± 0.50 a
	40	10.46 ± 2.94 e	16 ± 1.17 a

Table 4-3: pH, total soluble solids (TSS), titratable acid (TA), and TSS/TA ratio fresh fruit parameters of two strawberry cultivars, Elsanta and Elsinore, grown in different NaCl concentrations (0, 10, 20 and 40 mM NaCl). Mean values ± SD (n = 6).

Cultivar	NaCl(mM)	pH	TSS (%)	TA(g/100ml)	TSS/TA
Elsanta	0	3.59 ± 0.09	9.9 ± 0.53	0.88 ± 0.11 a	11.4 ± 0.91 c
	10	3.51 ± 0.02	10.2 ± 1.37	0.99 ± 0.04 a	10.3 ± 1.45 c
	20	3.55 ± 0.05	10.0 ± 1.06	0.95 ± 0.05 a	10.6 ± 1.11 c
	40	3.70 ± 0.09	9.1 ± 1.59	0.81 ± 0.05 ab	11.3 ± 1.94 c
Elsinore	0	3.54 ± 0.16	10.5 ± 0.79	0.64 ± 0.04 b	16.6 ± 2.01 a
	10	3.73 ± 0.12	10.3 ± 0.93	0.64 ± 0.06 b	16.1 ± 0.60 ab
	20	3.67 ± 0.08	8.2 ± 1.55	0.67 ± 0.04 b	12.3 ± 2.56 abc
	40	3.72 ± 0.07	8.0 ± 0.31	0.68 ± 0.02 b	11.7 ± 0.24 bc

Table 4- 4: Influence of salinity treatments (0, 10, 20 and 40 mM NaCl) sensory panel test of fresh fruit of two strawberry cultivars, Elsanta and Elsinore. Mean values ± SD (n=10).

Cultivar	NaCl (mM)	Appearance	Aroma	Taste
Elsanta	0	6.3 ± 0.42 ab	8.0 ± 0.21 a	6.2 ± 0.61
	10	6.9 ± 0.35 a	5.2 ± 0.76 bc	6.0 ± 0.49
	20	5.6 ± 0.34 bc	5.4 ± 0.60 bc	5.7 ± 0.60
	40	5.2 ± 0.25 cd	4.9 ± 0.55 c	4.8 ± 0.55
Elsinore	0	6.2 ± 0.29 ab	6.6 ± 0.72 ab	5.1 ± 0.50
	10	6.6 ± 0.31 a	5.9 ± 0.46 bc	6.0 ± 0.65
	20	4.9 ± 0.38 cd	5.3 ± 0.67 bc	6.3 ± 0.40
	40	4.5 ± 0.37 d	4.6 ± 0.79 c	5.1 ± 0.57

Table 4- 5: Influence of salinity treatments (0, 10, 20 and 40 mM NaCl) on fruit content of total phenoloic, total flvonoids, total anthocyanins and antioxidant capacity by using both DPPH and FRAP assays of two strawberry cultivars, Elsanta and Elsinore.

Cultivar	NaCl (mM)	Phenolics mg GA*	Anthocyanin mg pg-3-glu E*	Flavonoids Mg CE*	DPPH µmol Trolox*	FRAP mmol FeII*
Elsanta	0	208.5 ± 02.5 c	20.9 ± 0.2 e	18.3 ± 0.4 b	216 ± 11 bc	0.73 ± 0.03 c
	10	231.6 ± 11.6 b	22.7 ± 0.7 de	20.3 ± 0.6 a	238 ± 09 ab	0.79 ± 0.03 abc
	20	244.9 ± 02.5 b	23.5 ± 0.9 cd	20.2 ± 0.2 a	243 ± 04 ab	0.85 ± 0.00 a
	40	269.0 ± 03.3 a	25.8 ± 1.2 b	20.1 ± 0.3 a	263 ± 11 a	0.85 ± 0.04 a
Elsin -ore	0	170.3 ± 14.3 d	25.8 ± 1.8 b	16.1 ± 0.3 c	191 ± 11 c	0.77 ± 0.10 bc
	10	196.8 ± 06.0 c	25.3 ± 1.5 bc	14.7 ± 1.0 c	203 ± 16 c	0.79 ± 0.03 abc
	20	213.8 ± 11.6 c	29.0 ± 0.7 a	15.9 ± 0.4 c	214 ± 29 bc	0.79 ± 0.06 abc
	40	244.2 ± 04.6 b	27.3 ± 0.9 ab	18.7 ± 0.5 ab	261 ± 27 a	0.83 ± 0.01 ab

*Data are expressed per 100 g FW. Mean values ± SD (n = 6).

Table 4- 6: Pearson's correlation coefficients of antioxidant parameters for two strawberry cultivars under different salt concentrations.

Cultivar	Variables	TPC	TAC	TFC
Elsanta	S	0.93**	0.82**	0.72**
	DPPH	0.77**	0.65*	0.68*
	FRAP	0.71**	0.79**	0.77**
Elsinore	S	0.95**	0.39	0.62*
	DPPH	0.70*	0.41	0.52
	FRAP	0.25	0.41	0.089

TPC= total phenolic content, TAC = total anthocyanin content, TFC= total flavonoids content, S= salt treatments, * = significant differences at P≤0.05; ** = significant differences at P≤0.01

7 Figures

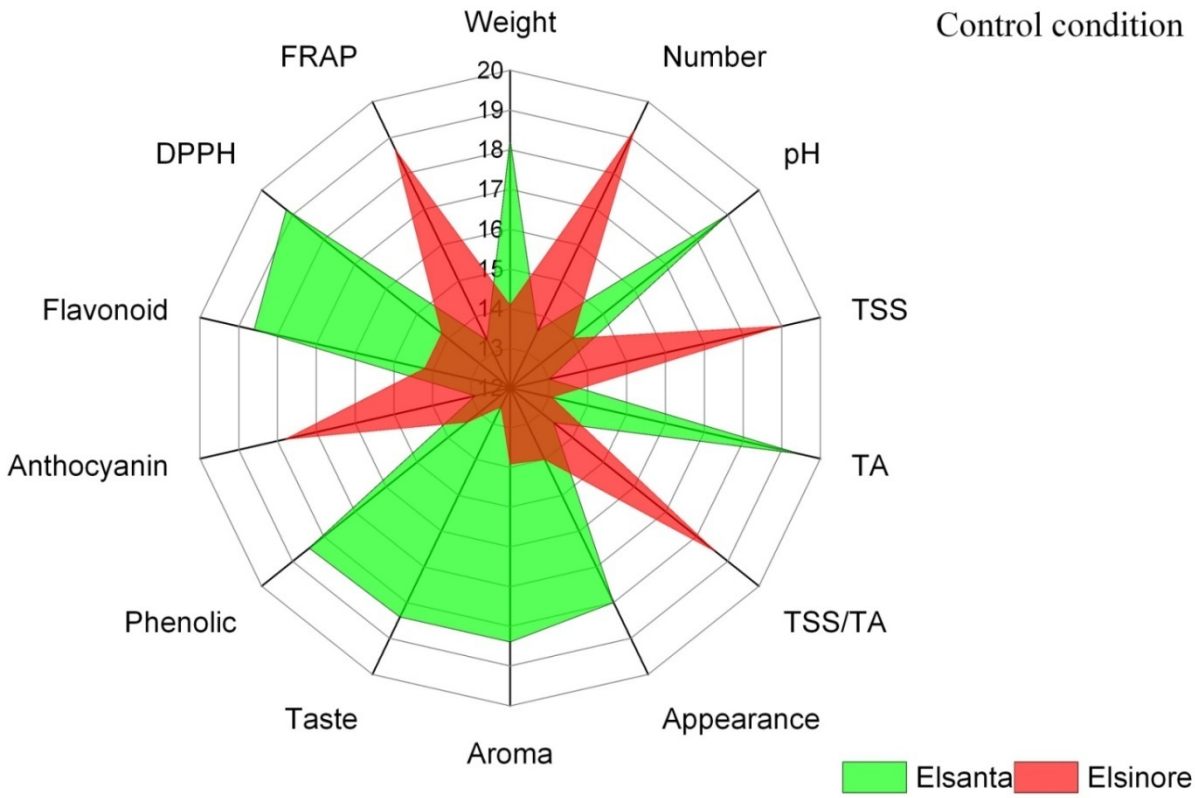

Figure 4- 1: Radar chart of fruit weight, fruit number per plant, organoleptic attributes (appearance, aroma and taste), biochemical content (TA, TSS, TSS/TA and pH) and health promoting compounds (phenolics, flavonoids, anthocyanin and antioxidant activity) in two cultivars of strawberry (Elsanta and Elsinore) at control condition.

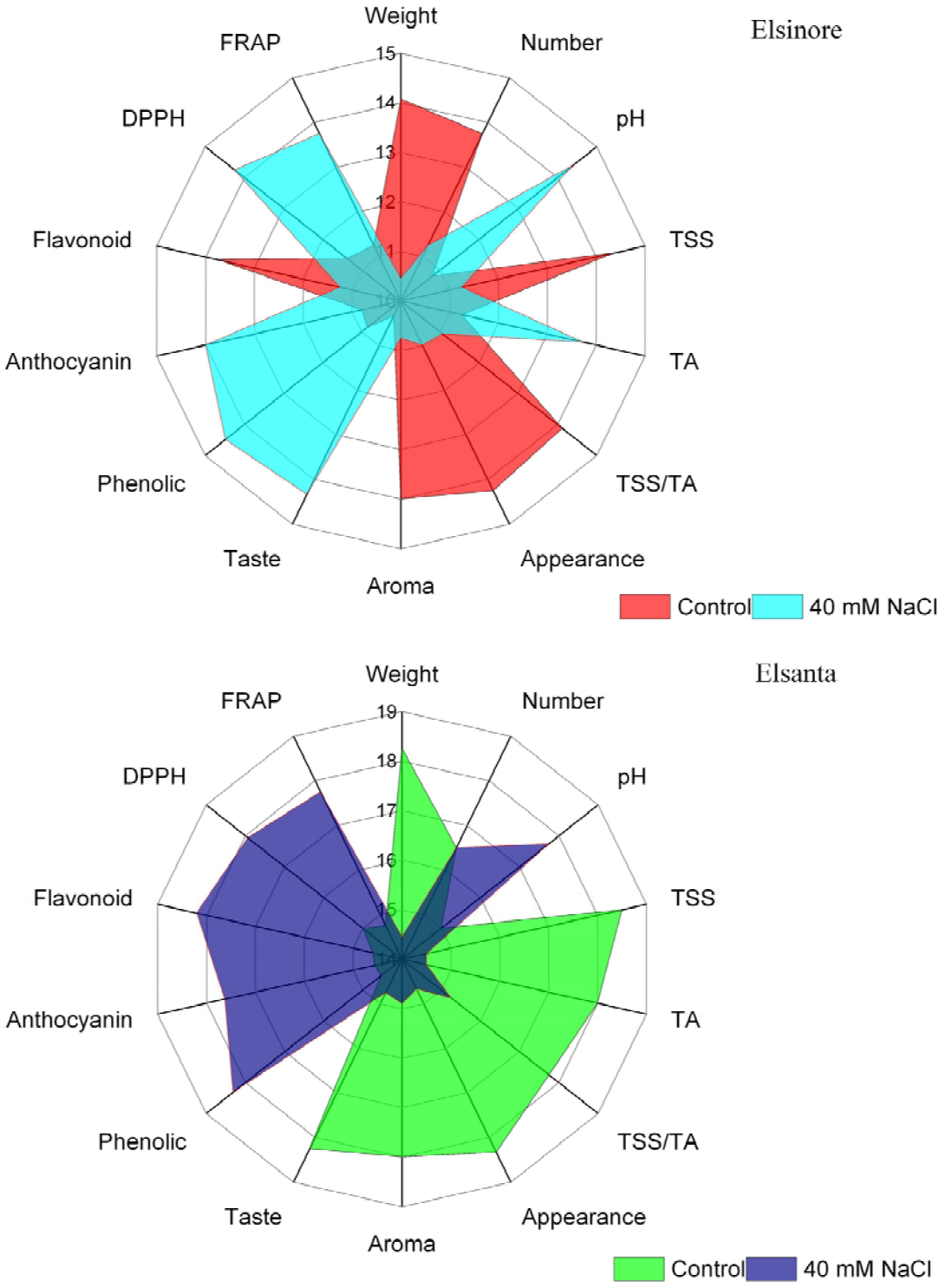

Figure 4- 2: Radar chart of fruit weight, fruit number per plant, organoleptic attributes (appearance, aroma and taste), biochemical content (TA, TSS, TSS/TA and pH) and health promoting compounds (phenolics, flavonoids, anthocyanin and antioxidant activity) in two cultivars of strawberry (Elsanta and Elsinore) at control and 40 mM NaCl conditions.

