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Salinity Effect on Horticultural Crops: Morphological, Physiological, and Biomolecular Elements of Salinity Stress Response

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PhD thesis. 2011-2014

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Title

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PREFACE

The current PhD thesis is the result of the work carried out from March 2011 to March 2014 in the Dipartimento di Scienze Agrarie- DipSA- ALMA MATER STUDIORUM- Università di Bologna and financially supported by Erasmus Mundus External Cooperation Window Program. It has been a great challenge for me to deal with different aspects of plant physiological, biochemical and molecular responses toward salinity stress. It was great efforts to shift myself from biochemical laboratory to molecular one and be familiar with all laboratory techniques in short tighten time. Also, it was challenging for me to perform the grafting processing for the first time trying to apply what I have read of textbooks and scientific articles about this technique. Also, it was not easy to conduct all morphological, biochemical, physiological analysis in different plant species from different families in less than three years of full working hours between greenhouse and the laboratories.

The thesis is structured in four main chapters. The first chapter is a general introduction about the adverse effect of salt stress and functional plant adaptations for salinity tolerance. The second chapter is devoted to dealing with grafting as a way to improve salt tolerance in two plant species of muskmelon and tomato. The third chapter addressed the determination salinity thresholds, genotypic variability, and morphological, physiological and biochemical adaptation of cabbage and radish genotypes grown under saline stress. The fourth chapter addresses the possible functional role of salt overly sensitive gene (SOS) and some elongation factors in *Brassica* under the salt stress condition.

Salinity exerts detrimental effects on crop cultivation worldwide. At present, most important advances on the understanding of salt tolerance in plants are achieved through the use of model plants (eg. *Arabidopsis thaliana*, *Thellungiella halophila*). However, the transfer of knowledge to the cropped species is a slow process, and most of the physiological/biochemical processes involved in stress response in cultivated crops are still unknown. The role of several morphological traits (eg. stomatal and root morphologies) was highlighted in both model and crop species as important features in determining plant response to the stress, but still little connection have been found on their related physiological consequences. This is also explained by the difficulties found in the transfer of results from model plants and the only recent application of an inter-disciplinary approach to agronomic experiments on crop species. During the PhD, several aspects of horticultural crop response to salinity have been addressed with the final aim of combining physiological and

biomolecular elements of functional stress response in plants. Species adopted in the trial were vegetable crops (eg. tomato, melon, cabbage and radish). Overall, the PhD project has achieved the following goals: identifying the methods for the measurement of salt stress response, screening and assessment of stress perception in plants; and creation of a database of plant biochemical and physiological features improving salinity tolerance.

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SUMMARY

Among abiotic stresses, high salinity stress is the most severe environmental stress. High salinity exerts its negative impact mainly by disrupting the ionic and osmotic equilibrium of the cell. In saline soils, high levels of sodium ions lead to plant growth inhibition and even death. Salt tolerance in plants is a multifarious phenomenon involving a variety of changes at molecular, organelle, cellular, tissue as well as whole plant level. In addition, salt tolerant plants show a range of adaptations not only in morphological or structural features but also in metabolic and physiological processes that enable them to survive under extreme saline environments. The main objectives of my dissertation were understanding the main physiological and biomolecular features of plant responses to salinity in different genotypes of horticultural crops that are belonging to different families *Solanaceae* (tomato) and *Cucurbitaceae* (melon) and *Brassicaceae* (cabbage and radish). Several aspects of crop responses to salinity have been addressed with the final aim of combining elements of functional stress response in plants by using several ways for the assessment of plant stress perception that ranging from destructive measurements (eg. leaf area, relative growth rate, leaf area index, and total plant fresh and dry weight), to physiological determinations (eg. stomatal conductance, leaf gas exchanges, water use efficiency, and leaf water relation), to the determination of metabolite accumulation in plant tissue (eg. Proline and protein) as well as evaluation the role of enzymatic antioxidant capacity assay in scavenging reactive oxygen species that have been generated under salinized condition, and finally assessing the gene induction and up-down regulation upon salinization (eg. SOS pathway).

Grafting is an integrative reciprocal process and an interesting tool to avoid or reduce yield losses caused by salinity stress in high-yielding genotypes belonging to *Solanaceae* and *Cucurbitaceae* families. In this research, we have investigated the role of grafting in alleviating the drastic effect of salt stress in muskmelon plant (*Cucumis melo*). The performances of several grafting combinations of interspecific rootstock-scion, self-grafted and non-grafted plants have been traced with the aim of highlighting the plant differential morphological and physiological responses toward salt stress and address the comprehension of which features are responsible of improving salinity tolerance in grafted plants. Additionally, particular attention has been given to the role of the root system in altering stress perception in the shoot. Furthermore, some contradictory issues have been addressed, which on many occasion remained overlooked or conflicted, whether the positive effect of

grafting in alleviating the deleterious effect of salt and increase plant tolerance was attributed to rootstock characteristics or scion genotypic differences, and/or belong to the scion-rootstock interaction. It is known that the capacity of some rootstocks is essential for salt resistance in some species, while for other species the salt resistance conferred by rootstock depends on complex physiological interactions which are not well understood yet, that involving the type of scion genotype or the complexity of specific interaction between the scion genotype and rootstock. Thus, in this work, for comprehending whether the conferred salt tolerance of grafted plants was depended on the rootstock character or is affected by scion genotypic differences and/or exchangeable effects of scion-rootstock interaction, different tomato cultivars (*Lycopersicon esculentum* Mill) were used as scions and grafted against different tomato rootstock genotypes. The main objectives of this work were determining the contribution of rootstock in inducing useful salt tolerance to the shoot growth and fruit productivity depending on salt tolerance mechanisms of the shoot genotype; identifying the main salt defence mechanisms that induced in the shoot by different rootstock genotypes based on rootstock potential to regulate the absorption of ions from saline solution and regulate their transportation into shoot in long-term salt stress; and identifying the conditioned significant effect of the scion genotypes on physiological and biochemical shoot performance that involved in plant salt tolerance. Moreover, the second part of this study was devoted to investigate the salinity thresholds, genotypic variability, morphological productive performances, and physiological and biochemical adaptation of cabbage (*Brassica oleracea*) and seven red radish genotypes (*Raphanous sativus*) grown under saline stress in order to identify the most tolerant genotype to be used in further breeding programme.

Reactive oxygen species (ROS) are important toxic and regulatory agents in plants. They are produced in response to salt stresses. The accumulation of ROS during abiotic stresses causes an additional challenge for plants that is called oxidative stress which induces oxidative damage to lipids, proteins, and nucleic acids. In plant cells, specific ROS-producing and scavenging systems are found in different organelles and coordinated by enzymatic and non-enzymatic defence system. The enzymatic components of the antioxidative defense system comprise several enzymes that operate in different subcellular compartments and respond when cells are exposed to oxidative stress. In this work, we assessed the plant differential responses upon salinization in term of secondary metabolite (proline and protein accumulation), occurring of oxidative stress (measuring the products of lipid peroxidation such as H_2O_2 and MDA) and determining the activities of enzymatic antioxidative defense

systems (superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR)) in the leaves of two botanical varieties of cabbage (white and savoy) and in seven different genotypic root radish cultivars in order to understand the biochemical elements of salt adaptive mechanisms and choose the most promoting cultivar(s) under stressed conditions. Moreover, the evaluation of the effect of scion and rootstock genotypes in conferring the salt resistance in term of enhancing the tomato fruit antioxidant defence system under NaCl stress have been under investigation.

Moreover, in response to high salinity stress, various genes get up-regulated, the products of which are involved either directly or indirectly in plant protection. Some of the genes encoding osmolytes, ion channels, receptors, components of calcium signalling, and some other regulatory signalling factors or enzymes are able to confer salinity-tolerant phenotypes when transferred to sensitive plants. Overall, the susceptibility or tolerance to high salinity stress in plants is a coordinated action of multiple stress responsive genes, which also cross talk with other components of stress signal transduction pathways. Salt Overly Sensitive (SOS) pathway is known to be defined by three protein components, SOS1, SOS2 and SOS3. At the cellular level, the SOS signaling pathway has been proposed to mediate cellular signaling under salt stress, to maintain ion homeostasis. In the third part of my PhD project, we investigated the possible functions of some elongation factor genes and the salt overly sensitive SOS3 family of Ca^{2+} sensors and their associated SOS2 family of protein Kinase as well as the plasma membrane Na^{+}/H^{+} exchanger (antiporter) encoded by the *SOS1* gene in conferring the salt tolerance of two varieties of *Brassica*.

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III. Salinity thresholds and genotypic variability of Cabbage (*Brassica oleracea* L.) grown under saline stress: physiological adaptation and nutritional value. *Journal of the Sciences of Food and Agriculture*. (submitted).

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II. Salinity response in melon scions as affected by grafting. In: International Symposium on Vegetable Grafting Book of Abstract. Viterbo (Italy), 3-5 October 2011, VITERBO: G. Colla, p. 89.

Annex 3: Poster

I. Stomatal regulation in grafted melon improves salinity tolerance. International Symposium on Vegetable Grafting. University of Tuscia, Viterbo, Italy. 3th -5th October 2011.

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CHAPTER 1

ADVERSE EFFECT OF SALT STRESS AND FUNCTIONAL PLANT ADAPTATIONS FOR SALINITY TOLERANCE

CHAPTER 1

Adverse Effect Of Salt Stress And Functional Plant Adaptations For Salinity Tolerance

1. INTRODUCTION

World agriculture is facing a lot of challenges like producing 70% more food for an additional 2.3 billion people by 2050 while at the same time fighting with poverty and hunger, consuming scarce natural resources more efficiently and adapting to climate change (FAO 2009). However, the productivity of crops is not increasing in parallel with the food demand. The lower productivity in most of the cases is attributed to various abiotic stresses. Curtailing crop losses due to various environmental stressors is a major area of concern to cope with the increasing food requirements (Shanker and Venkateswarlu 2011).

As a sessile organism, plants often experience abiotic stress like salinity, drought, high or low temperature, flooding, metal toxicity, ozone, UV-radiations, herbicides, etc., which pose serious threat to the crop production (Bhatnagar-Mathur et al. 2008; Ahmad and Prasad 2012a, b). The complex nature of the environment along with its unpredictable conditions and global climate change are increasing gradually which is creating the situation more adverse (Mittler and Blumwald 2010). Abiotic stresses remain the greatest constraint to crop production worldwide. It has been projected that more than 50% of yield reduction is the direct result of abiotic stresses (Rodriguez et al. 2005; Acquaah 2007). The major abiotic stresses like drought, high salinity, cold, and heat negatively influence the survival, biomass production and yield of staple food crops up to 70% (Kaur et al. 2008; Ahmad et al. 2010a; Ahmad et al. 2012); hence, threaten the food security worldwide.

Soil salinity is among the major abiotic stresses that limits crop productivity worldwide (Hu et al. 2005) since most crops are sensitive to soil salinization (Munns 2002). There are two major processes of soil salinization; geo–historical processes and man–made. Most of the worldwide salt–affected lands are the result of natural causes, i.e., from accumulation of salts over long time period, and this occurs mainly in arid and semiarid zones (Rengasamy 2002). One way of soil salinization is weathering of the rocks that releases soluble salts, which is mainly in the form of sodium chloride and calcium chloride (Szabolcs 1989), other being salt

accumulation due to the deposition of salts from oceans by wind or rain (Munns and Tester 2008). Man–made saline soils are mostly found in (semi) arid lands as a result of over-irrigated agriculture, and hence in the rise of water tables. This is the main factor of increasing salinity in agricultural lands (Munns et al. 2002).

There is a wider range of salt tolerance in natural populations, which is reported to be evolved naturally in numerous grass species like *Agrostis*, *Festuca*, *Lolium*, and *Poa* (Acharya et al. 1992). Such plants provide outstanding materials for studying the mechanisms of adaptations they use to tolerate high concentrations of salt (Ashraf 2003). Such adaptations have been evaluated in several grass populations from quite diverse habitats such as estuaries and coastal areas, marine and fresh water salt marshes, and dry–land salinities. Examples are *Sporobolus virginicus* (Naidoo and Mundree 1993), *Cynodon dactylon* (Hameed and Ashraf, 2008), *Ochthochloa compressa* and *Aeluropus lagopoides* (Naz et al. 2009), and *Imperata cylindrica* (Hameed et al. 2009).

2. ADVERSE EFFECT OF SALINITY STRESS

High salinity causes both hyperionic and hyperosmotic stresses and can lead to plant death (Hasegawa et al. 2000). It is reported that plants growing under saline conditions are affected in three ways: reduced water potential in root zone causing water deficit, phytotoxicity of ions such as Na^+ and Cl^- and nutrient imbalance depressing uptake and transport of nutrients. Na^+ competes with K^+ for binding sites essential for cellular functions (Munns 2002a). Excess salt concentration also enhances the osmotic potential of soil matrix which restricts the water uptake by plants. Sodium is the primary toxic ion, because it interferes with K^+ uptake as well as and disturbs stomatal regulation which ultimately causes water loss and necrosis. On the other hand, Cl^- induces chlorotic toxicity symptoms due to impaired production of chlorophyll (Chl). Although both Na^+ and Cl^- are the major ions which produce many physiological disorders in plants, especially Cl^-, which is the most dangerous than Na^+ (Tavakkoli et al. 2010). In plant cells, Cl^- is required for the regulation of some enzyme activities in the cytoplasm. It is also a co-factor in photosynthesis and is involved in turgor and pH regulation. However, it is toxic to plants at high concentrations, with critical levels for toxicity reported to be 4-7 mg g^{-1} for Cl^- -sensitive species and 15-50 mg g^{-1} for Cl^- tolerant species (White and Broadley 2001). Higher accumulation of Cl^- led to a significant reduction in growth and water use efficiency in plants.

2.1. Growth

One of the initial effects of salt stress on plant is the reduction of growth rate. Salinity can affect growth of plant in various ways. First, the presence of salt in the soil reduces the water uptake capacity of the plant, and this causes quick reduction in the growth rate. This first phase of the growth response is due to the osmotic effect of the soil solution containing salt, and produces a package of effects similar to water stress (Munns 2002b). The mechanisms by which salinity affects growth of a plant depend on the time scale over which the plant is exposed to salt. Munns (2002b) summarized the sequential events in a plant grown in saline environment. He stated that "In the first few seconds or minutes, water is lost from cells and shrinked. Over hours, cells recover their original volume but the elongation rates are still reduced which led to lower growth rates of leaf and root. Over days, cell division rates are also affected, and contribute to lower rates of leaf and root growth. Over weeks, changes in vegetative development and over months changes in reproductive development can be seen". Later on, Munns (2005) developed the 'two-phase growth response to salinity' for better understanding the temporal differences in the responses of plants to salinity. The first phase of growth reduction is a quicker process which is due to osmotic effect. The second phase, on the other hand, is much slower process which is due to the salt accumulation in leaves, leading to salt toxicity in the plants. The later one may results in death of leaves and reduce the total photosynthetic leaf area which reduce the supply of photosynthate in plants and ultimately affect the yield. With annual species, the timescale is day or week, depending on species and salinity level. With perennial species, the timescale is months or year. During phase 1, growth of both genotypes is reduced due to the osmotic effect of the saline solution adjacent to roots. During phase 2, leaves of more sensitive genotype are died and the photosynthetic capacity of the plant is greatly reduced which imposes an additional effect on growth. Upon addition of salt at one step, the growth rate plummets to zero or below and takes 1-24 h to regain the new steady rate, depending on the extent of the osmotic shock (Munns 2002a).

In plants, where Na^+ and Cl^- build up in the transpiring leaves over a long period of time, resulting in high salt concentration and leaf death. Leaf injury and death are attributed to the high salt load in the leaf that exceeds the capacity of salt compartmentation in the vacuoles, causing salt to build up in the cytoplasm to toxic levels (Munns 2002a, 2005; Munns et al. 2006). Under saline condition, some crops are most sensitive during vegetative and early

reproductive stages, less sensitive during flowering and least sensitive during the seed filling stage.

Salinity increased the number of sterile florets and viability of pollen, becoming more pronounced with increased salinity. Seed set was reduced by 38% when female plants were grown in as low as 10 mM NaCl. In *Suaeda salsa* , plant height, number of branches, length of branches and diameter of shoot were significantly affected by salt stress which was due to the increased content of Na^+ and Cl^- (Guan et al. 2011). Also, Dolatabadian et al. (2011) observed that salinity stress significantly decreased shoot and root weight, total biomass, plant height and leaf number.

2.2. Photosynthesis

The reduction in photosynthetic rates in plants under salt stress is mainly due to the reduction in water potential. Photosynthesis is also inhibited when high concentrations of Na^+ and/or Cl^- are accumulated in chloroplasts. As photosynthetic electron transport is relatively insensitive to salts, either carbon metabolism or photophosphorylation may be affected due to salt stress (Sudhir and Murthy 2004). A positive correlation between salt stress induced photosynthetic rate and yield has been obtained in different crops (Sudhir and Murthy 2004). Fisarakis et al. (2001) reported a positive growth inhibition caused by salinity associated with a marked inhibition of photosynthesis. In fact, the effect of salinity on photosynthetic rate depends on salt concentration as well as plant species or genotypes. There is evidence that at low salt concentration salinity sometimes stimulate photosynthesis. For instance, in *Bruguiera parvi fl ora* , Parida et al. (2004) observed that rate of photosynthesis increased at low salinity while decreased at high salinity, whereas stomatal conductance remained unchanged at low salinity and decreased at high salinity. There are some other factors that reduced photosynthetic rates under salt stress are: enhanced senescence, changes in enzyme activity, induced by alterations in cytoplasmic structure and negative feedback by reduced sink activity (Iyengar and Reddy 1996). The reduction in stomatal conductance which results in restricting the availability of CO_2 for carboxylation reactions is also a factor that reduces photosynthesis under stress (Brugnoli and Bjorkman 1992). It was reported that stomatal closure minimizes loss of water through transpiration and this affects light-harvesting and energy-conversion systems thus leading to alteration in chloroplast activity (Iyengar and Reddy 1996). Higher stomatal conductance in plants is known to increase CO_2 diffusion into the leaves and thereby favor higher photosynthetic rates. One of the most notable effects of salt stress is the alteration of photosynthetic pigment biosynthesis (Maxwell and Johnson

2000). The decrease in Chl content under salt stress is a commonly reported phenomenon and in various studies and the Chl concentration were used as a sensitive indicator of the cellular metabolic state (Chutipaijit et al. 2011). Saha et al. (2010) observed a linear decrease in the levels of total Chl, Chl*a*, Chl*b*, Car and xanthophylls as well as the intensity of Chlfluorescence in *Vigna radiata* under increasing concentrations of NaCl treatments. Compared to control, the pigment contents decreased on an average, by 31% for total Chl, 22% for Chl*a*, 45% for Chl*b*, 14% for carotene and 19% for xanthophylls (Saha et al. 2010).

2.3. Water relation

According to Romero-Aranda et al. (2001) increase of salt in the root medium can lead to a decrease in leaf water potential and, hence, may affect many plant processes. Osmotic effects of salt on plants are the result of lowering of the soil water potential due to increase in solute concentration in the root zone. At very low soil water potentials, this condition interferes with plant's ability to extract water from the soil and maintain turgor. However, at low or moderate salt concentration (higher soil water potential), plants adjust osmotically (accumulate solutes) and maintain a potential gradient for the influx of water. Salt treatment caused a significant decrease in relative water content (RWC) (Ghoulam et al. 2002). According to Katerji et al. (1997), a decrease in RWC indicates a loss of turgor that results in limited water availability for cell extension processes. Steudle (2000) reported that in transpiring plants, water is thought to come from the soil to the root xylem through apoplastic pathway due to hydrostatic pressure gradient. However, under salt stressed condition, this situation changes because of the restricted transpiration. Under these situations, more of water follows cell-to-cell path, flowing across membranes of living cells (Vysotskaya et al. 2010).

2.4. Nutrient imbalance

It is well-established that crop performance may be adversely affected by salinity induced nutritional disorders. However, the relations between salinity and mineral nutrition of crops are very complex (Grattan and Grieve 1999). The nutritional disorders may result from the effect of salinity on nutrient availability, competitive uptake, transport or distribution within the plant. Numerous reports indicated that salinity reduces nutrient uptake and accumulation of nutrients into the plants (Rogers et al. 2003; Hu and Schmidhalter 2005). However, very few evidences exist that addition of nutrients at levels above those considered optimal in non-saline environments, improves crop yield (Grattan and Grieve 1999). In fact, these processes may occur simultaneously and whether they affect the crop yield or quality depends on the toxic level, composition of salts, the crop species and surrounding environment (Grattan and

Grieve 1999). Numerous plant studies have demonstrated that salinity could reduce N accumulation in plants. Decreased N uptake under saline conditions occurs due to interaction between Na^+ and $NH4^+$ and/or between Cl^- and NO_3^- that ultimately reduce the growth and yield of the crop (Rozeff 1995). This reduction in NO_3^- uptake is associated with Cl^- antagonism (Bar et al. 1997) or reduced water uptake under saline conditions (Lea-Cox and Syvertsen 1993). The availability of P was reduced in saline soils due to (a) ionic strength effects that reduced the activity of PO_4^{3-} , (b) phosphate concentrations in soil solution was tightly controlled by sorption processes and (c) low solubility of Ca-P minerals. Hence, it is noteworthy that phosphate concentration in field grown agronomic crops decreased as salinity increased (Qadir and Schubert 2002). Different plant studies indicated that high level of external Na^+ caused a decrease in both K^+ and Ca^{2+} concentrations in plant tissues of many plant species (Hu and Schmidhalter 2005; Asch et al. 2000). This reduction in K^+ concentration in plant tissue might be due to the antagonism of Na^+ and K^+ at uptake sites in the roots, the influence of Na^+ on the K^+ transport into xylem or the inhibition of uptake processes (Suhayda et al. 1990). The availability of micronutrients in saline soils is dependent on the solubility of micronutrients, the pH of soil solution, redox potential of the soil solution and the nature of binding sites on the organic and inorganic particle surfaces.