Plant growth, gas exchange, water relations and osmotic adjustment as screening tools of salinity tolerance in nine cultivars of strawberry

1 Introduction

Abiotic stress, such as drought, cold and salinity, restrains the optimal growth of plants and needs a certain level of adaptation to such adverse environments. Thus, new sources of salinity tolerance are required for crops grown on salt-affected land, which allows for effective use of poor quality water and increases the ability to grow high return crops (Munns and James, 2003). Improving the salt tolerance of crops requires access to new genetic diversity, either natural or transgenic, efficient techniques for physiological understanding of mechanisms of adaptation, and traits that confer it and how best to screen for these traits (Munns, 2011). The screening approach of a large number of genotypes of a crop is needed to identify the salt-tolerant cultivars for breeding to evolve the salt-tolerant and high-yielding crop varieties. Flowers and Yeo (1995) reported that using the variation which is already present in existing crops is considered as one of the important ways to develop salt-tolerance crops in a shorter time.

Strawberry (*Fragaria* **x** *ananassa* Duch.) is considered as salt-sensitive crop, and 1 dS m^{-1} is proposed to be the threshold of strawberry relative salt tolerance (Ehlig and Bernstein, 1958). However, cultivars vary in salt tolerance especially in response to long-term, high NaCl concentrations (Keutgen and Pawelzik, 2009 and Saied et al., 2005). Considerable numbers of strawberry genotypes have been screened (mostly in couples/ triples) for salt tolerance in open field and glasshouse conditions. The criteria have been based on growth and yield in different salt conditions relative to the same parameters in non-saline condition. A screen by Kepenek and Koyuncu (2002) of six cultivars of strawberry showed diverse response to different levels of salinity among cultivars in glasshouse conditions. Also, a screen by Keutgen and Pawelzik (2009) and Saied et al

(2005) of two strawberry cultivars (Elsanta and Korona), based on exposing strawberry plants to different levels of salinity in greenhouse and open field in respect to researcher (40 and 80 mM NaCl), showed considerable genetic diversity among both cultivars. Although salinity reduced fresh and dry matter and leaf area in both cultivars, cv. Korona revealed more capability to tolerate salinity compared to cv. Elsanta.

Because of the complex nature of salinity tolerance, as well as the difficulties in maintaining long-term growth experiments, trait-based selection criteria are recommended for screening techniques (Munns, 2008). Traits used for screening germplasm for salinity tolerance have included osmotic adjustment, stomatal conductance, and photosynthesis and transpiration rates. It is known that salinity affects plants by osmotic and ionic stress (Munns and Tester, 2008). The initial response of a plant in relatation to osmotic stress is demonstrated by a reduction in stomatal conductance (g_s), which is mediate by root/shoot hormone signaling and, subsequently, causes a considerable reduction in photosynthesis and transpiration rates. Turhan and Eris (2007) reported that salinity caused a reduction in stomatal conductance and transpiration rate in cv. Camarosa, but salinity did not affect g_s of cv. Chandler. Osmotic adjustment is one of the essential strategies which enable plants to thrive under salinity either by accumulating inorganic compounds, such as ions in halophytic plants, or by synthezising organic compounds, such as carbohydrates, and amino acids primarily in glycophytic plants. Genetic variation for this trait has been found in strawberry cultivars. Keutgen and Pawelzik (2009) reported that free amino acids, proline and asparagines, accumulated in salt conditions in both cultivars, Elsanta and Korona, but the latter revealed higher free amino acid content. However, higher accumulation of amino acids, proline, and soluble sugars has been linked with better osmotic adjustment and, consequently, higher adaptation to salt conditions (Rahimi and Biglarifard, 2011; Turhan and Eris, 2009; Saied et al., 2005).

Knowledge about physiological traits and new molecular tools to identify key genes has the potential to improve crop salt tolerance. Therefore, our study focused on validation of some screening methods based on growth and physiological mechanisms as selection markers on nine cultivars of strawberry in order to differentiate the cultivars upon their responses towards salinity for further breeding program.

2 Materials and methods

2.1 Plant material and growth conditions

The experiment was conducted in a growth chamber under closed hydroponic system with a photosynthetic photon flux of 500 μmol m^{-2} s^{-1} from cool-white fluorescent bulbs and a 16-h light/8-h dark photoperiod. Day and night temperatures were set at 22 ºC and 19 ºC, respectively. Nine cultivars of strawberry plantlets (Elsanta, Elsinore, Camarosa, Clery, Kamila, Marmolada, Madeleine, Naiad, and Siba), kindly donated by a local nursery (Salvi vivai, Ferrara, Italy), were transplanted on September 20th, 2010 into plastic pots of 1.5 l volume (1 plant each pot) filled with a mix of perlite and pumice (2:1 v/ v). Plants were placed randomly in a block design with three replications for each treatment. Plants were automatically irrigated three times per day, Mineral nutrients were supplied twice per week by using modified Hoagland solution at the following concentration: $N\text{-}NO_3$ = 6.0 mM; $N\text{-}NH^4$ = 1.0 mM; PO_4^{3-} = 3.0 mM; K^+ = 4.0 mM; SO_4^{2-} = 7.0 mM; Ca^{2+} = 5.0 mM; Mg^{2+} = 4.0 mM; microelements in traces, at a final EC = 1.75 dS m^{-1}. The salt stress treatment was initiated on October 18st (28 days after transplanting, when the plants had 5-6 leaves), by irrigating plants with a water solution of 0 mM NaCl (control, EC_w = 0.45 dS m^{-1}), 10 mM NaCl (EC_w = 1.95 dS m^{-1}), 20 mM NaCl (EC_w = 3.90 dS m^{-1}) and 40 mM NaCl (EC_w = 7.80 dS m^{-1}). The EC_w values were maintained weekly by replacing the water for each salt treatment. At the end of the experiment (November 22th, 34 DAS), three plants per treatment were randomly selected and harvested. Shoot and root fresh and dry weights were measured and shoot/root ratio was calculated. The leaf area (LA) was determined by using a scanner and the image processor software *Image J* (Abramoff et al., 2004).

2.2 Leaf gas exchanges

Leaf transpiration (E), stomatal conductance (g_s) and net photosynthesis (A) were measured at 2 and 7 DAS on the youngest fully expanded leaf of two plants per plot, using a CIRAS-2 infrared gas analyser (PPSystem, Hitchin, UK) with a Parkinson's Automatic Universal Leaf Cuvette equipped with 2.5-cm^2 area cuvette inserts (environmental conditions inside the cuvette were set as follows: PAR=1000 μmol m^{-2} s^{-1}; leaf

temperature=26 ºC, CO_2 =450 ppm). Water use efficiency (WUE) of photosynthesis was determined as net photosynthesis (A) / leaf transpiration (E) ratio.

2.3 Plant water relations

Total leaf water potentials (Ψ_t) were determined on three samples per plot at 15 DAS with a dew-point psychrometer (WP4, Decagon Devices, Washington, WA). The osmotic potential (Ψ_π) was estimated on frozen/thawed leaf samples and the pressure potential (Ψ_p) as the difference between Ψ_t and Ψ_π, assuming a matrix potential equal to 0. Leaf osmotic adjustment (*LOA*) was determined as follows: $LOA = \Psi_{\pi 0}\ V_0 - \Psi_\pi\ V$, where $\Psi_{\pi 0}\ V_0$ is the product of (osmotic potential) × (osmotic volume) of unstressed plants and $\Psi_\pi\ V$ is the product of (osmotic potential) × (osmotic volume) of leaves from salinized plants. For each measurement, the osmotic volume was approximated by the corresponding relative water content (*RWC*) value calculated as: *RWC* = (leaf fresh weight – leaf dry weight) / (leaf saturated weight – leaf dry weight). Leaf saturated weight was determined after leaf immersion in distilled water for 24 h (Orsini et al., 2010b).

3 Statistical analysis

Statistical analyzes were performed by using SPSS statistical program. The significance of differences between treatments and between cultivars, and of the interaction between these two factors, was determined using two-way ANOVA. Where significant effects were found, ANOVA was followed by LSD test.

4 Results

4.1 Growth analysis

All cultivars were significantly ($P \leq 0.001$) affected by salinity (Table 5-1, 5-2). Among studied cultivars Madeleine and Clery cultivars had the highest values of growth reduction. Dry weight of shoot, root, and total plant reduced by 72%, 54%, 68% and 65%, 61% and 63% in respect to both cultivars at 80 mM NaCl compared to control plants, followed by Naiad, Camarosa, Kamila, Marmolada, and Siba, while the lowest growth

reduction values were recorded in Elsanta and Elsinore cultivars at 19% and 17% respectively.

Salinity significantly influenced (P≤0.001) leaves area of all cultivars (Table 5-1, 5-2). The highest reduction values were recorded in Naiad, Madeleine, Kamil, and Camarosa cultivars at 76%, 76%, 63, and 59% respectively at 80 mM NaCl, while leaves area of Elsinore, Clery, and Marmolada cultivars reduced at 61%, 44% and 33 % at 40 mM NaCl (Table 5-2). However, these cultivars did not tolerate the highest NaCl concentration (80 mM). Kamila and Elsanta cultivars were the most tolerant to salt stress in term of leaves area, whose reduction values were 5% and 26% at 40 mM NaCl respectively compared to control plants. On the other hand, cv.Siba was the most sensitive cultivar in response to salinity where the plant died at 40 mM NaCl.

The genotypic factor significantly influenced (P≤0.001) all growth parameters (Table 5-1, 5-2). Naiad and Madeleine cultivars had the highest values of shoot, total dry weight and leaves area as follows: 4.5 g, 10.43 g, 1294 cm^2 plant $^{-1}$ and 3.45 g, 7.94 g, 1318 cm^2 $plant^{-1}$ respectively followed by Elsinore, Camarosa, Clery, Elsanta, Marmolada cultivars, while the lowest values were recorded in Kamila and Siba cultivars in control plants. Significant interaction was observed between cultivars and salt treatments over all growth parameters. On the other hand, a significant correlation was found between leaf area and leaf gas exchange and water status regardless of cultivars and treatments (Table 5-6).