2.5. Yield

The above mentioned effects of salt stress on plants ultimately lead to reduction of yield of crop which is most countable effect of salt stress in agriculture. Except some halophytes, yield of most of the crops reduced greatly due to salt stress. Tolerance and yield stability are multigenic traits that are complicated to establish in crops since salt stress may be imposed continuously or intermittently, or become gradually more severe and at any stage during development (Yokoi et al. 2002). Crop species have exhibited substantial differences in salt tolerance based on their relative yields. Relative yield often exhibits a linear decrease after a threshold salinity has been reached, and salt tolerance has been defined in terms of two parameters: the threshold electrical conductivity and the percent decrease in relative yield per unit of electrical conductivity in dS m^{-1} above the threshold. It was observed that relative yield varied greatly depending on the salinity levels and the degree of tolerance (Mass 1986). Number of pods per plant, seeds per pod and seed weight were negatively correlated with salinity levels. This reduction of yield and its component rated under salt stress condition may also be attributed to low production, expansion, senescence and physiologically less active green foliage (Wahid et al. 1997), thus reduced photosynthetic rate might be a supplementary effect (Seemann and Critchley 1985). The severe inhibitory effects of salts on

fertility may be due to differential competition in carbohydrate supply between vegetative growth and constrained supply of these to the developing panicles (Murty and Murty 1982). Also reduced viability of pollen under stress condition could result in failure of seed set (Abdullah et al. 2001). As reported by Greenway and Munns (1980), after some time in 200 mM NaCl, a salt-tolerant species such as sugar beet might have a reduction of only 20% in dry weight, a moderately tolerant species such as cotton might have a 60% reduction, and a sensitive species such as soybean might be dead. On the other hand, a halophyte such as *Suaeda maritima* might be growing at its optimum rate (Flowers et al. 1986).

3. ADAPTIVE COMPONENTS OF SALT TOLERANCE

Salt tolerance is a complex phenomenon involving a variety of mechanisms. It can be defined as the ability of the plants to complete their growth cycle with an acceptable growth and yield (Flowers et al. 1986; Colmer and Flowers 2008). Three major factors affect the plant growth under salinity, water stress, ion toxicity, and nutrient uptake and translocation, and as a result, disturbance of ionic balances such as K^+ and Ca^{2+}. Physiological drought may play a crucial role, which restricts the water uptake by plants. On contrary, excess salt uptake by plants interrupts the cellular functions and this damages vital physiological processes, i.e., photosynthesis and respiration (Marschner 1995). Furthermore, mechanisms like increased leaf resistance (fewer stomata, increased cuticle and epidermis thickness, and mesophyll resistance) could prevent turgor loss from leaf and root surface, and hence better water efficiency.

Plant tolerance to saline environments is of broad spectrum ranging from glycophytes (that are sensitive to salt) to halophytes (that tolerate high concentrations of salt). The acquired salt tolerance may be of hereditary nature in some species (Niknam and McComb 2000), i.e., passed along to offspring. Halophytic or salt tolerant species can adopt multiple strategies to survive under high salinities by controlling the levels of ions their shoots or particularly in leaves. The mechanisms involved are restricting or excluding the ion uptake at root level, and hence minimizing the translocation of salts to the shoot (Flowers and Colmer 2008). Genkel (1954) divided the halophytes into three groups: euhalophytes, crinohalophytes, and glycohalophytes, but this classification has been modified by Nagalevskii (2001) and Zhao et al. (2002). Salt tolerance in euhalophytes is based on accumulation, as they accumulate salts in their tissues, crinohalophytes depend on excretion of toxic ions like Na^+ and Cl^- as they are capable of excreting salts out of the plant body, and glycohalophytes rely on avoiding mechanism by preventing the accumulation of excess salts (Voronkova et al. 2008). The

growth rate can be linked to the accumulation of salts in the plant leaves that plant takes up from the roots, so the continuation of growth under saline environments is an indication of high degree of salt tolerance.

Plants generally use two mechanisms to tolerate high salt concentrations. Firstly, the avoidance, i.e., keeping the salts away from the metabolically active tissues (Munns and Tester 2008). This is through passive exclusion of ions (by a permeable membrane), active expelling of ions (by ion pumps), or by dilution of ions in plant tissues (Allen et al. 1994). Secondly, compartmentalization of accumulated salts in the vacuoles of plant cells (Munns 2002). These two methods are vital for preventing toxic ions to accumulate or causing damage to the plant tissues, and therefore, they could be employed for identifying markers for genetic manipulation of salinity tolerance in plants.

Salt tolerant or halophytic plants can minimize the detrimental effects of salts (i.e., ion toxicity, nutritional disorder, osmotic stress) by modifying morphological, anatomical and physiological mechanisms of salt tolerance (Poljakoff-Mayber 1975; Hameed et al. 2009). Extensive root system (root length and proliferation) and the presence of salt secreting structures (e.g., salt glands) on the leaf surface may prove vital in plants (Naz et al. 2009). The salt tolerance of plants may involve: (a) restricted or controlled uptake of salts, (b) tissue tolerance, (c) accumulation of salt in inert areas (e.g., vacuoles), (d) ion discrimination (e.g., uptake and translocation of ions like K^+, Na^+, Cl^- and SO_4^{2-}), (e) production of low molecular weight protective osmolytes like enzymes, hormones, antioxidants, etc. (Munns and Tester 2008). These mechanisms may be responsible for variations in the salt tolerance within plant genotypes or species.

When a plant is exposed to increased soil salinity, a primary response is decreased plant water potential, and this is due to a decrease in both osmotic and water potentials of the soil. Accumulation of osmotically compatible cellular solutes (e.g., sugars, proteins, free amino acids) is one of the well–characterized responses of plants to such low water potential. In salt tolerant species, accumulation of osmotically compatible solutes directly correlates with Na gradients in soil and thereby reduces the detrimental effect of salt stress (Briens and Larher 1982; Lee et al. 2007). Mechanisms involved in salinity tolerance or adaptations crucial for the plant survival are still not well understood. Therefore, there is a need to identify appropriate morpho-anatomical or physio-biochemical indicators of salinity tolerance in halophytic and other salt tolerant plants (Ashraf and Harris 2004).

3.1. Morphological and anatomical traits

Both halophytes and non–halophytes exhibit remarkable anatomical changes when exposed to elevated levels of salinity (Maas and Nieman 1978). However, most conspicuous changes are notable in leaf. Many salt tolerant plants, particularly dicotyledonous halophytes are characterized by xeromorphic characteristics such as thick succulent leaves, which apparently aid sufficient water supply (Vakhrusheva 1989). Smaller reduced leaves with dense covering of pubescence are also a characteristic of xerophytes, which accounts for a successful survival of halophytes under dryland salinities (Mokronosov and Shmakova 1978).

Stomatal features like density and size are critical for controlling transpirational loss from leaf surface and even more critical under physiological droughts (Hameed et al. 2009). The importance of stomatal characteristics in avoiding water loss through leaf surface has been reported several species like wheat (Akram et al. 2002). Succulence (both leaf and stem) is one of the most noticeable features in halophytes, which provides not only more space for dumping off toxic ions in the plant body, but also increasing the total plant water content (Drennan and Pammenter 1982), and this is crucial for balancing out ion toxicity.

It is not very much clear as succulence is simply a response to salinity or is the response of adaptive value of halophytic plants (Waisel 1972). Increased succulence in halophytes in response to increasing salinity is presumed to be of adaptive nature (Waisel 1972). Succulence is very much greater in halophytic dicotyledonous species than in monocotyledonous ones (Flowers et al. 1986). There is also evidence of a rapid increase in vacuolar volume and in the concentration of Na^+ (Mimura et al. 2003) in the cells of mangrove *Bruguiera sexangula*, which is a potential mechanism to cope with a rapid increase in external salt concentration.

3.2. Salt excretion

Halophytes utilize salts in osmotic adjustment, which lowers water potentials of their tissues. Accumulation of toxic ions in large quantities in leaves, while avoiding their toxic effects seems to be an important strategy for growth and survival under harsh climates (Greenway and Munns 1980). Balancing of growth and ion accumulation is the major phenomenon of salt tolerance in some species, while in others excess of toxic ions is secreted via secretory structures like salt glands and micro-hairs (Drennan and Pammenter 1982). *Spartina* spp. are the example where shoot mineral content is regulated by the ionic secretion through

specialized salt glands. Salts are also released by the leaf surface through cuticle or in guttation fluid; but they also become concentrated in salt hairs (Stenlid 1956).

Many species exude Na^+ salts onto the leaf surface (Naidoo and Naidoo 1998), which is effective in reducing Na^+ concentration in plant tissues, i.e., *Sporobulus* spp. (Marcum and Murdoch 1992). Salt secretory trichomes, characteristic of *Atriplex* spp., are bladder–like hairs projecting out of leaf surface. They consist of a large secretory or bladder cells on the top and a stalk consisting of one or sometimes a few cells (Dickison 2000). All these cells contain mitochondria, dictyosomes, ribosomes, endoplasmic reticulum and a large flattened nucleus. The chloroplasts are rudimentary or partially developed. The only difference lies in that a single large vacuole is present in bladder cell and many small vacuoles in the stalk cell (Osmond et al. 1969). A symplastic continuum exists from the mesophyll cells to the bladder cells for the movement of ions. The external walls of bladder and stalk cells are cutinized, while inner primary walls are not (Thomson and Platt-Aloia 1979).

3.3. Physiological/biochemical traits

Salinity causes many adverse effects on plant growth which may be at physiological or biochemical levels (Munns 2002; Munns and James 2003), or at the molecular level (Mansour 2000; Tester and Davenport 2003). In order to assess the tolerance of plants to salinity stress, growth or survival of the plant is measured because it integrates up– or down–regulation of a variety of physiological mechanisms (Niknam and McComb 2000). Cell growth rate depends on cell wall extensibility as well as turgor (Lockhart 1965).

3.3.1. Osmotic adjustment

Accumulation of exceptionally high concentrations of inorganic ions as well as organic solutes is an important physiological adaptation in both halophytic and salt tolerant species (Pitman 1984). In salt excretory plants, salt is kept away from photosynthesizing or meristematic cells. In these plants, osmotic balance is generally achieved via extensive accumulation of organic solutes and/or inorganic ions. However, in plants where salt inclusion is the prime mechanism, accumulation of some inorganic ions (predominantly Na^+ and Cl^-) regulates the osmotic adjustment (Ashraf 2004). Both organic and inorganic solutes are essential for osmoregulation in plants, especially under saline environments. However, their relative contribution to osmotic adjustment varies from plant to plant or species to species, or even within different tissue of the same plant (Hameed and Ashraf 2008).