4.2 Leaf gas exchange

Stomatal conductance (g_s), leaf transpiration (E), net photosynthesis (A), and intercellular CO_2 (C_i) were significantly (P≤0.001) reduced over all cultivars in response to salinity (Table 5-1, 5-3, and 5-4). At 2 DAS, the highest reduction values of (g_s) were recorded in Kamila, Naiad, and Camarosa cultivars at 70%, 61% and 51% respectively, while, the (g_s) reduction values ranged between 41% and 50% for other cultivars at 80 mM NaCl compared to control plants. At 7 DAS, the highest (g_s) reduction values were recorded in Kamila and Naiad cultivars at 60%, 59% respectively at 80 mM NaCl compared to control plants. Elsinore, Elsanta, Marmolada, Madeleine and Clery cultivars did not tolerate high salt concentration (80 mM NaCl). Subsequently, the plants died. The (g_s) reduction value, however, ranged between at 35% and 63% in these cultivars at 40

mM NaCl compared to control plants, while Camarosa showed the lowest (g_s) reduction among all cultivars at 19% at 80 mM NaCl compared to control plants.

In terms of leaf transpiration (E), at 2 DAS, Kamila and Naiad cultivars showed the highest (E) reduction values at 49% for both of them at 80 mM NaCl compared to control plants, while (E) reduction values ranged between 26% and 30% for Elsanta, Camarosa, Madeleine, Marmolada, and Siba cultivars. The lowest (E) reduction value was recorded in Clery and Elsinore cultivars at 20% and 17% respectively at 80 mM NaCl compared to control plants. At 7 DAS, high (E) reduction values were observed in Kamila, Naiad at 51% and 48% respectively, while Camarosa recorded the lowest (E) reduction value at 27% at 80mM NaCl compared to control plants. Cv.Siba did not tolerate high concentration of salinity at 40 and 80 mM NaCl.

Net photosynthesis (A) values at 2 DAS of Clery, Naiad, Kamila and Siba were decreased by 56%, 46%, 43% and 37% at 80mM NaCl. The (A) reduction values ranged between 21% and 35% for Elsanta, Elsinore, Madeleine, Marmolada cultivars, while the (A) value did not change in response to salinity at 80 mM NaCl compared to control plants. At 7 DAS, Naiad and Elsinore had the highest reduction values of (A) at 40 mM NaCl compared to control plants, while the lowest (A) reduction value was recorded in Camarosa at 19% at 80 mM NaCl compared to control plants. Intercellular CO_2 (C_i) values decreased by 37%, 30% and 30% in Naiad, Camarosa, and Elsinore at 80 mM NaCl compared to control plants. Generally, (C_i) reduction values ranged between 10% and 24 % for other cultivars except for cv. Clery where the value increased by 7% at 80 mM NaCl. At 7 DAS (C_i) reduction values ranged between 53% and 75% over all cultivars at 40 mM NaCl compared to control plants. cv. Siba was the most sensitive cultivar, where the plants died by time at high salt concentrations (40 and 80 mM NaCl). The genotypic factor did not significantly affect (E) at 2DAS. Furthermore, the interaction of salt and cultivars was insignificant at 2DAS. On the other hand, the genotypic factor significantly influenced the (E) ($P \leq 0.001$) value at 7 DAS and (g_s) ($P \leq 0.05$), (C_i) ($P \leq 0.001$) at both dates. Significant interactions were observed in (A), (g_s) ($P \leq 0.01$) at 7DAS and (C_i) ($P \leq 0.01$) at both dates (Table 5-1).

4.3 Plant water relations

Salinity significantly affected total water potential (ψ_t), osmotic potential (ψ_π), relative water content (RWC), and LOA= leaf osmotic adjustment (LOA) (Table 5-1, 5-5). Variation in water potential (ψ_t), and osmotic potential (ψ_π) between cultivars suggested that some cultivars tolerated salt stress better than others. Naiad, Kamila, Camarosa tended to decrease their (ψ_t), and (ψ_π) values by 190%, 151%, and 92% and 51%, 51% and 45% in respect to cultivars and parameters at 80 mM NaCl compared to control plants, while the other cultivars, such as Elsinore, Clery, Madeleine, and Marmolada cultivars, did not tolerate high salt concentrations and died at 80 mM NaCl. Their (ψ_t) values, however, decreased between 43% and 248%, while (ψ_π) values varied between 26% and 77% at 40mM NaCl compared to control plants. In cv. Elsanta, the (ψ_t) value was not affected by salinity, while cv. Siba (ψ_t) value decreased by 24% at 20 mM NaCl compared to control plants. Similar RWC and LOA trend were found over all cultivars in response to salinity.

The genotypic factor significantly influenced total water potential (ψ_t), osmotic potential (ψ_π), and relative water content (RWC) (Table 5-1). On the other hand, a significant interaction between treatments and cultivars was remarkable in total water potential (ψ_t), and relative water content (RWC) (Table 5-1). Also, a significant correlation ($P< 0.05$) was found between plant water status (water potential and osmotic potential) and leaf gas exchange (E, A, g_s and C_i) (Table 5-6).

5 Discussion

Strawberry (***Fragaria*** x ***ananassa*** Duch.) is considered as a glycophytic (salt sensitive) crop, T = 1.0 dS m^{-1}, S = 33% (Ehlig and Bernstein, 1958), but a variation in salt tolerance has been demonstrated (Bisko et al., 2010; Keutgen and Pawelzik, 2009; Saied et al., 2005; Kaya et al., 2002). In the present study, presence of NaCl in irrigation water reduced leaves area and shoot mass, root mass, total plant mass, and shoot: root ratio in all strawberry cultivars. Nevertheless, Kamila, Elsanta, Naiad cultivars were characterized by lower leaf area reduction at 40 NaCl condition. Moreover, cv. Naiad had the highest leaf area at 40 mM NaCl compared to other cultivars which means that there are enough photosynthesizing leaves for the plant to produce flowers and fruit (Munns,

2011).The decreased rate of leaf area in response to salinity is primarily due to the osmotic effect which reduces plant water uptake, which quickly reduces the rate of cell expansion (Kaya et al., 2002). Accordingly, a significant correlation was found between leaf area and water potential (0.42**) and osmotic potential (0.43**) (Table 5-6). The slower formation of photosynthetic leaf area in turn reduces the flow of assimilates to plant shoots and roots, (Munns and Sharp, 1993). With time, the toxic level of Na^+ and Cl^- in the older transpiring leaves causes premature yellowing and death (Munns, 2011; Bisko et al., 2010; Keutgen and Pawelzik, 2009). In our experimental condition, water potential reduction, at 80 mM NaCl condition, and perhaps in coordination with the higher accumulation of Na^+ and Cl^-, caused dramatic leaf area reduction in Naiad, Kamila, Camarosa cultivars, whereas other cultivars did not tolerate high salt concentration, which resulted in the death of the plants. Kaya et al (2003) proposed that the plant growth reduction was due to nutrient uptake suppression by Na^+ and Cl^- competition with other nutrients. On the other hand, root mass was reduced to a lesser extent than shoot mass under salt conditions (Saied et al., 2005; Munns, 1993). Accordingly, shoot/root ratio was reduced due to higher reduction in shoot than root. Consequently, total plant mass was reduced in all cultivars at 80 mM NaCl compared to control plants. However, in our experimental condition, Elsinore and Elsanta cultivars were characterized by lower total mass reduction at 40 mM NaCl. on the contrary, cv. Naiad had the highest dry mass value compared to other cultivars at control, 20 and 40 mM NaCl, while cv.Siba was the most sensitive among cultivars.

Decreases in water uptake can be gradually reversed by the a accumulation of additional solutes through the process of osmotic adjustment (Keutgen and Pawelzik, 2009; Munns and Tester, 2008), which, in turn, reduces the rate of leaf senescence as it increases both avoidance and tolerance to dehydration (Joshi, 2011). In the present study, salinity decreased the (ψ_t) and (ψ_π) values in all cultivars (Table 5-4). Saied et al (2005) reported that salt at 30, and 60 mM NaCl concentrations reduced leaf water potential and osmotic potential in Elsanta and Korona cultivars compared to control plants, but cv.Korona was characterized by better osmotic control than cv.Elsanta. In addition, relative water content reduced by 15% in plants grown at 40 mM NaCl compared to control plants (Yildirim, 2009). However, in our experimental condition, Naiad, Kamila, and Camarosa cultivars were characterized by a higher ability to reduce water potential

and osmotic potential and increase the (LOA) value in response to high salt concentration (80mM NaCl). This may give an interpretation why these cultivars tolerated a high salinity concentration of 80 mM NaCl more than other cultivars. Generally, the osmotic adjustment can be used as a selective marker to distinguish between salt-sensitive and salt-tolerant cultivars.

Poor osmotic adjustment may lead to turgor loss and stomata1 closure, which is soon followed by reduced gas exchange and photosynthesis (Shannon, 1998). In addition, a high accumulation of Na^+ and Cl^- at photosynthetic organs up to toxic levels severely inhibits enzymes activities, including photosynthetic ones (Munns et al., 2006). Stomatal conductance is essential for both CO_2 acquisition and dehydration prevention (Medici et al., 2007). In our experimental condition, at 2 DAS, higher reduction in stomatal conductance was recorded in Kamila, Naiad, Camarosa cultivars compared to other cultivars at 80 mM NaCl. The inhibition of gas exchange is mostly mediated by shoot- and root- generated hormons (Chaves et al., 2009). These rapid responses to salinity in these cultivars may reflect higher adaption features allowing the plants to maintain water potential by reducing transpiration water losses. On the other hand, a significant correlation was found between water potential and leaf gas exchange (E, A, g_s and C_i) 0.48**, 0.30**, 0.53** and 0.44** respectively at 2 DAS (Table 5-6), suggesting that stomata plays an important role in order to maintain plant water status. It is known that prolonged water stress or salinity, especially during plant development, may cause profound modifications in leaf anatomy, such as thickened cell walls and smaller and more densely-packed leaf cell (Qiu et al., 2007). This may explain the long-term reduction of mesophyll conductance (g_m) in salt stressed plants and consequently photosynthesis inhibition (Niinemets et al., 2009). However, the relative contribution of stomatal and nonstomatal limitations to photosynthesis depends on the severity, velocity and type of stress being imposed (Chaves et al., 2009). A study on red raspberry showed that increasing the salt concentrations in the nutrient solution caused the reduction of photosynthesis and stomatal conductance (Neocleous and Vasilakakis, 2007). Biomass production of a plant has always to be seen in connection with the water use efficiency of photosynthesis (Koyro et al., 2011). At 2 DAS, strawberry cultivars varied in their WUE of photosynthesis (in term of A/E) values in response to salinity, where an insignificant effect on WUE in Elsinore, Elsanta, and Madeleine cultivars was observed. On the other

hand, WUE values significantly increased in Camarosa, Kamila, Naiad, and Siba at 80 mM NaCl compared to control plants. In addition, the WUE value decreased in Clery and Marmolada cultivars in response to salinity. In mild to moderate salt stress, stomata limits CO_2 access to the mesophyll, but the photosynthetic demand for CO_2 remains the same, and intercellular CO_2 partial pressure (p_i) values may decrease at 60–70% of external CO_2 partial pressure (p_a) (Chaves et al., 2004). This explains why in mild to moderate water deficits an increase in WUE is observed (Chaves and Oliveira., 2004). This may explain the significant reduction in intercellular CO_2 in all strawberry cultivars in response to salinity, especially in salt-tolerance cultivars (Kamila, Camarosa and Naiad) at 2 DAS. Several halophytic plants, such as *Aster tripolium* reveal a combination of low net photosynthesis, minimum transpiration, high stomatal resistance, and minimum intercellular CO_2 at their threshold salinity tolerance (Koyro and Huchzermeyer, 2004).