There is a variety of compatible osmolytes in higher plants. Important among these are soluble sugars, organic acids, and soluble proteins. The important amino acids that accumulate in the plants are alanine, arginine, glycine, leucine, serine, and valine, along with the imino acid proline, citrulline and ornithine (Mansour 2000; Ashraf 2004). Osmoregulation via accumulation of free amino acids and in particular, glycinebetaine is the principal strategy in many plant species to tolerate salt stress (Martino et al. 2003). Amides such as glutamine and asparagine (Mansour 2000), and proline (Abraham et al. 2003) have also been reported to accumulate in large amounts in higher plants in response to salt stress.

3.3.2. Ion selectivity

A major feature of the solute transport by plants in saline conditions is the degree of selectivity, particularly between potassium and sodium (Ashraf et al. 2005). One of the most important physiological mechanisms of salt tolerance is the selective absorption of K^+ by plants from the saline media (Ashraf et al. 2006). Halophytic or salt tolerant species differ from salt–sensitive ones in having restricted uptake or transport of Na^+ and Cl^- to the leaves despite an effective compartmentalization of these ions. This is critical in preventing the build–up of toxic ions in cytoplasm (Munns 2002; Ashraf 2004). Ion imbalance, particularly that caused by Ca^{2+} and K^+ is the most important and widely studied phenomenon affected by salt stress, which is directly influenced by the uptake of Na^+ and Cl^- ions (Munns 2002 Munns et al. 2006). Maintaining better concentrations of K^+ and Ca^{2+} and limiting the Na^+ uptake are vital for the salt stress tolerance in plants (Karmoker et al. 2008). Higher K^+/Na^+ or Ca^{2+}/Na^+ ratios are characteristic to the tissue salt tolerance, and are often used as a screening criteria for the salt tolerance (Ashraf 2004; Song et al. 2006).

3.3.3. Salt exclusion

Halophytes or highly salt tolerant plants have both types of mechanisms that enable them to survive and grow for long times in saline soils. They exclude salts efficiently in addition to effective compartmentalization of the salts in vacuoles. Glycophytes, on the other hand, exclude the salts but they are unable to compartmentalize them. The mechanism of salt exclusion involves transport of salts to the leaves and subsequently excreted out of the plant body thereby reducing salt concentration in plant tissues. Salts translocated in the transpiration stream are deposited and their concentration increases with time. This results in much higher salt concentrations in older leaves than those in younger leaves. Salt exclusion is the most important adaptive strategy regulating the internal salt load of halophytes. As an example, about 98% of salt was reported to be excluded in the mangrove species *Avicennia marina* growing in 500 m*M* NaCl (Ball 1988). In perennials, exclusion is particularly

important and it is more vital to regulate the incoming salt load in the plant body (Hasegawa et al. 2000).

3.3.4. Intracellular ion compartmentation

Sequestering of Na^+ and Cl^- in the vacuoles of the plant cells is ideal situation for plants under salt stress. Exceptionally, high concentrations of salts are found in leaves, which still function normally. Concentrations well over 200 mM are common in halophytic or highly salt tolerant species, and such concentrations will severely inhibit the activity of several enzymes in vivo (Munns and Tester 2008).

3.3.5. Stomatal responses

Although there are few data available on stomatal responses of different plant species, it is possible to identify two types of stomatal adaptations to increasing salinity (Flowers et al. 1997): the guard cells can utilize sodium instead of potassium to achieve their normal regulation of turgor (Ashraf 1994), or the ionic selectivity of the guard cells that use potassium and are capable of limiting the sodium intake (Robinson et al. 1997). This mechanism may be very important in nonsecretory halophytes that lack secretion mechanisms, and it may therefore be of particular interest as a potential contributor to the development of salt tolerance in crops. Sodium can substitute for potassium in the stomatal mechanism (Flowers and Colmer 2008). In *Suaeda maritima*, sodium is the major cation under salinity in the guard cells of closed stomata (Flowers et al. 1989). Stomatal regulation by sodium provides a vital regulatory mechanism for the control of excessive salt translocation in the shoot, when a plant capacity to compartmentalize increases. In glycophytes, accumulation of sodium ions damages the stomatal function, and this disruption supports their lack of survival under saline conditions (Robinson et al. 1997).

4. REACTIVE OXYGEN SPECIES AND ANTIOXIDATIVE DEFENSE SYSTEMS IN PLANTS GROWING UNDER SALT STRESS

4.1. Generation of ROS and oxidative stress

Like all aerobes, plants use O_2 as terminal electron acceptor. At ambient temperature, one-step full reduction of O_2 to H_2O could proceed very slowly due to the requirement of high activation energy. When oxygen is exposed to high-energy or electron-transfer chemical reactions, it gets converted to various highly reactive chemical forms collectively known as reactive oxygen species (ROS). Although, it has been shown that some of the ROS may function as important signalling molecules that alter gene expression and modulate the activity of specific defense proteins, all ROS are extremely harmful to organisms at high concentrations. In plants, ROS are continuously produced by the inevitable leakage of

electrons on to molecular oxygen from the electron transport activities of chloroplast, mitochondria, and plasma membrane (Pinto et al. 2003), or as a byproduct of various metabolic pathways such as respiration and photosynthesis (Apel and Hirt 2004). These ROS include highly reactive species such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) which disrupt the homeostasis of the organism by oxidatively damaging membrane lipids, proteins, chlorophylls, and nucleic acids (Sharma and Dubey 2007; Konieczny et al. 2008; Maheshwari and Dubey 2009).

In its ground state, molecular O_2 is relatively unreactive due to the presence of two unpaired electrons having parallel spin, which makes it paramagnetic (Apel and Hirt 2004). Activation of O_2 may occur by two different mechanisms: monovalent reduction or absorption of sufficient energy to reverse the spin on one of the unpaired electrons. The stepwise monovalent reduction of O_2 leads to the formation of $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$, whereas energy transfer to O_2 leads to the formation of $^1O_2$. Singlet oxygen is a highly destructive ROS, which reacts with most of the biological molecules at near diffusion-controlled rates (Foyer and Harbinson 1994). It is much more reactive toward organic molecules and can last for 4 μs in water and 100 μs in a nonpolar environment (Foyer and Harbinson 1994). $O_2^{\bullet-}$ is produced if one electron is added to ground state oxygen. $O_2^{\bullet-}$ is a moderately reactive, short-lived ROS with a half-life of approx. 2-4 μs. H_2O_2 is produced in the SOD-catalyzed disproportionation of $O_2^{\bullet-}$, or from the reduction of $O_2^{\bullet-}$ by AsA, manganese ions, or ferredoxin (Hideg 1997). The H_2O_2 is one of the major and the most stable ROS that regulates basic acclimatization, defense, and developmental processes in plants (Slesak et al. 2007). It has been shown to act as a signal transduction molecule in several developmental processes. Nevertheless, at high concentrations, it causes oxidative stress marked by increased lipid peroxidation and the alteration of membrane permeability (Imlay 2003). The main source of $^{\bullet}OH$ in biological systems is the decomposition of H_2O_2 in the Haber–Weiss reaction. This reaction is enhanced by the presence of a transition metal, such as Fe^{2+} (Fenton reaction) (Hideg 1997). $^{\bullet}OH$ is the most reactive among all ROS. $^{\bullet}OH$ can potentially react with all biological molecules, can initiate self-perpetuating lipid peroxidation, and, because cells have no enzymatic mechanism to eliminate this highly reactive ROS, its excess production would eventually lead to cell death (Pinto et al. 2003). $^{\bullet}OH$ interacts with all biological molecules and causes subsequent cellular damages such as lipid peroxidation, protein damage, and membrane destruction (Foyer et al. 1997). The well-known reactivity of H_2O_2 is not due to its

reactivity per se, but due to the formation of highly reactive $^{\bullet}OH$, which is strong oxidizing agent, and is formed in the presence of metal reductants (Boo and Jung 1999). The production of ROS under normal growth conditions in cells is low (240 μM s−1 $O_2^{\bullet-}$ and a steady state level of 0.5 μM H_2O_2 in chloroplasts) (Polle 2001). However, the various stressful conditions of the environment that disrupt the cellular homeostasis enhance the production of ROS (up to 720 μM s−1 $O_2^{\bullet-}$ and a steady state level of 5–15 μM H_2O_2) (Polle 2001). When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of "oxidative stress." Oxidative stress is defined as a shift of the balance between prooxidative and antioxidative reactions in favour of the former. There is enhanced production of ROS in plants growing under various abiotic stresses such as drought, salinity, extremes of temperature, excessive levels of metals, anaerobiosis, gaseous pollutants, and UV-B radiation. The enhanced level of these ROS causes oxidative damage to biomolecules such as membrane lipids, proteins, enzymes, nucleic acids, chloroplast pigments, etc. (Verma and Dubey 2003; Sharma and Dubey 2007; Maheshwari and Dubey 2009).

4.1.1. Lipid peroxidation

Lipid peroxidation is a normal metabolic process associated with the developmental processes of plants, including the juvenile stage of growth, the production of odor volatiles, senescence, and the formation of compounds like jasmonic acid under normal aerobic conditions (Anderson 1995). Both free radicals as well as enzymes can lead to the initiation of lipid peroxidation in both cellular and organellar membranes. Increased peroxidation (degradation) of lipids has been reported in plants growing under salt stressful conditions (Tanou et al. 2009). Increase in lipid peroxidation under this stresses parallels with the increased production of ROS.

Lipid peroxidation, in both cellular and organellar membranes, takes place when above-threshold ROS levels are reached, thereby not only directly affecting normal cellular functioning, but also aggravating the oxidative stress through production of lipid-derived radicals (Montillet et al. 2005). The level of lipid peroxidation has been widely used as an indicator of free radical mediated damage to cell membranes under stressful conditions. Malondialdehyde (MDA) is one of the final products of the peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage (Halliwell and Gutteridge 1985).

The polyunsaturated fatty acids (PUFA) present in membrane phospholipids are particularly sensitive to attack by $^{\bullet}OH$ and other oxidants. When PUFA in biomembranes are peroxidized, a great diversity of aldehydes is formed, some of which are highly reactive. The peroxidation of PUFA by ROS attack can lead to chain breakage and thereby increase in membrane fluidity and permeability. Phospholipids are essential components of the membrane that surrounds the cell as well as other cellular structures, such as nucleus and mitochondria, and therefore damage to phospholipids can affect the viability of the cells (Woessmann et al. 1999). There are two common sites of oxygen free radical attack on the phospholipid molecules—the unsaturated (double) bond between two carbon atoms that can be easily obtained in chemical reaction and interaction with other substances and the ester linkage between glycerol and the fatty acid. It has been suggested that decrease in cell membrane stability or increase in membrane permeability reflects the extent of lipid peroxidation caused by ROS (Sairam et al. 2002).

4.1.2. Protein modification

The attack of ROS on proteins results in the site-specific amino acid modification, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electric charge, and increased susceptibility of proteins to proteolysis. ROS may cause the modification of proteins in a variety of ways, some are direct and others indirect. Direct modification involves modulation of a protein's activity through nitrosylation, carbonylation, disulfide bond formation, and glutathionylation. Proteins can be modified indirectly by conjugation with the breakdown products of fatty acid peroxidation (Yamauchi et al. 2008). As a consequence of excessive ROS production, tissues injured by oxidative stress generally contain the increased concentrations of carbonylated proteins (Dean et al. 1993). The enhanced modification of proteins has been reported in plants under various stresses (Sharma and Dubey 2007; Maheshwari and Dubey 2009). The amino acids in a peptide differ in their susceptibility to attack by ROS and the various forms of ROS differ in their potential reactivity. $^{\bullet}OH$ and alkoxyl radicals are mainly involved in the oxidation of proteins. Sulfur-containing amino acids and thiol groups specifically are very susceptible sites for attack by ROS. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form a disulfide bridge.

4.1.3. DNA damage

DNA is cell's genetic material and any damage to the DNA can result in changes (i.e., mutation) in the encoded proteins, which may lead to malfunctions or the complete inactivation of the encoded proteins. Thus, it is essential for the viability of cell that the DNA

remains intact. ROS are a major source of DNA damage (Imlay and Linn 1986) and cause strand breaks, removal of nucleotides, and a variety of modifications in the organic bases of the nucleotides. Changes in the nucleotides of one strand can result in mismatches with the nucleotides in the other strand, yielding subsequent mutations. Although cells have developed repair mechanisms to correct naturally occurring changes in the DNA, additional or excessive changes caused by ROS or other agents can lead to permanent damage to the DNA with potentially detrimental effects for the cell (Chen et al. 2002). Mitochondrial and chloroplast DNA are more susceptible to oxidative damage than nuclear DNA due to the lack of protective protein, histones, and close locations to the ROS-producing systems in the former. Enhanced DNA degradation has been observed in plants exposed to various environmental stresses such as salinity and metal toxicity. The principle cause of single strand breaks is the oxidation of the sugar moiety by the $^{\bullet}OH$. When $^{\bullet}OH$ attacks either DNA or proteins associated with it, DNA protein cross-links are formed. DNA protein cross-links cannot be readily repaired and may be lethal if replication or transcription precedes repair. $^{\bullet}OH$ reacts with free carbohydrates, such as sugars, and polyols (Smirnoff and Cumbes 1989). The oxidation of sugars with $^{\bullet}OH$ often releases formic acid as the main breakdown product.

4.2. Antioxidative defense system in plants

The balance between production and quenching of ROS may be perturbed by a number of adverse environmental factors, giving rise to rapid increases in intracellular ROS levels (Noctor et al. 2002; Pitzschke and Hirt 2006). The accumulation of ROS during abiotic stresses induces oxidative damage to lipids, proteins, and nucleic acids. In order to avoid the oxidative damage, higher plants possess a complex antioxidative defense system comprising of nonenzymatic and enzymatic components. In plant cells, specific ROS-producing and scavenging systems are found in different organelles such as chloroplasts, mitochondria, and peroxisomes, and the ROS-scavenging pathways from different cellular compartments are coordinated (Pang and Wang 2008). Plants have the capability to scavenge or detoxify ROS by producing different types of antioxidants. Antioxidants can generally be categorized into two different types: enzymatic and non-enzymatic compounds. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). The most commonly known nonenzymatic antioxidants are glutathione (reduced form, GSH), ascorbate (reduced form, AsA), carotenoids, and tocopherols (Apel and Hirt 2004; Ashraf 2009).

4.2.1. Enzymatic defense system

The enzymatic components of the antioxidative defense system comprise several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), enzymes of ascorbate-glutathione (AsA-GSH) cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Noctor and Foyer 1998). These enzymes operate in different subcellular compartments and respond in concert when cells are exposed to oxidative stress.

4.2.1.1. Superoxide dismutase

The enzyme SOD belongs to the group of metalloenzymes and catalyzes the disproportionation of $O_2^{\bullet-}$ to O_2 and H_2O_2. Within the cell, SOD constitutes the first line of defense against ROS (Alscher et al. 2002). This enzyme neutralizes the very reactive $O_2^{\bullet-}$ produced in the different compartments of plant cells into O_2 and H_2O_2 with the rate 104 times faster than the spontaneous dismutation reaction. Since SOD is present in all aerobic organisms and in most of the subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in defense against oxidative stress. While all compartments of the cell are possible sites for $O_2^{\bullet-}$ formation, chloroplasts, mitochondria, and peroxisomes are thought to be the most important generators of ROS (Alscher et al. 2002).

4.2.1.2. Catalase

Catalase is a tetrameric, heme-containing enzyme found in all aerobic organisms and catalyzes the dismutation of H_2O_2 into water and oxygen. H_2O_2 has been implicated in many stress conditions. In plants, CAT scavenges H_2O_2 generated during mitochondrial electron transport, β-oxidation of fatty acids, and, most importantly, during photorespiratory oxidation (Scandalios et al. 1997). CAT is unique among H_2O_2 degrading enzymes as it degrades H_2O_2 without consuming cellular reducing equivalents. Therefore, when cells are stressed for energy and are rapidly generating H_2O_2 through catabolic processes, H_2O_2 is degraded by CAT in an energy efficient manner. Abiotic stresses cause either the enhancement or depletion of CAT activity (Sharma and Dubey 2007; Noreen and Ashraf 2009). The properties of CAT suggest that the enzyme is inefficient in removing low concentration of H_2O_2.

4.2.1.3. Enzymes of Ascorbate–Glutathione cycle

Efficient scavenging/destruction of ROS generated during abiotic stresses require the action of several antioxidant enzymes. The AsA–GSH cycle, also referred to as Halliwell–Asada pathway, present in at least four different subcellular locations including the cytosol, chloroplast, mitochondria, and peroxisomes, scavenges H_2O_2. The AsA–GSH cycle involves successive oxidation and reduction of AsA, GSH, and NADPH catalyzed by the enzymes

APX, MDHAR, DHAR, and GR. APX uses two molecules of AsA to reduce H_2O_2 to water with a concomitant generation of two molecules of MDHA. Many workers have reported enhanced expression of APX in response to abiotic stresses such as drought, salinity, heat, chilling, metal toxicity, anaerobiosis, UV irradiation, gaseous pollutants, etc. (Sharma and Dubey 2007; Han et al. 2009; Maheshwari and Dubey 2009).

In plant cells, AsA is a major antioxidant that is part of the AsA–GSH cycle. MDHAR, the enzymatic component of this cycle, is involved in the regeneration of reduced AsA. MDHA radical produced in APX catalyzed reaction has a short lifetime and if not rapidly reduced, it disproportionates to AsA and DHA (Ushimaru et al. 1997). Within the cell, such as at the plasmalemma or at the thylakoidmembrane, MDHA can be reduced directly to AsA. The electron donor for MDHA reduction may be b-type cytochrome, reduced ferredoxin, or NAD(P)H. The reaction is catalyzed by the enzyme MDHAR, which is found in several cellular compartments (Miyake and Asada 1994). Despite the possibility of enzymic and nonenzymic regeneration of AsA directly from MDHA, some DHA is always produced when AsA is oxidized in leaves and other tissues. DHA is reduced to AsA by the action of DHAR using GSH as the reducing substrate (Ushimaru et al. 1997). DHAR is a key component of the AsA recycling system. Reaction catalyzed by DHAR generates GSSG that in turn gets rereduced to GSH using NADPH in a reaction catalyzed by enzyme GR. GR is a NAD(P)H-dependent enzyme ubiquitously present in mesophyll cells. Although it is located in chloroplasts, cytosol, and mitochondria, around 80% of GR activity in photosynthetic tissues is accounted for by chloroplastic isoforms (Edwards et al. 1990). Several authors have reported the increased activity of this enzyme under abiotic stresses (Sharma and Dubey 2007; Yoshida et al. 2006; Maheshwari and Dubey 2009).

5. GRAFTING AS A WAY TO ALLEVIATE SALT-INDUCED DAMAGE AND RAISE THE PLANT SALT TOLERANCE

Numerous attempts have been made to improve the salt tolerance of crops by traditional breeding programmes. However, commercial success has been very limited due to the complexity of the trait: salt tolerance is complex genetically and physiologically (Flowers, 2004). At present, major efforts are being directed towards the genetic transformation of plants in order to raise their tolerance (Borsani et al. 2003) and in spite of the complexity of the trait, the transfer of a single gene or a few genes has led to claims of improvement in salt tolerance, such as occurs with the expression of some genes involved in the control of Na^+ transport (Zhang and Blumwald 2001). However, the nature of the genetically complex

mechanisms of abiotic stress tolerance, and potential detrimental side effects, make this task extremely difficult (Wang et al. 2003; Flowers 2004). Solving a problem as complex as the profitable use of saline water in irrigated agriculture requires more than one strategy. In addition to tolerant cultivars, several cultural practices, each contributing to a small extent to allow plants to withstand better the deleterious effects of salt, needs to be applied (Cuartero and Fernandez-Muñoz 1999).

One environment-friendly technique for avoiding or reducing losses in production caused by salinity in high-yielding genotypes belonging to *Solanaceae* and *Cucurbitaceae* families would be to graft them onto rootstocks capable of ameliorating salt-induced damage to the shoot (Santa-Cruz et al. 2002; Fernández-García et al. 2002, 2004; Estaň et al. 2005; Colla et al. 2005, 2006a,b; He et al. 2009; Yetisir and Uygur 2010; Zhen et al. 2010). This strategy could also enable plant breeder to combine desired shoot characteristic with good root characteristic (Zijlstra et al. 1994). Proposed explanations for grafting-induced salt tolerance are: (1) higher accumulation of proline and sugar in the leaves (Ruiz et al. 2005); (2) higher antioxidant capacity in the leaves (López-Gómez et al. 2007); (3) lower accumulation of Na^+ and/or Cl^- in the leaves (Estaň et al. 2005; Zhu et al. 2008a,b).

5.1. Mechanisms of salt tolerance in grafted plants

5.1.1. Morphological root characteristics

Root characteristics which may play an active role in ions and water uptake are root length and density (Krasilnikoff et al. 2003), number of root hairs and their length and hence their surface area (Dvoralai and Jens, 1999). The enhanced salt tolerance of grafted vegetables has often been associated with the root system. In fact, the root systems are the most critical parts of the plant faced with soil-related stress factors such as salinity. Therefore, root characteristics are the main reason for the alleviation of deleterious effect of salt stress on the shoot growth.