Recovery of photosynthesis following stress determines plant tolerance to water dificits and salinity (Chaves et al., 2011). Recovery depends on the intensity of photosynthesis decline under stress (Chaves et al., 2009). At 7 DAS, salt-tolerance cultivars (Camaros, Naiad, Kamila) revealed higher stomatal conductance and 80-92% of photosynthesis when compared to stomatal conductance and photosynthesis at 2 DAS, while (A) values varied between 61-65% in other cultivars at 40 mM NaCl. Turhan et al (2008) reported that by time stomatal conductance either remained almost unchanged or slightly increased at 8.5, 17 and 34 mM NaCl of more tolerant Tioga and Chandler cultivars, while g_s reduction was significantly observed in cv. Camarosa, especially at 30 DAS.

In conclusion, although Elsanta and Elsinore cultivars had the lower reduction of dry mass at 40 mM NaCl, Kamila, Naiad, Camarosa cultivars tolerated salinity up to 80 mM NaCl and were characterized by better osmotic adjustment and rapid response to salinity. Therefore, they revealed better capability to tolerate salinity at 80 mM NaCl. Finally, we can consider that Kamila, Naiad, and Camarosa cultivars are less sensitive among the studied nine cultivars of strawberry.

6 Tables

Table 5- 1: Summary ANOVA table for the parameters under assessment in two strawberry cultivars, Elsanta and Elsinore, in response to four salinity treatments (0, 10, 20 and 40 mM NaCl). LA= leaf area, g_s = stomatal conductance; E = leaf transpiration; A = net photosynthesis, ψ_t = total water potential, ψ_π = osmotic potential, RWC= relative water content, LOA= leaf osmotic adjustment; ns = non significant differences; * = significant differences at P≤0.05; ** = significant differences at P≤0.01; *** = significant differences at P≤0.001

Variables		**Salt stress**	**Cultivar**	**S x C**
LA		***	***	***
Shoot		***	***	**
Root		ns	***	*
dry weight		*	***	***
Shoot/Root		***	***	**
E	2 DAS	***	ns	ns
	7 DAS	***	***	*
A	2 DAS	***	***	ns
	7 DAS	***	***	**
g_s	2 DAS	***	*	ns
	7 DAS	***	*	**
C_i	2 DAS	***	***	**
	7 DAS	***	***	***
WUE	2 DAS	ns	**	ns
	7DAS	ns	ns	**
ψ_t		***	**	**
ψ_π		***	*	ns
RWC		***	***	*
LOA		*	ns	ns

Table 5- 2: Effect of salt stress (0, 20, 40 and 80 mM NaCl) on leaves area, shoot, root dry weight and shoot/ root ratio of nine cultivars of strawberry. Means ± SE. nd = non determined, since plants were dead. Different letters indicate significant difference at the 5% level by Duncan test.

Cv	Na	LA (cm^2 $plant^{-1}$)	Shoot (g)	Root (g)	DW (g)	Shoot/root ratio
ELSA -NTA	0	556 ± 116	3.05 ± 0.86	2.23 ± 0.18	5.28 ± 1.02	1.32 ± 0.32
	20	613 ± 072	4.52 ± 0.34	2.92 ± 0.43	7.44 ± 0.73	1.58 ± 0.14
	40	408 ± 117	3.43 ± 1.26	2.17 ± 0.23	5.60 ± 1.45	1.51 ± 0.43
	80	nd	2.13 ± 0.36	2.14 ± 0.33	4.27 ± 0.97	0.99 ± 0.08
ELSI -NORE	0	717 ± 087 a	2.92 ± 0.77	4.93 ± 0.16 ab	7.85 ± 0.61	0.60 ± 0.17
	20	566 ± 214 a	4.02 ± 1.80	3.92 ± 0.44 ab	7.94 ± 1.46	1.15 ± 0.63
	40	280 ± 006 b	3.27 ± 0.07	3.28 ± 1.05 b	6.55 ± 1.03	0.99 ± 0.52
	80	nd	1.81 ± 0.48	4.67 ± 0.66 ab	6.48 ± 0.75	0.39 ± 0.10
CAMA -ROSA	0	700 ± 108 a	4.89 ± 0.46 ab	2.62 ± 0.38	7.51 ± 0.73	1.93 ± 0.25 b
	20	686 ± 191 a	6.22 ± 1.01 a	1.93 ± 0.29	8.15 ± 1.29	3.21 ± 0.06 a
	40	445 ± 081 ab	3.25 ±1.08 b	2.85 ± 0.39	6.10 ± 1.30	1.14 ± 0.39 b
	80	286 ± 000 b	2.24 ± 0.65 b	1.82 ± 0.15	4.06 ± 0.78	1.19 ± 0.28 b
CLE -RY	0	603 ± 182	3.40 ± 0.16 a	3.88 ± 1.96	7.28 ± 1.81 a	0.98 ± 0.44 ab
	20	600 ± 121	3.61 ± 0.73 a	1.81 ± 0.76	5.42 ± 1.08 ab	1.99 ± 0.07 a
	40	339 ± 043	3.36 ± 0.94 a	2.60 ± 0.58	5.96 ± 1.36 ab	1.30 ± 0.29 ab
	80	nd	1.18 ± 0.03 b	1.50 ± 0.40	2.68 ± 0.43 b	0.84 ± 0.20 b
KAM -ILA	0	486 ± 160 ab	3.11 ± 1.15 ab	1.92 ± 0.70	5.04 ± 1.77 ab	1.80 ± 0.60
	20	659 ± 198 a	4.01 ± 0.56 ab	1.96 ± 0.39	5.97 ± 0.89 ab	2.12 ± 0.28
	40	458 ± 070 ab	4.90 ± 0.88 a	2.19 ± 0.12	7.09 ± 0.77 a	2.29 ± 0.51
	80	177 ± 000 b	1.36 ± 0.20 b	1.48 ± 0.01	2.84 ± 0.19 b	0.92 ± 0.14
MADEL -EINE	0	1319 ± 060 a	6.11 ± 1.45	1.83 ± 0.36	7.94 ± 1.80 a	3.27 ± 0.26
	20	592 ± 210 b	3.20 ± 1.31	1.67 ± 0.12	4.87 ± 1.19 ab	1.98 ± 0.92
	40	617 ± 184 b	5.06 ± 2.10	2.07 ± 0.65	7.13 ± 2.74 a	2.34 ± 0.29
	80	317 ± 026 b	1.74 ± 0.19	0.84 ± 0.11	2.58 ± 0.19 b	2.17 ± 0.47
MARMO -LADA	0	490 ± 082	3.09 ± 0.80	2.37 ± 1.13	5.46 ± 1.93 ab	1.57 ± 0.31
	20	531 ± 156	4.23 ± 1.59	2.51 ± 0.86	6.73 ± 2.45 a	1.66 ± 0.05
	40	327 ± 010	3.28 ± 0.07	2.98 ± 0.72	6.26 ± 0.70 ab	1.22 ± 0.25
	80	nd	1.76 ± 0.24	2.02 ± 0.32	3.78 ± 0.08 b	0.95 ± 0.23
NAI -AD	0	1294 ± 022 a	6.91 ± 0.32 bc	3.52 ± 0.13 b	10.43 ± 0.45 b	1.96 ± 0.03 b
	20	1403 ± 012 a	15.45 ± 3.43 a	4.23 ± 0.89 b	19.67 ± 4.26 a	3.68 ± 0.28 a
	40	865 ± 091 ab	10.60 ± 1.40 ab	6.81 ± 0.74 a	17.46 ± 2.01 a	1.57 ± 0.13 bc
	80	314 ± 028 b	2.97 ± 0.52 c	2.53 ± 0.51 b	5.50 ± 0.97 c	1.20 ± 0.13 c
SIBA	0	402 ± 030	1.87 ± 0.20	2.85 ± 0.47	4.73 ± 0.63 ab	0.67 ± 0.06
	20	439 ± 166	3.26 ± 1.12	2.71 ± 0.60	5.98 ± 1.55 a	1.24 ± 0.32
	40	nd	1.51 ± 0.13	2.23 ± 0.85	3.74 ± 0.91 ab	0.84 ± 0.22
	80	nd	1.76 ± 0.01	2.37 ± 0.57	3.25 ± 1.45 b	0.60 ± 0.01

Table 5- 3: Leaf transpiration (E), net photosynthesis (A), stomatal conductance (g_s), intercellular CO_2 (C_i), and water use efficiency (WUE) in response to 2 days of salt stress (0, 20 40 and 80 mM NaCl) application in seedlings of nine cultivars of strawberry. Means ± SE. Different letters indicate significant difference at the 5% level by Duncan test.

Cv	NaCl	E (mmol m^2 s^{-1})	A (mmol m^2 s^{-1})	g_s (mmol m^2 s^{-1})	C_i ppm	WUE A/E
ELSA -NTA	0	1.79 ± 0.17	7.57 ± 0.18 a	172 ± 09 a	268 ± 07	4.29 ± 0.32
	20	1.36 ± 0.05	6.93 ± 0.23 a	178 ± 13 a	277 ± 06	5.10 ± 0.21
	40	1.51 ± 0.41	6.53 ± 0.23 ab	127 ± 39 ab	233 ± 24	4.96 ± 1.14
	80	1.31 ± 0.14	5.47 ± 0.65 b	97 ± 15 b	236 ± 09	4.18 ± 0.11
ELSI -NORE	0	1.51 ± 0.02	8.03 ± 0.68 a	170 ± 20 a	284 ± 06 a	5.32 ± 0.42
	20	1.47 ± 0.02	8.00 ± 0.21 a	152 ± 13 ab	247 ± 08 ab	5.44 ± 0.21
	40	1.26 ± 0.12	7.33 ± 0.07 ab	96 ± 13 b	211 ± 31 ab	5.92 ± 0.50
	80	1.25 ± 0.20	6.37 ± 0.23 b	95 ± 32 b	200 ± 30 b	5.31 ± 0.67
CAMA -ROSA	0	1.49 ± 0.11ab	5.93 ± 1.84	173 ± 18 a	308 ± 25 a	3.91 ± 1.18
	20	1.68 ± 0.09 a	6.77 ± 0.54	165 ± 08 a	258 ± 10 a	4.08 ± 0.48
	40	1.50 ± 0.21ab	6.63 ± 0.41	140 ± 16 a	287 ± 10 ab	4.60 ± 0.66
	80	1.11 ± 0.12 b	5.93 ± 0.46	84 ± 12 b	214 ± 07 b	5.39 ± 0.22
CLE -RY	0	1.38 ± 0.04 ab	6.93 ± 0.33 a	137 ± 09 a	243 ± 08 bc	5.05 ± 0.39 a
	20	1.43 ± 0.03 a	6.73 ± 0.43 ab	155 ± 08 a	296 ± 03 a	4.71 ± 0.21 a
	40	1.22 ± 0.02 ab	5.60 ± 0.31 b	86 ± 06 b	223 ± 0.7 c	4.60 ± 0.20 a
	80	1.11 ± 0.15 b	3.03 ± 0.47 c	73 ± 08 b	259 ± 10 b	2.74 ± 0.12 b
KAM -ILA	0	1.74 ± 0.19 a	7.40 ± 0.53 a	202 ± 49 a	274 ± 19 a	4.43 ± 0.81
	20	1.28 ± 0.01 ab	5.27 ± 0.07 b	155 ± 10 a	289 ± 03 a	4.11 ± 0.06
	40	0.99 ± 0.20 b	4.70 ± 0.76 b	61 ± 14 b	201 ± 10 b	4.92 ± 0.64
	80	0.89 ± 0.06 b	4.23 ± 0.38 b	60 ± 04 b	212 ± 17 b	4.83 ± 0.68
MADEL -EINE	0	1.74 ± 0.34	6.27 ± 1.17 ab	212 ± 13 a	337 ± 19 a	3.73 ± 0.75
	20	1.39 ± 0.11	6.30 ± 0.67 ab	139 ± 17 b	253 ± 04 b	4.53 ± 0.20
	40	1.56 ± 0.13	7.47 ± 0.09 a	158 ± 20 ab	285 ± 09 b	4.84 ± 0.37
	80	1.21 ± 0.14	4.53 ± 0.59 b	107 ± 21 b	256 ± 23 b	3.89 ± 0.76
MARM -LADA	0	1.62 ± 0.02 a	7.93 ± 0.41 a	172 ± 04 a	265 ± 03 ab	4.89 ± 0.30
	20	1.44 ± 0.13 ab	7.07 ± 0.07 b	163 ± 09 ab	270 ± 03 a	4.98 ± 0.44
	40	1.41 ± 0.15 ab	6.90 ± 0.21 b	131 ± 22 b	238 ± 15 b	4.99 ± 0.49
	80	1.17 ± 0.05 b	5.17 ± 0.24 c	89 ± 06 c	237 ± 07 b	4.44 ± 0.31
NAI -AD	0	1.53 ± 0.04 a	8.23 ± 0.23 a	136 ± 09 a	261 ± 06 a	5.40 ± 0.15
	20	1.29 ± 0.04 a	7.43 ± 0.52 ab	147 ± 08 a	257 ± 10 a	5.78 ± 0.41
	40	1.18 ± 0.02 a	6.63 ± 0.52 b	95 ± 07 b	214 ± 17 ab	5.62 ± 0.44
	80	0.78 ± 0.23 b	4.47 ± 0.53 c	53 ± 21 c	164 ± 43 b	6.43 ± 1.16
SIBA	0	1.74 ± 0.12	4.97 ± 1.01 ab	148 ± 17	281 ± 14	2.83 ± 0.50
	20	1.51 ± 0.07	6.23 ± 0.64 a	166 ± 33	286 ± 08	4.18 ± 0.60
	40	1.28 ± 0.11	5.00 ± 0.25 ab	97 ± 11	244 ± 14	4.00 ± 0.52
	80	1.21 ± 0.32	3.13 ± 0.49 c	87 ± 32	252 ± 41	3.22 ± 1.37

Table 5- 4: Leaf transpiration (E), net photosynthesis (A), stomatal conductance (g_s), intercellular CO_2 (C_i), and water use efficiency (WUE) in response to 7 days of salt stress (0, 20 40 and 80 mM NaCl) application in seedlings of nine cultivars of strawberry. Means ± SE. nd = non determined, since plants were dead. Different letters indicate significant difference at the 5% level by Duncan test

Cv	NaCl mM	E (mmol m^2 s^{-1})	A (mmol m^2 s^{-1})	g_s (mmol m^2 s^{-1})	C_i ppm	WUE A/E
ELSA -NTA	0	1.50 ± 0.14	6.50 ± 0.21 a	184 ± 17 a	82 ± 05.8 a	4.40 ± 0.41
	20	1.25 ± 0.15	5.70 ± 0.36 ab	116 ± 19 b	19 ± 07.7 b	462 ± 0.34
	40	1.17 ± 0.15	4.30 ± 0.76 b	102 ± 26 b	21 ± 19.2 b	3.75 ± 0.63
	80	nd	nd	nd	nd	nd
ELSI -NOR	0	1.65 ± 0.08 a	7.63 ± 0.32 a	188 ± 10	104 ± 04.1 a	4.61 ± 0.16
	20	1.65 ± 0.28 a	5.80 ± 0.20 b	156 ± 27	57 ± 08.1 b	3.67 ± 0.47
	40	1.06 ± 0.19 b	4.43 ± 0.43 c	122 ± 36	42 ± 7.0 b	4.43 ± 0.79
	80	nd	nd	nd	nd	nd
CAMA -ROSA	0	1.79 ± 0.08	6.60 ± 0.21 a	184 ± 26	113 ± 06.6 a	3.70 ± 0.15
	20	1.51 ± 0.09	4.90 ± 0.87 ab	166 ± 08	56 ± 09.1 b	3.29 ± 0.64
	40	1.34 ± 0.29	4.20 ± 0.06 c	136 ± 37	34 ± 14.4 b	3.42 ± 0.64
	80	1.31 ± 0.07	5.33 ± 0.52 ab	148 ± 11	66 ± 08.1 b	4.14 ± 0.58
CLE -RY	0	1.38 ± 0.04 a	5.50 ± 0.20 a	167 ± 11 a	91 ± 0.9 b	3.98 ± 0.10
	20	1.11 ± 0.04 b	5.20 ± 0.00 a	147 ± 04 a	112 ± 01.8 a	4.70 ± 0.01
	40	0.87 ± 0.07 c	3.73 ± 0.30 b	87 ± 09 b	43 ± 11.1 c	4.30 ± 0.31
	80	nd	nd	nd	nd	nd
KAM -ILA	0	1.41 ± 0.06 a	5.77 ± 0.15 a	170 ± 11 a	87 ± 02.9 ab	4.11 ± 0.12
	20	1.23 ± 0.06 a	5.10 ± 0.40 a	150 ± 16 a	114 ± 06.7 a	4.15 ± 0.34
	40	0.67 ± 0.06 b	4.10 ± 0.10 b	58 ± 13 b	29 ± 00.1 b	6.20 ± 0.45
	80	0.69 ± 0.10 b	3.37 ± 0.22 b	67 ± 13 b	28 ± 07.5 b	5.13 ± 0.97
MADEL -EINE	0	2.08 ± 0.34 a	5.70 ± 0.64 a	320 ± 84 a	141 ± 10.8 a	2.96 ± 0.67
	20	1.02 ± 0.25 b	3.90 ± 0.60 b	85 ± 25 b	23 ± 13.7 b	4.02 ± 0.48
	40	1.16 ± 0.15 b	4.57 ± 0.35 ab	140 ± 09 b	40 ± 01.8 b	4.02 ± 0.29
	80	nd	nd	nd	nd	nd
MARMO -LADA	0	1.63 ± 0.08 b	6.43 ± 0.32 b	224 ± 02 a	94 ± 03.2 b	3.98 ± 0.36
	20	1.86 ± 0.09 a	7.13 ± 0.18 a	196 ± 06 b	102 ± 00.0 a	3.86 ± 0.27
	40	0.88 ± 0.02 c	4.73 ± 0.13 c	83 ± 06 c	26 ± 01.0 c	5.40 ± 0.16
	80	nd	nd	nd	nd	nd
NAI -AD	0	1.69 ± 0.15 a	7.43 ± 0.41 a	174 ± 31 a	85 ± 20.7 a	4.50 ± 0.66
	20	1.32 ± 0.06 b	5.57 ± 0.03 b	178 ± 21 a	49 ± 06.9 ab	4.25 ± 0.22
	40	0.94 ± 0.15 c	3.93 ± 0.41 c	95 ± 17 b	35 ± 08.0 b	4.47 ± 0.95
	80	0.89 ± 0.07 c	4.13 ± 0.03 c	71 ± 12 b	32 ± 08.3 b	4.72 ± 0.38
SIBA	0	1.26 ± 0.02	3.70 ± 0.49	159 ± 04	112 ± 5.9 b	2.93 ± 0.37
	20	1.07 ± 0.22	3.33 ± 0.72	122 ± 36	128 ± 4.6 a	3.09 ± 0.09
	40	nd	nd	nd	nd	nd
	80	nd	nd	nd	nd	nd

Table 5- 5: Total leaf water potential (ψ_t), leaf osmotic potential (ψ_π), relative water content (RWC) and leaf osmotic adjustment (LOA) in response to 7 days salt stress (0, 20 40 and 80 mM NaCl) in seedlings of nine cultivars of strawberry. Means ± SE. nd = none determined, since plants were dead. Different letters indicate significant difference at the 5% level by Duncan test

Cv	NaCl	ψ_t MPa	ψ_π MPa	RWC %	LOA
ELSANTA	0	-0.63 ± 0.07	-1.16 ± 0.11	94.1 ± 1.32	
	20	-0.68 ± 0.04	-1.35 ± 0.10	93.9 ± 0.44	0.18 ± 0.17
	40	-0.62 ± 0.05	-1.55 ± 0.20	83.8 ± 6.92	0.18 ± 0.12
	80	nd	nd	nd	nd
ELSINOR	0	-0.49 ± 0.04 a	-1.45 ± 0.02	90.2 ± 8.54	
	20	-0.79 ± 0.11 a	-1.55 ± 0.27	89.4 ± 4.17	0.08 ± 0.18
	40	-1.70 ± 0.35 b	-2.22 ± 0.39	90.3 ± 1.94	0.70 ± 0.52
	80	nd	nd	nd	nd
CAMAROSA	0	-0.60 ± 0.03 a	-1.44 ± 0.05 ab	93.7 ± 2.28 a	
	20	-0.57 ± 0.05 a	-1.26 ± 0.15 a	90.7 ± 1.37 a	0.21 ± 0.16 b
	40	-1.03 ± 0.01 b	-1.74 ± 0.08 bc	89.0 ± 0.92 a	0.19 ± 0.04 ab
	80	-1.15 ± 0.18 b	-2.11 ± 0.20 c	75.7 ± 2.18 b	0.25 ± 0.12 a
CLERY	0	-0.41 ± 0.06 a	-1.36 ± 0.07 a	93.3 ± 2.11 a	
	20	-0.63 ± 0.10 ab	-1.34 ± 0.04 a	90.7 ± 2.82 a	0.06 ± 0.19
	40	-0.90 ± 0.16 b	-1.71 ± 0.11 b	81.2 ± 2.08 b	0.12 ± 0.06
	80	nd	nd	nd	nd
KAMILA	0	-0.62 ± 0.13 a	-1.33 ± 0.09 a	90.5 ± 2.49 a	
	20	-0.79 ± 0.11 a	-1.38 ± 0.02 a	92.1 ± 1.17 a	0.07 ± 0.08
	40	-0.92 ± 0.07 a	-1.67 ± 0.10 b	86.6 ± 1.70 a	0.25 ± 0.21
	80	-1.57 ± 0.27 b	-2.01 ± 0.08 c	65.6 ± 1.05 b	0.12 ± 0.13
MADELEINE	0	-0.68 ± 0.08	-0.99 ± 0.08 a	90.1 ± 3.91 a	
	20	-0.80 ± 0.14	-1.73 ± 0.19 b	79.1 ± 3.03 b	0.48 ± 0.19
	40	-1.04 ± 0.12	-1.77 ± 0.15 b	80.1 ± 3.50 b	0.54 ± 0.20
	80	nd	nd	nd	nd
MARMOLADA	0	-0.77 ± 0.10 a	-1.35 ± 0.16 a	93.7 ± 1.69	
	20	-0.71 ± 0.05 a	-1.40 ± 0.08 a	90.7 ± 7.52	0.21 ± 0.03
	40	-1.11 ± 0.04 b	-2.15 ± 0.06 b	89.0 ± 4.56	0.19 ± 0.15
	80	nd	nd	nd	nd
NAIAD	0	-0.47 ± 0.01 a	-1.22 ± 0.05 a	89.5 ± 1.57 a	
	20	-0.62 ± 0.04 ab	-1.34 ± 0.09 a	92.1 ± 1.19 a	0.14 ± 0.11 b
	40	-0.88 ± 0.19 b	-1.78 ± 0.17 b	83.5 ± 1.13 b	0.40 ± 0.14 a
	80	-1.36 ± 0.08 c	-1.85 ± 0.07 b	73.9 ± 1.87 c	0.28 ± 0.09 ab
SIBA	0	-0.54 ± 0.04	-1.35 ± 0.09	93.7 ± 1.53	
	20	-0.73 ± 0.05	-1.37 ± 0.04	91.7 ± 0.63	0.01 ± 0.06
	40	nd	nd	nd	nd
	80	nd	nd	nd	nd