5.1.2. Physiological and biochemical mechanisms

High salt concentrations cause ion imbalance, ion toxicity, and hyperosmotic stress in plants. As a consequence of these primary effects, secondary stresses such as oxidative damage often occur (Zhu 2001a). Grafted plants develop numerous physiological and biochemical mechanisms to cope with salt stress. These strategies include (i) salt exclusion in the shoot and retention of salt ions in the root, (ii) better maintenance of potassium homeostasis, (iii) compartmentation of salt ions in the vacuole, accumulation of compatible solutes and

osmolytes in the cytosol, (iv) activation of an antioxidant defense system, and (v) induction of hormonesmediated changes in plant growth.

5.1.2.1. Salt exclusion in the shoot and retention of salt ions in the root

The most common effect of soil salinity is the growth inhibition due to direct Na^+ and Cl^- toxicity at biochemical level. For some plants, particularly woody perennials such as citrus and grapevines, Na^+ is retained in the woody roots and stems, while Cl^- is accumulated in the shoot and causes the most damage to the plant (Flowers 1988). However, for many plants, including vegetables such as cucumber, melon, watermelon, tomato, and eggplant, Na^+ is the primary cause of ion-specific damage (Tester and Davenport 2003; Varlagas et al. 2010). Plants grafted onto appropriate rootstocks restricted the transport of Na^+ from root to shoot (Estaň et al. 2005; Zhu et al. 2008a).

Salt tolerance mechanisms can occur in a wide range of organizational levels from the cellular level (e.g., compartmentation of Na^+ within cells) to the whole plant (e.g., exclusion of Na^+ from the plant and exclusion of Na^+ from the shoot) (Tester and Davenport 2003; Møller et al. 2009). The enhanced salt tolerance of grafted vegetables has often been associated with lower Na^+ and/or Cl^- contents in the shoot. Apart from the level of Na^+ in the shoot, another component of plant salinity tolerance is the capability of the tissue to tolerate Na^+ (Munns and Tester 2008). Tissue tolerance to Na^+ involves the storage of Na^+ in vacuoles, which can protect cytosolic enzymes from the toxic action (Apse et al. 1999). Electrochemical H^+ gradients, generated by H^+-pumps at the plasma membrane (H^+-ATPase) and the tonoplast (H^+-ATPase,H^+-PPase), provide the energy used by the plasma membrane- and tonoplast-bound Na^+/H^+ antiporters to couple the passive movement of H^+ to the active movement of Na^+ out of the cell and into the vacuole, respectively (Blumwald 1987). This mechanism suggests that the roots of grafted plants have a higher capacity for vacuolar Na^+ sequestration (Chen et al. 2008).

5.1.2.2. Better maintenance of potassium homeostasis

The metabolic toxicity of Na^+ is largely a result of its capability to compete with K^+ for binding sites essential for cellular function. More than 50 enzymes are activated by K^+, and Na^+ cannot be used as a substitute in this role (Bhandal and Malik 1988). It should be emphasized that a decrease in the K^+/Na^+ may result in a deficiency of K^+. Therefore, K^+ homeostasis is an important factor in salt tolerance (Munns and Tester 2008). Grafted plants have a higher K^+ content which seems to relate to the higher salt tolerance compared with

self-grafted plants (Zhu et al. 2008a; Huang et al. 2009a). The salt tolerance of grafted tomato plants was associated with xylem K^+ but not Na^+ (Albacete et al. 2009).

5.1.2.3. Accumulation of compatible solutes and osmolytes

Plants need to maintain internal water potential below that of the soil in order to maintain turgor and water uptake for growth. This requires an increase in osmotica either through uptake of inorganic ions or synthesis of metabolically compatible solutes. The compounds that most commonly accumulate include sucrose, proline, and glycine betaine (Munns and Tester 2008). Unlike inorganic, inorganic solutes such as Na^+ and Cl^- ions, however, these organic solutes are not harmful to enzymes and other cellular structures even at high concentrations. They are often referred to as compatible osmolytes (Zhu 2001b). At high concentrations, compatible solutes function in osmotic adjustment. The high concentrations of compatible osmolytes accumulate in the cytosol and organelles to balance the osmotic pressure of the ions in the vacuole (Munns and Tester 2008). In addition, data suggest that an increased amount of compatible osmolytes may protect plants by scavenging oxygenfree radicals caused by salt stress (Zhu 2001b; Huang et al. 2009c).

5.1.2.4. Induction of the antioxidant defense system

Salt stress reduces stomatal conductance, thereby limiting CO_2 supply to the leaf (Apel and Hirt 2004). This in turn causes the over-reduction of the photosynthetic electron transport chain, resulting in the production of reactive oxygen species (ROS). These ROS are highly reactive and can seriously disrupt normal metabolism through oxidative damage to lipids, proteins, and nucleic acids (Apel and Hirt 2004). Antioxidants can be used as markers of salinity tolerance in grafted vegetables. An efficient antioxidant system is an important factor for the enhanced salt tolerance of grafted plants. This is achieved by obtaining higher activities of anti-oxidative enzymes and contents of non-enzymatic antioxidants to scavenge ROS, thereby reducing oxidative damage.

6. SOS PATHWAYS IN RELATION TO SALINITY STRESS

6.1. Electrochemical gradients and fluxes

The driving force for Na^+ into root cells is the combined gradient of voltage and chemical activity across the plasma membrane (electrochemical gradient). In a typical plant cell, the difference in electrical potential between the cytoplasm and the apoplast (membrane potential) is in the order of –120 to –180 mV. According to the Nernst equation, this provides a driving force for 100–1000-fold accumulation of Na^+ in the cytoplasm. The combined evidence suggests that cytoplasmic Na^+ concentrations are generally in the low millimolar

range. This is in accordance with the notion that cytoplasmic Na^+ concentrations above 100 mM are toxic due to the detrimental effects of a high Na^+ environment to protein stability (Serrano et al. 1999) and displacement of K^+ from essential co-factor binding sites on K^+-dependent enzymes (Wyn Jones and Pollard 1983). Thus in both low and high salt environments, living cells have to balance passive influx of Na^+ with Na^+ efflux, either across the plasma membrane back into the apoplast or across the tonoplast into the vacuole. The energy requirement for Na^+ efflux is considerable; approximately -5.7 kJ/mol per tenfold concentration gradient or per -60 mV of membrane potential. In addition to energy, time is an important factor for salt tolerance because the rate of Na^+ uptake will determine how quickly Na^+ reaches toxic levels inside the cell. It is clear then that limiting Na^+ influx into root cells is a fundamental requisite for plant life in high salt conditions. Balancing Na^+ influx with Na^+ export from the cytoplasm back into the apoplast (also sometimes termed 'futile cycling') is one way of reducing the Na^+ load (Malagoli et al. 2008).

A second strategy for removing Na^+ from the cytoplasm is to compartmentalise it in the vacuoles. Na^+ uptake into the vacuole also requires energy but has a dual benefit in saline conditions; it avoids Na^+ build-up in the apoplast (Oertli 1968) and enhances the intracellular solute potential thereby contributing to turgor adjustment. The importance of Na^+ allocation into vacuoles is evident in the fact that over-expression of NHX-type vacuolar Na^+/H^+ antiporters, (vacuolar Na^+/H^+ exchanger, NHX), enhances salt tolerance in plants (Zhang et al. 2001). However, the vacuolar Na^+ storage as a means to remove Na^+ from the cytoplasm relies on growth. Only if the vacuolar lumen is constantly enlarged can rapid saturation of this mechanism be avoided. Or, putting it the other way round, when the vacuolar storage space is exhausted Na^+ will accumulate in the cytoplasm, and its toxic effect will slow down growth thereby exacerbating the problem. The ability of plants to cope with high Na^+ concentrations in the soil therefore relies on maintaining a positive balance between the rate of growth (enlargement of the vacuolar lumen) and the rate of Na^+ uptake across the root plasma membrane. Two important points should be taken into considerations:the rate of Na^+-uptake (the size of Na^+-influx) is critical for the ability of plants to avoid the build-up of toxic Na^+ concentrations in the cytoplasm; and the driving force for Na^+ uptake into roots is directed inward and therefore Na^+ uptake can proceed through passive transport.

6.2. Ion pumps, calcium, and SOS pathways in relation to salinity stress

The adaptation of plants to a saline environment must be due to some salt-related changes in the pattern of gene(s) expression (Foodlad 1997). More than 100 genes were estimated to be

expressed when subjected to salt stress (Meyer et al. 1990). There are several reports of alterations in protein accumulation due to salinity (Meyer et al. 1990). High salinity stress causes an imbalance in sodium ions (Na^+) homeostasis, which is maintained by the coordinated action of various pumps, ions, Ca^{2+} sensors, and its downstream interacting partners, which ultimately results in the efflux of excess Na^+ ions. Certain channels show more selectivity to K^+ over Na^+. These include the K inward-rectifying channel, which mediates the influx of K^+ upon plasma membrane hyperpolarization and selectively accumulates K^+ over Na^+ ions. The nonspecific cation channel is a voltage-independent channel, which acts as a gate for the entry of Na^+ into plant cells. Moreover, there is the K^+ outward-rectifying channel, which opens during the depolarization of the plasma membrane and mediates the efflux of K^+ and the influx of Na^+ ions, leading to Na^+ accumulation in the cytosol. The vacuolar Na^+/H^+ exchanger (NHX) helps push excess Na^+ ions into vacuoles. Na^+ extrusion from plant cells is powered by the electrochemical gradient generated by H^+-ATPases, which permit the NHX to couple the passive movement of H^+ inside along the electrochemical gradient and extrusion of Na^+ out of the cytosol. Another pump, the H^+/Ca^{2+} antiporter (CAX1), helps in Ca^{2+} homeostasis (Mahajan et al. 2006a; Zhang et al. 2004; Zhu 2002).

Calcium is one of the principal candidates for functioning as a central node in the overall ‘‘signaling web’’ and plays an important role in providing salinity tolerance to plants. High salinity leads to increased cytosolic Ca^{2+}, which initiates the stress signal transduction pathways for stress tolerance. Ca^{2+} release may result from the activation of phospholipase C, leading to the hydrolysis of phosphatidylinositol bisphosphate to inositol trisphosphate and the subsequent release of Ca^{2+} from intracellular Ca^{2+} stores. Furthermore, calcium-binding proteins (calcium sensors) can provide an additional level of regulation in calcium signaling. These sensor proteins recognize and decode the information provided in the calcium signatures and relay the information downstream to initiate a phosphorylation cascade, leading to regulation of gene expression. Wu et al. (1996) commenced a mutant screen for *Arabidopsis* plants, which were oversensitive to salt stress. As a result of this screen, three genes, SOS1, SOS2, and SOS3 (salt overlay sensitive), were identified. The SOS3 gene (also known as AtCBL4) encodes a calcineurin B-like protein (CBL, calcium sensors), which is a Ca^{2+}-binding protein and senses the change in cytosolic Ca^{2+} concentration and transduces the signal downstream. A loss of function mutation that reduces the Ca^{2+}-binding capacity of SOS3 (*sos 3–1*) renders the mutant hypersensitive to salt (Zhu 2002). SOS2 (AtCIPK24)

encodes a novel serine/threonine protein kinase known as the CBL-interacting protein kinase (CIPK). SOS3 activates SOS2 protein kinase activity in a calcium-dependent manner (Mahajan et al. 2006a). The first target of the SOS3–SOS2 pathway was identified by genetic analysis of the *sos1* mutant of *Arabidopsis*. SOS1 is a Na^+/H^+ antiporter, and the *sos1* mutant was hypersensitive to salt and showed an impaired osmotic/ ionic balance. Genetic analysis confirmed that SOS3, SOS2, and SOS1 function in a common pathway of salt tolerance (Mahajan and Tuteja 2005; Zhang et al. 2004; Zhu 2002). The SOS3–SOS2 kinase complex was found to phosphorylate SOS1 directly. The SOS pathway also seems to have other branches, which help remove excess Na^+ ions out of the cell, thereby maintaining cellular ion homeostasis. SOS2 also interacts and activates the NHX, resulting in the sequestration of excess Na^+ ions and pushing it into vacuoles, thereby contributing further to Na^+ ion homeostasis. Some other calcium-binding proteins, such as calnexin and calmodulin, also sense the increased level of calcium and can interact and activate the NHX. CAX1 has been identified as an additional target for SOS2 activity, reinstating cytosolic Ca^{2+} homeostasis. This reflects that the components of SOS pathway may cross talk and interact with other branching components to maintain cellular ion homeostasis, which helps in salinity tolerance.

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CHAPTER 2

GRAFTING AS A WAY TO IMPROVE SALT TOLERANCE IN *SOLANACEAE* (TOMATO) AND *CUCURBITACEAE* (MUSKMELON) FAMILIES

I. Improved stomatal regulation and ion partitioning boosts salt tolerance in grafted melon
II. Ionic partitioning and stomatal regulation Dissecting functional elements of the genotypic basis of salt stress adaptation in grafted melon
III. Physiological and biochemical alteration modulated by different rootstock and scion genotypes in tomato plant under salinized condition

CHAPTER 2

I. Improved Stomatal Regulation And Ion Partitioning Boosts Salt Tolerance In Grafted Melon

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ABSTRACT

Grafted plants are often more tolerant to salinity than nongrafted controls. In order to distinguish differential response components in grafted melon (*Cucumis melo* L.), salt stress was imposed on several rootstock–scion combinations in four experiments. The rootstock used was an interspecific squash (*Cucurbita maxima* Duch.× *Cucurbita moschate* Duch.), RS841, combined with two cantaloupe (*C. melo* var. *cantalupensis*) cultivars, namely London and Brennus, against both self-grafted and nongrafted controls. Physiological, morphological and biochemical adaptations to 0, 40 and 80mM NaCl were monitored. Upon salinity, plant biomass and leaf area were improved by grafting *per se*, since self-grafted plants performed similarly to the heterografted ones. However, improvements in the exclusion of Na^+ and the uptake of K^+ were due only to the rootstock genotype, since ionic composition was similar in self-grafted and nongrafted plants. These results indicate that the favourable effects of grafting on plant growth cannot be ascribed to a more efficient exclusion of Na^+ or enhanced nutrient uptake. On the other hand, growth improvements in both self- and heterografted plants were associated with a more efficient control of stomatal functions (changes in stomatal index and water relations), which may indicate that the grafting incision may alter hormonal signalling between roots and shoots.

Additional keywords: cantaloupe, interspecific rootstock, nutritional imbalance, salt stress, water relations.

1. INTRODUCTION

Soil salinisation is limiting the future of agriculture in many areas of the world and the improvement of salt tolerance in crops is becoming an imperative of agricultural research. Scientists have therefore approached different aspects of salinity related to both soil and plant issues (Munns 2002). For the latter, much effort over the last decades has been dedicated to understanding the fundamental biology of plant stress adaptation with the ultimate aim of identifying key stress tolerance functions (Orsini *et al*. 2010*a*). One of the main elements that, so far, has not earned enough interest is understanding the role played by the root system in conferring tolerance. Under salt stress, toxic, osmotic and nutritional factors deplete plant growth. The adoption of tolerant rootstocks in vegetable grafting has been suggested to improve plant performances under stress. Grafting results in more efficient water and nutrient use, increased yield, extended harvest periods and improved fruit quality (Romero *et al*. 1997; Estan *et al*. 2005). Rootstocks can also influence tolerance to extreme temperature and moisture (drought, flooding) and salt stress (Colla *et al*. 2010). In cucurbits, grafting experiences addressed the induction of resistance against disease infestation (Cohen *et al*. 2002), low root-zone temperature (Bulder *et al*. 1990), soil alkalinity (Edelstein *et al*. 2011) and the enhancement of both water and nutrient uptake (Huang *et al*. 2010). Indications on the induction of tolerance to salinity by grafting have been provided in the last decade by several authors (Estan *et al*. 2005; Oztekin *et al*. 2007; Edelstein *et al*. 2011), although this research was often limited to the evaluation of salt-tolerant genotypes, rather than the identification of the main elements reducing the effects of salinity on the shoot. Morphological adaptations to salinity in grafted cucurbits have been widely described, mainly resulting in more vigorous root systems (Romero *et al*. 1997; Zhu *et al*. 2008) and greater root : shoot ratios (Colla *et al*. 2006*a*; Yang *et al*. 2006; Yetisir and Uygur 2010; Huang *et al*. 2010). However, although the available literature provides excellent guidance about the agronomic performance of grafted plants, comprehension of the physiological basis of improved tolerance in grafted plants is still unclear, mainly due to a lack of controls in experiments (interspecific graftings are often compared with either nongrafted or self-grafted plants, but rarely with both of them simultaneously), or to partial coverage in the analysis of the stress factors (rarely taking into consideration the nutritional elements of the stress). This paper aims to identify the functional morphological and physiological responses to salinity that are most responsible for the increased tolerance of grafted plants. In this study, particular attention is given to the role of the root system in altering stress perception in the shoots.

2. MATERIAL AND METHODS

2.1. Plant growth conditions

Four independent experiments were conducted in order to compare the effect of grafting on the response of melon (*Cucumis melo* L.) to salinity. From a preliminary screening, two melon scions, Brennus (*C. melo*, Zöldségtermesztési Kutató Intézet (ZKI), Kecskemét, Hungary; salt-sensitive) and London (*C. melo*, Nunhems Bayer Vegetables, Nunhem, The Netherlands; salt tolerant), were selected and combined with an interspecific squash rootstock of *Cucurbita maxima* Duch.× *Cucurbita moschate* Duch., namely 'RS841 improved' (Monsanto, St Louis, MO, USA; salt-tolerant). Six grafting combinations were assessed for their response to salinity: nongrafted (either London or Brennus), self-grafted (London–London or Brennus–Brennus) or interspecific grafting (London–RS841 or Brennus–RS841).

Seeds of the rootstock and scions were sown in trays filled with peat moss and when the rootstock seedlings reached at least one true leaf, and scion seedlings presented one or two true leaves, the grafting was performed and plants were then incubated for 1 week. The one-cotyledon splice grafting technique was used and plants were grown in soilless systems, watered with a nutrient solution with the following composition: 16.5mM NO_3^-, 1mM NH_4^+, 1.50mM $H_2PO_4^-$, 1.50mM SO_4^{2-}, 7.0mM K^+, 5.0mM Ca^{2+}, 1.5mM Mg^+, 15mM Fe^{2+}, 10mM Mn^{2+}, 25 mM B^+, 5.0 mM Zn^{2+}, 0.5 mM Cu^{2+} and 0.5 mM Mo^+. Salinity was supplied by adding 0 (2.55 dS m^{-1}), 40 (5.50 dS m^{-1}) or 80 (7.10 dS m^{-1}) mM NaCl in the nutrient solution. The experimental protocols were as follows:

Experiment 1

Experiment 1 was conducted at the research station of Bologna University (44° 30′ 54″ N, 11° 24′ 24″ E, 39m above sea level (a.s.l.)), in an experimental glasshouse under controlled conditions (maximum temperature, 25° C; minimum temperature, 18° C; relative humidity (RH), 60%). Seedlings were transplanted into 5-L pots filled with perlite: vermiculite (1 : 1, v : v). Seedlings were irrigated with nutrient solution in a closed-loop soilless system and, starting from 10 days after transplanting, salt stress was applied. At 15 days after salt supply (DAS) leaf gas exchange, water potential and overnight plant water loss (WL) measurements were performed. On the same day, samples for stomatal morphology determinations were also collected. Destructive measurements for biometric determinations were performed at 30 DAS. The experimental design was a strip block with grafting combinations within the strip and the salt treatments in the main plots. For each treatment, six replicates (individual plant pots) were considered.

Experiment 2

Experiment 2 was conducted at the experimental station of Corvinus University of Budapest (47° 29′ 09″ N, 19° 03′ 28″ E, 106m a.s.l.). Plants were grown in 1-L pots, filled with perlite : vermiculite (1 : 1, v : v) in a growth chamber under controlled environmental conditions (12 h light and 22°C, 500 mmol m^{-2} s^{-1} from cool white fluorescent bulbs, and 12 h dark and 18° C; RH 75%). At 14 days after transplanting, salt stress was imposed. At 10 DAS, leaf gas exchange and WL measurements were performed. The experiment was then closed at 15 DAS and plants were harvested for biometric determination. The experimental design was a strip block with grafting combinations within the strip and the salt treatments in the main plots. For each treatment, three replicates were considered.

Experiment 3

Experiment 3 was conducted at the Bologna University experimental station under the same conditions as Experiment 1. Salt stress treatments were applied when plants were 2 months old, and destructive measurements for stomatal morphology and other plant biometric determinations were performed at 20 DAS. The experimental design was a strip block with grafting combinations within the strip and the salt treatments in the main plots. For each treatment, six replicates were considered.

Experiment 4

Experiment 4 was conducted at the Faculty of Agriculture, Ege University of Izmir, Turkey (38° 27′ 16″ N, 27° 13′ 17″ E, 33m a.s.l.) in an experimental greenhouse (maximum temperature, 33.3°C; minimum temperature, 9.9°C; maximum RH, 88.8%; minimum RH, 34.4%). Plants were grown in 9-L pots filled with perlite. At 16 days after transplanting, salt stress was imposed. The experiment was then closed at 60 DAS and plants were harvested for biometric determination. The experimental design was a strip block with grafting combinations within the strip and the salt treatments in the main plots. For each treatment, three replicates were considered.

2.2. Measurements

A summary table showing dates of all measurements performed is shown in Table S1, available as Supplementary Material to this paper.

2.2.1. Plant growth measurements

Plant vegetative growth measurements were performed in all experiments. Morphological determinations included plant FW and DW (after drying at 60°C), root : shoot (R : S) ratio on a DW basis and total leaf area (LA). LA was measured on digital images by Image J processing software (Orsini *et al*. 2011). All measurements were on three replicates per plot.