Table 5- 6: Pearson's correlation coefficients between biometric measurements (LA= leaf area and DW= dry weight) and gas exchange (E, A, g_s, and C_i) and water status (ψ_t, ψ_π and RWC) on nine cultivars of strawberry. * = significant at $P < 0.05$; ** = significant at $P < 0.01$; *** significant at $P < 0.001$: ^{ns} = not significant differences

	2DAS				7DAS						
	E	A	g_s	C_i	E	A	g_s	C_i	ψ_t	ψ_π	RWC
LA	0.20^{ns}	0.30**	0.36**	0.30**	0.37**	0.35**	0.42**	0.23*	0.42**	0.43**	0.31**
DW	0.15^{ns}	0.15^{ns}	0.28**	0.13^{ns}	0.24*	0.29**	0.19^{ns}	0.03^{ns}	0.05^{ns}	0.10^{ns}	0.04^{ns}
ψ_t	0.48**	0.30**	0.53**	0.44**	0.39**	0.39**	0.32**	0.37**	-	0.75**	0.50**
ψ_π	0.46**	0.26*	0.57**	0.49**	0.47**	0.36**	0.41**	0.47**	-	-	0.51**

Morphological and physiological characterisation of barley plants that overexpress the vacuolar two-pore K^+ channel (*Hv*TPK1)

1 Introduction

Most of the past research on abiotic stress tolerance has compared the physiological status of stressed plants with unstressed plants in order to understand the tolerance mechanisms (Singh and Flower, 2011). However, recent developments in the field of genetic and molecular biology opened exciting new possibilities in understanding the physiology of abiotic stresses (Ismail et al., 2007). Salinity has a long lasting effect on plant productivity by imposing both osmotic stress and specific ion toxicity associated with long salt exposure (Munns and Tester, 2008). Nevertheless, plants differ significantly in their tolerance of salinity. Of the cereals, barley (*Hordeum vulgare*) is considered as the most salt tolerant (Munns and Tester, 2008). It has been suggested that plant ability to maintain a high cytosolic K^+/Na^+ ratio is linked to plant salt tolerance (Shabala and Pottosin, 2010). Potassium has several important functions, such as electrical neutralization of anions groups and osmoregulation Lebaudy et al (2007), and also it is essential for all types of plant movements, including stomatal opening. The two major pools of potassium in plant cells are in the vacuole and in the cytosol (Shabala and Pottosin, 2010). Potassium retention in the cytosol has been shown to be a key determinant of salinity tolerance in glycophytes (Chen et al., 2008).

Vacuolar K^+ plays important roles in maintaining cell turgor through maintaining of cytosolic K^+ when plants are exposed to adverse conditions (Shabala and Pottosin, 2010). Regarding K^+ channels active in the tonoplast, there are three major types of cation conductance, namely the Fast Vacuolar (FV), Slow Vacuolar (SV) and Vacuolar K^+ (VK) channel (Lebaudy et al., 2007). TPK channels represent VK channels and it is proposed that the VK conductance might be involved in stomatal functioning due to its high K^+ selectivity and its presence in guard cell (Gobret et al., 2010). Also vacuolar TPK channels

could function as cellular osmosensors during rapid change in external osmotic pressure through activation of osmosensitive TPKs, which in turn would ensure the rapid release of K^+ from the vacuole (Maathuis, 2011). The function of the tonoplast K^+-selective channel, VK, could be beneficiary during salt stress by providing a shunt conductance for H^+-pumping, and exporting K^+ from the vacuole to improve the cytosolic K^+/Na^+ ratio (Pottosin et al. 2003). In *Arabidopsis*, 5 TPK isoforms have been reported, four isoforms are highly homologous (TPK1, 2, 3, and 5) and are expressed at the tonoplast (Voelker et al., 2006). In mesophyll and guard cells of *Arabidopsis* vacuoles VK channels are encoded by TPK1, a tandem pore K^+ channel. These channels mediate vacuolar K^+ release during stomatal closure, seed germination, and K^+ accumulation during seedlings growth (Gobert et al., 2007). The function of *At*TPK2, *At*TPK3, *At*TPK5, has not yet been demonstrated (Maathuis, 2010), while TPK4 is expressed at the plasma membrane (Becker et al., 2004). TPK families have been found in genomes of other species, such as rice, tobacco and *Physcomitrella* (Dunkel et al., 2008). In rice, there are two close homologous of *At*TPK1, *Os* TPKa and *Os*TPKb. However, *Os*TPKa is localized to the tonoplast of the central lytic vacuole (LV), whereas OsTPKb is localized to protein storage vacuoles (PSV) suggesting particular roles of different TPKs (Isayenkov et al., 2010). K^+ ion channels of the two pore K^+ (TPK) family have been extensively studied in Arabidopsis and tobacco, and are proposed to play an important role in K^+ homeostasis and seed germination.

However, the role of various TPKs during salt stress is not clear, particularly in cereal crops. In barley *Hv*TPK1 has been cloned and was overexpressed. The objectives of this project are to identify overexpression of *Hv*TPK1 in transgenic plants using leaf test methods, PCR and RT-PCR analysis and to characterize these transgenic plants with respect to growth and tolerance to a range of stresses, such as salinity stress and drought stress by measuring growth parameters and ion contents.

2 Materials and methods

2.1 Plant materials

Seeds of 35 lines of putative transgenic barley (*Hordeum vulgare)* in addition to wild type as a control were germinated using terra-green® substrate in growing trays at 20ºC for 10 days. Seedlings were used for leaf test, DNA and RNA analysis.

2.2 Screening of homozygous *Hv*TPK1-overexpressed lines using a leaf test method

Segregation analysis was carried out based on a leaf antibiotic resistance assay method (Wang and Waterhouse, 1997). Hygromycin resistance is assessed by the ability of leaf tissues to survive on media containing hygromycin. Briefly; two healthy green leaf tips about 2 cm long were cut off from each plant (on average 35 plants per line were tested). Leaf tips were immediately placed on labeled plates with the cut ends embedded in the growth medium with the angled top edge sticking out of the medium. Growth medium composed of 1/2 Murashige and Skoog salts (MS) medium (Murashige and Skoog, 1962), 200 mg/L hygromycin, and 5% of gelling agent (5 g/L of phytagel™, SIGMA) was used. The assays were continued for 5 to 7 days in growth room at 24^{o}C/18^{o}C (day/night) temperature under a 16/8 h light/dark cycle. The results were recorded based on green or green/yellow leaves indicating resistance and sensitive plants with bleached, or partly bleached. To confirm the results, homozygous resistance lines were tested in duplicate.

2.3 DNA extraction from leaf tissue of barley

DNA extraction was carried out according to the CTAB method with some modification. Plant material up to 200 mg was ground to fine powder in liquid nitrogen. Ground tissues were quickly mixed with pre-warmed 500 µl of 2 x CTAB buffer and incubated at 65 °C for 30–60 min. After vortex the mixture, 300 µl of chloroform:isoamylalcohol solution (24:1) was added. The mixture was vigorously shaken and spun for 5 min. The top aqueous layer was transferred to clean sterilized eppendorf tubes and DNA was precipitated by adding 2 volumes of 96 % ethanol and 4 % 3 M Na acetate (pH 5.2). The mixture was mixed well and left at room temperature for 30 minutes

to precipitate the DNA. The mixture was then centrifuged for 10 min at 13000 rpm to obtain the DNA pellet. Finally, the pellet was rinsed twice in 70 % ethanol, dried for 10 min and resuspended in 100 µl TE buffer. CTAB extraction buffer composition: 2 % CTAB, 1.4 M NaCl, 100 mM Tris-HCL (pH 8) and 20 mM Na-EDTA.

2.4 PCR analyses for screening of *Hv*TPK1-overexpressed lines

To confirm the results of leaf test and the integration of the transformed gene in transgenic barley plants, PCR was performed on 5 µl of gDNA extraction for 30 cycles (30 sec. at 94°C, 30 sec. at 60°C, 1 min. at 72°C), using the Hygromycin gene specific primers (Table 6-1). The PCR products were ran by electrophoresis in a 1 % agarose gel.

Table 6-1: Primer sequences of Hygromycine-F and Hygromycin-R gene used for PCR analysis.

Gene ID	Primers sequence	Product size (bp)
Hygromycin-F	5'- ATTTGTGTACGCCCGACAGT-3'	944
Hygromycin-R	5'- GGATATGTCCTGCGGGTAAA-3'	944

2.5 Expression analyses of *Hv*TPK1 transcript level by RT-PCR

Total RNA was extracted from leaf tissues of plants, using an RNeasy® mini KIT (Qiagen, UK), according to the manufacturer's instructions. The first-strand synthesis of cDNA was carried out using M-MLV reverse transcriptase protocol (Promega, USA). PCR was applied to 5 µl of cDNA with gene-specific primers against *Hv*TPK1. The house-keeping gene, α-tubulin was used as the control. PCR was performed based on the following instruction: 25 cycles at 57 °C, 1 min at 72 °C, and 30 s at 95 °C. The primers used for RT-PCR analyses are listed in table 6-2.

Table 6-2: Primers used to detect gene expression in control and *Hv*TPK1-overexpressed lines using RT-PCR.

Gene ID	Primers sequence	Product size (bp)
αTubulin-F	5'-AGTGTCCTGTCCACCCACTC-3'	760
αTubulin-R	5'-AGCATGAAGTGGATCCTTGG-3'	760
*Hv*TPK1_cDNA-F	5'- TTAGACTAGCAGGGCTCCTCC-3'	442
*Hv*TPK1_cDNA-R	5'- GAAGGTTGAGAAGCTGAGCCT-3'	442

2.6 Growth and ion analysis of transgenic barley plants

Seeds of transgenic lines of barley (*Hordeum vulgare)* in addition to a non transgenic line (lines showed sensitivity against Hygromycin leaf test and did not have amplification during PCR test) as a control were germinated using terra-green® substrate in growing trays at 20ºC. After 10 days, transplanting was done using hydroponic culture system consisting of boxes containing 2 liters of medium with macronutrients (1.25 mM KNO_3, 0.5 mM Ca $(NO_3)_2.4H_2O$, 0.5 mM $MgSO_4.7H_20$, 42.5 µM Fe-EDTA and 0.625 mM KH_2PO_4) and micronutrients (0.16 µM $CuSO_4.5H_20$, 0.38 µM $ZnSO_4.5H_20$, 1.8 µM $MnSO_4.H\ H_20$, 45 µM H_3BO_3, 0.015 µM $(NH_4)_2Mo_7O_{24}.4H_20$ and 0.01 $CoCl_2$) as described by Miyamoto et al (2001). The hydroponic boxes were transferred to growth cabinet for one week under following growth condition (12h 28°C/12h 24°C, relative humidity 70 %).The first fresh weights were recorded for all plants then the treatments of Na^+ and K^+ were applied (100 and 150 mM NaCl, 0-K^+ and 100 mM KCl) and boxes were placed in a green house set to a light/dark cycle of 16/8 h daily (PAR 300 µmol/m^2 per second), 20-25ºC day/night temperature, and 40–60% relative humidity. Nutrient solution was changed every seven days. After 11 days, second fresh weight was recorded and RGR (mg g^{-1} d^{-1}) was calculated based on the following formula:

$$RGR= (\ln W_2 - \ln W_1)/ (t_2-t_1)$$

Where ln= natural logarithm, W_1= fresh weight at t_1, while W_2= fresh weight at t_2

Plants were divided into shoot and root, and were dried at 80°C for 2 days. Dry weight of shoot and root was recorded. For Na^+ and K^+ contents of leaf and root of barley

plant, around 300 mg of dried plant tissue were immersed in 30 ml of 20 mM $CaCl_2$ for 24 h, and then the contents were measured by a flame photometer (Sherwood flame photometer-410, Cambridge, UK).

3 Results

3.1 Segregation and over-expression analyses of putative transgenic lines of barley

35 lines of putative transgenic lines of barley with *Hv*TPK1 were tested. The results of leaf tests showed that 13 lines were found to be homozygous and resistant to hygromycin, 19 lines were heterozygous, and 4 lines were homozygous and not resistant to hygromycin (Fig. 6-1). These results have been confirmed by PCR analyses using hygromycin phosphotransferase (HPT) gene primers.

RT-PCR analyses were carried out on leaf tissue in order to investigate the expression level of *Hv*TPK1 in the homozygous *Hv*TPK1 overexpressing lines and control plants. The result showed that the expression of *Hv*TPK1 in all tested homozygous transgenic lines of barley slightly increased compared to wild type line with respect *Hv*TPK1/Tubulin ratio (Fig. 6-2).

3.2 Growth and ion analysis of transgenic barley plants

Three transgenic lines (OX1, OX4,OX6) and wild type plants were grown under hydroponic conditions at control, 100 mM NaCl, 150 mM NaCl, 0-K^+, 100 mM KCl, and 15% PEG conditions in order to assess the effect of overexpression *Hv*TPK1 on plant growth. The results showed that OX1 line displayed lower growth rate by 16% and 36% compared to wild type at 100 mM KCl and 15% PEG conditions respectively (Fig. 6-3). On the other hand, the other treatments did not result in any significant difference in growth rate between transgenic lines and wild type plants (Fig. 6-3).

3.3 K^+ and Na^+ contents of overexpressed *Hv*TPK1 lines and wild type

K^+ and Na^+ contents were assayed in root and shoot of the overexpressed lines and wild type plants at the end of 11 days treatments. The result showed different patterns of K^+ and Na^+ contents in shoot and root organs in overexpressed lines compared to wild

type plants. At control condition, OX4 and OX6 lines showed significantly lower K^+ root content than WT (Fig. 6-4A). It should also be noted that decrease in K^+ root content was accompanied by a decrease in Na^+ content (Fig. 6-5A). In saline conditions, OX1 had significantly higher K^+ root content, while OX4 line had significantly lower K^+ shoot content compared to wild type plants (Fig. 6-4B). In this treatment all transgenic lines significantly showed lower Na^+ root content compared to WT (Fig. 6-4B). In high saline condition (150 mM NaCl) OX6 line significantly showed lower K^+ root content (Fig. 6-4C) and significantly higher Na^+ root content than WT (Fig. 6-5C).

In absence of K^+ in the growth medium, OX4 and OX6 lines significantly displayed higher K^+ content in shoot (Fig. 6-4D) and significantly lower Na^+ content in both root and shoot compared to WT (Fig. 6-5D). With high K^+ present in the growing medium, OX1 line significantly showed lower K^+ content in root and shoot (Fig. 6-4E) and this was associated with lower Na^+ content in both root and shoot of transgenic lines compared to WT (Fig. 6-5E). In drought condition, OX6 displayed significantly higher K^+ content in root and shoot while OX4 had significantly higher K^+ root content compared to wild type plants (Fig. 6-4F). In contrast, OX1 and OX4 lines displayed significantly higher Na^+ shoot content than WT (Fig. 6-5F).

4 Discussion

Vacuoles play fundamental roles in many physiologically relevant processes in plants. They constitute the main site of turgor generation through their role as depository for minerals and water (Maathuis, 2010). They also play a role in plant nutrition; for example plants grown in K^+-rich media will deposit large quantities of this nutrients in the vacuoles of vegetative tissue Maathuis and Sanders (1993), allowing plants to reuse them in order to maintain cytoplasmic K^+ homeostasis when they are exposed to K^+ deficient conditions (Walker et al., 1996). Under adverse environmental condition, such as salinity, vacuoles can function as detoxification of Na^+ and Cl^- through sequestration process into vacuoles mediated by channels and carrier type transporters localized on tonoplast (Maathuis, 2010). Vacuolar K^+ (VK) cation channels, such as TPK1 have been recorded in many plants vacuoles where they are involved in turgor regulation and K^+ nutrition (Gobert et al., 2007). The barley TPK1 gene has been cloned, but the physiological role of

this gene is not well known. In this study, Potential phenotypic changes resulting from overexpressing TPK1 were observed in parameters, such as relative growth rate, Na^+ and K^+ tissue contents under different K^+, NaCl, and drought conditions. No significant difference was observed for transgenic lines in relative growth rate compared to wild type at control condition. Maathuis (2010) reported that no obvious morphological phenotypes are present in Arabidopsis *tpk1* mutants when plants are grown in normal conditions. However, comparisons of *tpk1* KO mutant, wild type and TPK1 overexpressing plants pointed to multiple functions for TPK1, a gene expressed in all tissues and cell types. In order to investigate the function of overexpressed *Hv*TPK1gene in transgenic lines in high K^+ concentration, addition of 100 mM KCl in hydroponic condition significantly reduced plant growth rate for both transgenic and wild type plants compared to control condition. Furthermore OX1 showed lower growth rate than wild type plants (Fig. 6-3). It is known that drought stress is one of the most significant abiotic stresses to limit plant growth and productivity. In the present study, 15% PEG significantly reduced plants growth rate compared to control condition and this is in accordance with studies that were carried on barley in different drought conditions (Jasmioson et al., 1995, Krcek et al., 2008). Moreover, OX1 line showed lower growth rate compared to control plants suggesting an effect of *Hv*TPK1 on OX1 growth in this condition (Fig. 6-3).

The cytosolic K^+/Na^+ ratio has been named as a key determinant of plant salt tolerance (Maathuis and Amtmann, 1999). The optimal cytosolic K^+/Na^+ ratio can be maintained by either restricting Na^+ accumulation in plant tissue or preventing K^+ loss from the cell (Shabala and Cuin, 2008). In the present study, a different response was observed among transgenic lines compared to wild type. At 100 mM KCl and 15% PEG conditions, OX1 line showed lower K^+/Na^+ root compared to control plants (Fig. 6-4E and 6-4F) and this was accompanied with lower growth rate suggesting that *Hv*TPK1 plays a role in cellular homeostasis and redistributing of K^+ within plant tissue. Shabala and Pottosin (2010) reported that higher K^+/Na^+ ratios play an important role in maintaining cellular metabolism and ultimately, the ability of a plant to survive in adverse conditions.

In conclusion, this study showed there are some promising transgenic lines where *Hv*TPK1 plays a role in improving K^+/Na^+ which allows the plants to thrive in adverse conditions. However, growth of transgenic lines and wild type plants during longer

periods may be necessary to reveal the real response of overexpressing lines under adverse conditions.

5 Figures

Figure 6- 1: Leaf antibiotic resistance assay method of *Hv*TPK1-overexpressed barley for selection of homozygous transgenic lines. Bleached leaves were considered not resistant to hygromycin, while green leaves were considered tolerant to hygromycin (Transgenic plants).

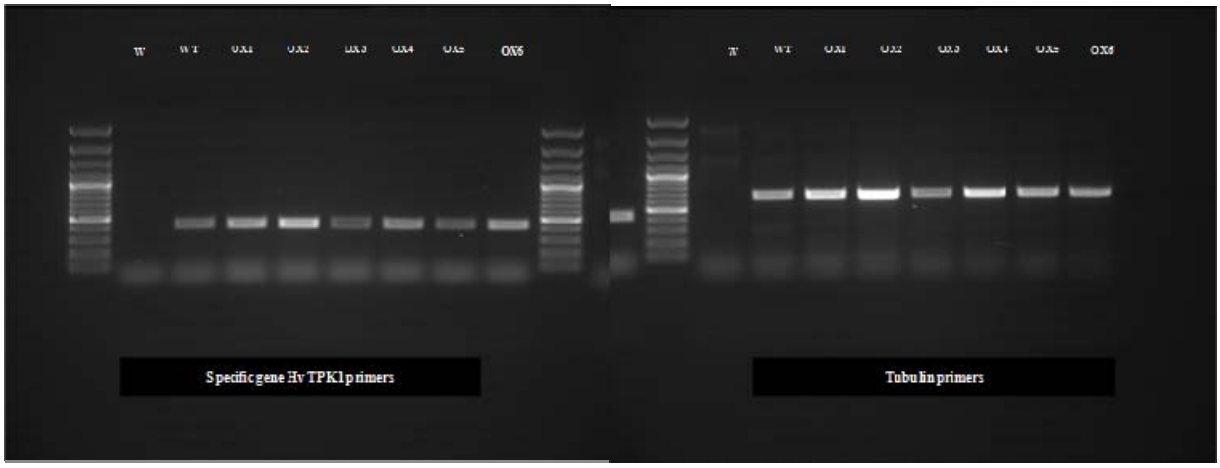

Figure 6- 2: RT-PCR analysis of *Hv*TPK1 over-expression in leaf tissue of transgenic lines of barley compared to wild type; on the left *Hv*TPK1 specific gene primers, on the right *Tubulin* primers.

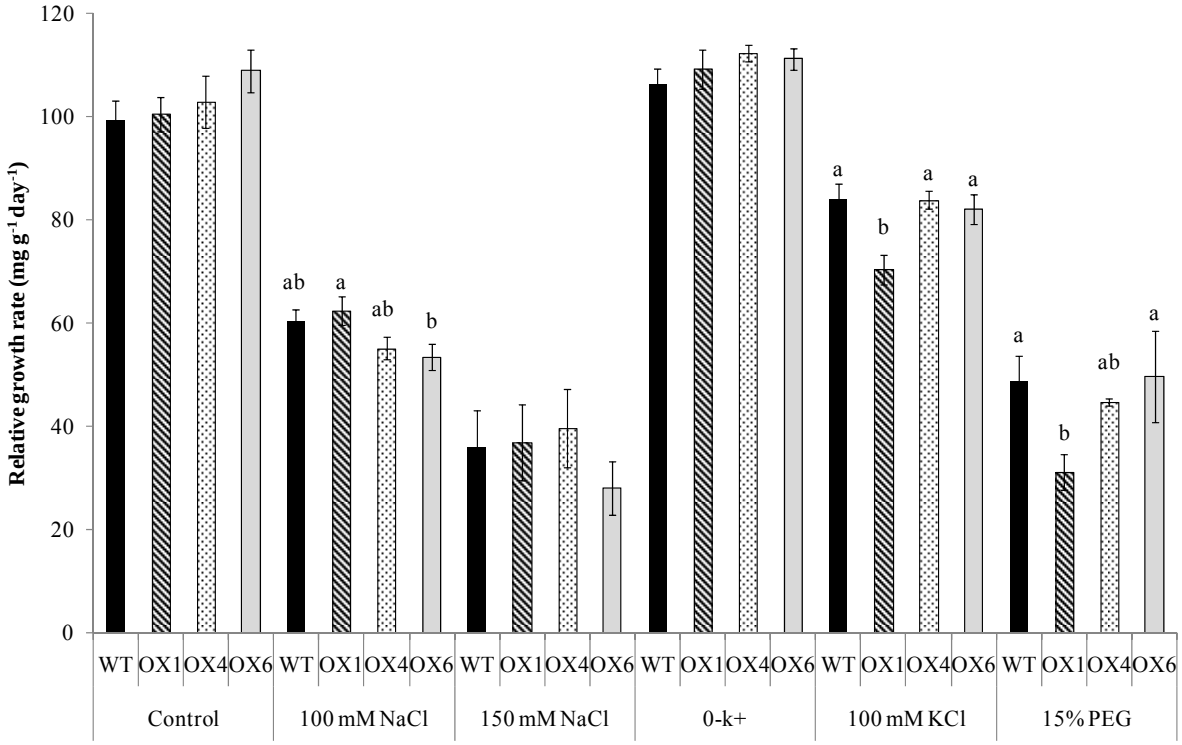

Figure 6- 3: Relative growth rate of *Hv*TPK1-overexpressed barley (OX1, OX4, and OX6) and wild type plants (WT). Plants were grown in control, 100 mM NaCl, 150 mM NaCl, 0-K^+, 100 mM KCl, and 15% PEG conditions for 11 days in hydroponic medium. Values are the mean ± SD (n=4). Different letters indicate significant difference at the 5% level by Duncan test.

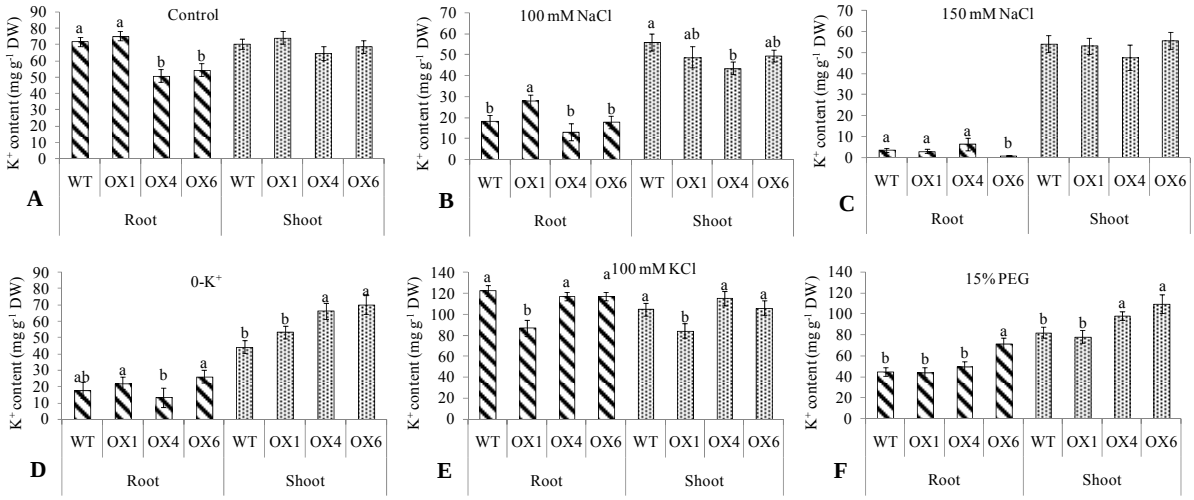

Figure 6- 4: K^+ content in root, shoot of *Hv*TPK1-overexpressed barley (OX1, OX4, OX6) and wild type plants grown in **A**) Control, B)100 mM NaCl, **C**) 150 mM NaCl, **D**) 0-K^+, **E**) 100 mM KCl, and **F**) 15% PEG conditions for 11 days in hydroponic medium. Values are the mean ± SD (n=4). Different letters indicate significant difference of K^+ content at the 5% level by Duncan test.

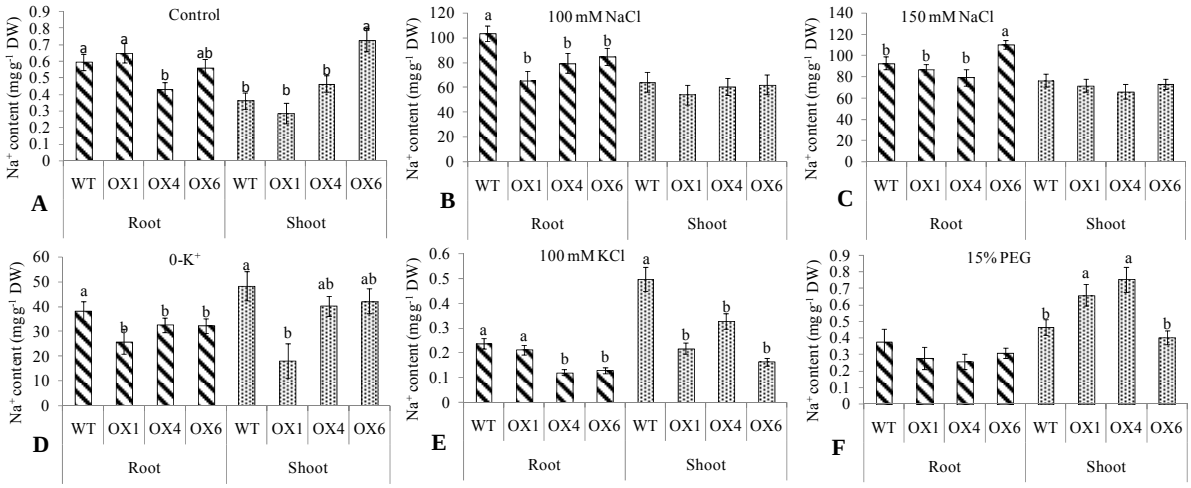

Figure 6-5: Na^+ content in root, shoot of *Hv*TPK1-overexpressed barley (OX1, OX4, OX6) and wild type plants grown in **A**) Control, B)100 mM NaCl, **C**) 150 mM NaCl, **D**) 0-K^+, **E**) 100 mM KCl, and **F**) 15% PEG conditions for 11 days in hydroponic medium. Values are the mean ± SD (n=4). Different letters indicate significant difference of K^+ content at the 5% level by Duncan test.

Conclusion

1 Low stomatal density and reduced transpiration facilitate strawberry adaptation to salinity

Water and soil salinizations are major constraints to agricultural productions because plant adaptation to hyperosmotic environments is generally associated to reduced growth and ultimate yield loss. Understanding the physiological/molecular mechanisms that link adaptation and growth is one of the greatest challenges in plant stress research since it would allow us to better define strategies to improve crop salt tolerance. In this study we attempted to establish a functional link between morphological/physiological traits in strawberry in order to identify margins to "uncouple" plant growth and adaptation in concrete agricultural systems. In this study, the results showed Upon salinization Elsanta plants maintained a larger and more functional leaf area compared to Elsinore plants, which were irreversibly damaged at 40 mM NaCl. The tolerance of Elsanta was correlated with a constitutive reduced transpirational flux due to low stomatal density (173 *vs.* 234 stomata mm^{-2} in Elsanta and Elsinore, respectively), which turned out to be critical to pre-adapt plants to the oncoming stress. The reduced transpiration rate of Elsanta (14.7 g H_2O $plant^{-1}$ h^{-1}) respect to Elsinore (17.7 g H_2O $plant^{-1}$ h^{-1}) most likely delayed the accumulation of toxic ions into the leaves, preserved tissues dehydration and consented to adjust more effectively to the hyperosmotic environment. Although we cannot rule out the contribution of other physiological and molecular mechanisms to the relatively higher tolerance of Elsanta, here we demonstrate that low stomatal density may be beneficial for cultivars prescribed to be used in marginal environments in terms of salinity and/or drought.

2 Response of endogenous proline, total soluble proteins, lipid peroxidation, and antioxidative enzymes in leaves of two strawberry cultivars (Elsanta and Elsinore) to long- terms of salt stress

Stressful conditions of the environment, such as drought and salinity lead to the enhanced genereation of ROS in plants, which in turn can impose a threat to cell by causing lipid peroxidation and oxidation of proteins and ultimately cell death. Scavenging

of ROS is achieved by an efficient of both non-enzymatic and enzymatic antioxidant systems. In this study, cv. Elsinore was characterized by higher enzymes activities than cv. Elsanta in control condition. In mild stress, initial decreasing in SOD, CAT, and POD activities were detected in both cultivars. Under severe salt stress, however, SOD, CAT, and POD activities were higher in cv. Elsinore than those in cv. Elsanta. Proline and total soluble protein contents increased in response to severe salt stress with distinct response in cv. Elsinore compared to cv. Elsanta. Accordingly, the coordinated function of enzymatic antioxidant system and non-enzymatic antioxidant system in terms of proline and soluble proteins contents may contribute efficiently in the detoxification of the damaging effect of ROS in cv. Elsinore rather than in cv. Elsanta. Subsequently, Lipid peroxidation which is considered as an indicator for ROS activity decreased in cv. Elsinore rather than in cv. Elsanta. Overall, although cv. Elsinore had an active enzymatic antioxidant system, that did not allow it to mitigate adverse effect of salinity, where the plants died at 40 mM NaCl. Contrariwise, cv. Elsanta was characterized by more ability to tolerate salinity at 40 mM NaCl due to some morphological adaptation (such as stomatal density). These results suggest that in our experimental conditions, enzymatic antioxidant system and proline accumulation in strawberry leaves were not substantial factors which allow palnt to tolerate salt stress and perhaps they were not the appropriate screening tools for salt tolerance in strawberry.

3 Organoleptic attributes, taste-relative, and bioactive compounds

Strawberry fruit have a highly desirable taste and flavor and are commonly considered to be a valuable nutritional resource of vitamins and photochemical compounds that concern human satisfaction and health. It is also reported that accumulation of taste-relative and photochemical compounds in many plants has been positively correlated with salt stress. In the present study, cv. Elsanta was characterized by higher fruit weight and less fruit number compared to cv. Elsinore in control condition, whereas fruit weight significantly reduced while fruit number did not change of both cultivars in salt conditions. According to the model of assessment, the results showed that in moderate salinity taste-related compounds (TA and TSS) were within a very good range of 8-9, and 0.80 respectively. On the other hand, these compounds were impaired and did not satisfy the panelist preference in severe salt conditions. Concerning organoleptic

attributes, a significant correlation between fruit weight and appearance and aroma was found, suggesting that high salinity impaired organoleptic attributes through its effect on fruit weight in both cultivars.Finally, bioactive compounds, such as phenolics, anthocyanin and flavonoids and antioxidant capacity were enhanced in salt conditions for both cultivars; however cv. Elsanta revealed higher antioxidant capacity than cv.Elsinore. Overall, in moderate salt conditions, fruit quality of both cultivars improved and this may economically compensate for the reduction of fruit yield at 14-18%. While, in severe salt conditions (40 mM NaCl), although the health related compounds were significantly enhanced in both cultivars, the organoleptic attributes and fruit yield significantly decreased, especially in cv. Elsinore than in cv. Elsanta and the fruit were completely rejected by the panelists. This kind of fruit might be functional for developing value-added products wealth with health promoting compounds particularly for elder people.

4 Screening nine cultivars of strawberry

The tolerance of strawberry cultivars have evaluated using various criteria, such as absolute growth, leaf area, plant water status, and leaf gas exchange under saline and none saline conditions. In this study, the results showed that Elsinore and Elsanta cultivars were characterized by lower total mass reduction at 40 mM NaCl. In contrast, cv. Naiad had the highest dry mass, while cv. Siba was the most sensitive among cultivars. In response to 80 mM NaCl, our data showed that Naiad, Kamila, Camarosa cultivars were characterized by rapid response to salt stress, where stomatal conductance reduced when plants were exposed to salinity maintaining water status through lowering water loss. Moreover these cultivars had lower osmotic potential than other cultivars. Thus, these cultivars tolerated salinity up to 80 mM NaCl, while the other cultivars did not tolerate high salt concentrations and died at 40 mM NaCl. Overall, in terms of plant growth under high NaCl salinity, Kamila, Naiad, Camarosa cultivars are considered as less-sensitive salt cultivars among nine studied cultivars at 80 mM NaCl.

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