2.2.2. Plant water relations

WL determination was performed in Experiments 1 and 2. At 15 DAS, three plant pots for each treatment were sealed with a plastic film to prevent water loss from the surface, leaving the shoot protruding from the film. Before sealing, plants were rewatered to pot capacity with water (control), or water plus 40 or 80mM NaCl. Each plant was then placed on an electronic balance under glasshouse conditions and the weight loss was measured after 24 h. WL values were normalised respect to the whole-plant DW taken at the end of the measurements (Orsini *et al*. 2012). Total leaf water potential (Ψw) and osmotic potential (Ψp) were determined in Experiments 1 and 3 at 15 DAS with a dewpoint potentiometer (WP4, Decagon Devices, Pullman, WA, USA). The Yp was estimated on frozen and thawed leaf samples. Relative water content (RWC) was determined in Experiments 1 and 3, calculated as showen in Eqn 1:

$$RWC\ (\%) = (FM - DM)/(TM - DM) \times 100, \quad (1)$$

where *FM*, *DM* and *TM* are the fresh, dry and turgid masses, respectively. Leaf saturated weight was determined after leaf immersion in distilled water for 24 h (Orsini *et al*. 2010*a*).

2.2.3. Stomatal size and index

Stomatal features were measured on the abaxial surface of the fully expanded fourth leaf from the apex of each plant in Experiments 1–3. Leaf surface imprints were obtained from the middle portion of the blade between the midrib and the leaf margin (areas in the vicinity of large veins were avoided) and laid on the microscope slide with a couple of water drops over the tissues. The stomatal count was performed in nine randomly chosen microscopic fields on each leaf on three plants per plot and the average was calculated. Each visual field consisted of 0.43mm^{-2} at 40× magnification. All the stomatal cell photographs were taken by using an eye-lens microscope (47–4620–9900, Zeiss, Oberkochen, Germany). Width and length of the stomata were determined in mm by using Image J processing software (Orsini *et al*. 2011). The stomatal area (SA) was calculated as shown in Eqn 2:

$$SA = \pi \times (SW \times 0.5) \times (SL \times 0.5), \quad (2)$$

where *SW* and *SL* are the width and length of the stomata (Orsini *et al*. 2010*b*). Stomatal and epidermal cells in a 1-mm^2 unit area were counted to determine the stomatal index, SI. SI was estimated according to Eqn 3:

$$SI\% = (NS \times 100)/ (EC + NS) \quad (3)$$

where *N*S is the number of stomata and *E*C is the number of epidermal cells (Orsini *et al*. 2011).

2.2.4. Leaf gas exchanges

According to the instruments available in each research station, leaf gas exchange determinations were performed on the first fully expanded young leaf (usually corresponding to the 8th to 10th leaf from the top) with either a CIRAS-2 infrared gas analyser (PP Systems, Hitchin, UK) (Experiments 1 and 3), a Li-Cor steady-state porometer (LI-1600 M, Li-Cor, Lincoln, NE, USA) (Experiment 4) and a LCi photosynthesis measurement system (ADC BioScientific Ltd, Hoddesdon, UK) (Experiment 2). Net photosynthesis (A), stomatal conductance (*g*s), and leaf transpiration rate (E) were measured at 15 DAS. All measurements were conducted between 1100 hours and 1500 hours on bright sunny days. Instantaneous water use efficiency (WUE) was calculated as the A: E ratio (Experiments 1 and 3).

2.2.5. Ion analysis

Ion concentration was determined in Experiments 1 and 4. Chemical analyses were carried out a DW basis on samples harvested by three randomly selected plants per replicate. For the estimation of anions (Cl^-,NO_3^-, SO_4^{2-} andPO_4^-) and cations (Na^+, K^+, Ca^{2+}, Mg^{2+} and $NH4^+$), 200 mg of root, stem and leaf dry matter were suspended in water (50 ml) and homogenised with a stirrer at 0.19*g* for 20 min. Samples were then filtered (grade 589 filter paper, Schleicher, Dassel, Germany) and extracts were further filtered through cellulose acetate syringe filters (0.20 mm) and analysed by ion chromatography. For quantification of ions and cations, the ion chromatography was performed using an ICS-900 Ion Chromatography System (Dionex Corporation, Sunnyvale, CA, USA) equipped with a dual piston pump, a, autosample (model AS-DV), an isocratic column at ambient temperature and a DS5 conductivity detector and a suppressor (4 mm). Chromeleon ver. 6.5 chromatography management software (Dionex Corporation) was used for system control and data processing. A Dionex IonPac AS23 analytical column (4mm × 250 mm) and an AG25 guard column (4mm × 50 mm) were used for separation of anions, and a Dionex IonPac CS12A analytical column (4mm × 250 mm) and a CG25 guard column (4mm × 50 mm) were used for separation of cations. The eluent consisted of 4.5mM sodium carbonate and 0.8mM sodium bicarbonate for anions, and mathanphoric acid (20mM) for cations at a flow rate for both cations and anions of 1mL min^{-1}.

2.2.6. Statistical analysis

Morphological and physiological data were analysed by ANOVA, performed using SPSS software (SPSS Inc., Chicago, IL, USA). Treatment means were compared using Duncan's multiple range tests at the 5% level of significance. Ion composition and partitioning data were analysed using principal component analysis (PCA), using StatGraphics software (Statpoint Technologies, Inc., Warrenton, VA, USA). ThePCAresults were graphically represented by the projection of the first two components.

3. RESULTS

3.1. Factors affecting plant response to salinity

When data from all experiments were pooled and analysed (Table 1), the results highlighted that differences in plant features were attributable to the cultivar for the R : S ratio only; the grafting for LA, Ψp, SI, *g*s, E, A and WUE; and salinity for all measured features. Experiments did not affect the plants' response to salinity, except for FW and LA due to the different ages of the plants at sampling. The only significant interaction observed was grafting_salinity, which was significant for all features under study. Consistently, the experimental results were successively analysed by correlating the response to salinity of each grafting type (nongrafted, selfgrafted and interspecific).

3.2. Morphological response

In order to compare morphological data from the different experiments, FW and LA were presented as relative values of their best performances for each grafting combination. A general decrease in both plant FW and LA was observed in all plants exposed to salinity, with a concomitant increase in the R : S ratio (Fig. 1). Relatively, the decrease in both FW (Fig. 1*a*) and LA (Fig. 1*b*) was lower in interspecific graftings (-31% and -42% respectively upon 80mM NaCl), but this was also due to suboptimal performances under control conditions. On the other hand, smaller changes in the R : S ratio (Fig. 1*c*) were observed in interspecific graftings under 40 (+43%) and 80 (+31%) mM NaCl, mainly due to the higher R : S ratio under control conditions (0.15 as compared with the mean value of 0.13 in nongrafted or self-grafted melons). The R : S ratio was generally higher in Brennus (+14%) compared with London (data not shown).

3.3. Water relations and stomatal response

Plant water relations were assessed by determination of Ψw and Ψp as well as the RWC (Fig. 2). Although salinity reduced both Ψw and Ψp in all treatments, Ψw appeared to be more negative in nongrafted plants compared with self-grafted plants under control conditions (Fig.

2*a*). On the other hand, Ψp was not significantly affected by grafting combination (Fig. 2*b*). Finally, RWC was lower in nongrafted plants compared with self- or interspecific graftings under control conditions or 80mM NaCl (Fig. 2*c*). At the leaf level, differences in the stomatal response to salinity were observed (Fig. 3). Although under control conditions, SI (Fig. 3*a*) was similar in nongrafted and interspecific graftings, and slightly lower in self-grafted plants, upon 80mM NaCl, the increase in SI was greater in self-grafted plants (+132%) compared to interspecific (+83%) and nongrafted plants (+61%). Although SI increased with salinity, the specific area of the stomata (Fig. 3*b*) was reduced. Instantaneous measurements of *g*s under the control treatment revealed higher values in self-grafted and interspecific plants (mean value: 553 ± 63.5mMm^{-2} s^{-1}), compared with nongrafted plants (377±73.0mM m^{-2} s^{-1}; data not shown). Grafting did not affect CO2 assimilation under control conditions; when salinity occurred, indeed, the reduction in A was greater in nongrafted plants (-68% and -92% at 40 and 80mM NaCl, respectively), compared with grafted plants (mean values: -49% and -68% at 40 and 80mMNaCl, respectively; data not shown). Transpiration measurements (WL, Fig. 3*c*) highlighted that although values were higher under control conditions (+69%) in nongrafted plants compared with grafted ones, a dramatic reduction in transpiration followed the increase in salinity (80mM NaCl), resulting in a 94%, 74% and 61% reduction in nongrafted, selfgrafted and interspecific graftings, respectively. WUE was preserved under salinity in all grafted plants and was always higher than in nongrafted ones (1.5-fold higher under control conditions and up to 9.4-fold higher under severe salinity, compared with nongrafted plants; Fig. 4).

3.4. Ion partitioning

Saline stress resulted in changes in the main ionic concentrations in all plant organs, with the most relevant variations being observed in Cl^-, Na^+ and K^+ concentrations. On a whole-plant basis, similar increases in C–content upon salinity were recorded for the grafting combinations (a threefold increase under 80mM NaCl compared with control conditions; Fig. 5). On the other hand, Na^+ accumulation was much lower (and was scarcely affected by the salinity of the growing medium) in the interspecific grafting (a 2.6-fold increase under 80mM NaCl compared with control conditions), compared with nongrafted or self-grafted melon plants (a 5.8-fold increase under 80mMNaCl compared with control conditions). However, a consistent trend of a reduction of the K^+ content (reduced by half under 80mM NaCl) and the K^+: Na^+ ratio under salt stress was observed in all treatments (Fig. 5). In order to properly describe the main changes in the ionic composition occurring in the different plant organs as

a consequence of salt application, the PCA analysis was conducted, as displayed in Fig. 6. In the chart, the size of the bubbles represents the s.d. for each treament. The bigger the bubble, the higher the variability. As PCA takes all nine ions at the same time into account, it allows us to identify the ions that were more relevant for justifying the behaviour of one grafting combination compared with another in response to salinity. The vectors relative to each ion represent the positive axes of the effect of that ion. For example, a bubble lying near the vector means that the relative ion has a positive effect for identifying the treatment relating to the bubble itself. The analysis identified the most relevant ions in each plant organ and allowed their representation ona chart. As bubbles move from one salt concentration to another, changes in the ionic composition may be identified. For all studied organs, variability was significantly explained by five variables, which were Cl^-, SO_4^{2-}, K^+, Na^+ and NH_4^+ concentrations (root); Cl^-, NO_3^-, PO_4^{3-}, K^+ and Na^+ concentrations (stem); and Cl^-, NO_3^-, PO_4^{3-}, K^+ and Na^+ concentrations (leaf). At glance, although a common ion accumulation and partitioning in response to salinity was observed in non- and self-grafted plants, interspecific graftings behaved differently. In roots of nongrafted and self-grafted plants, 80mM salinity caused accumulation of Cl^- (14.1 mg g^{-1} DW, 2.5-folds higher than the control); in the interspecific grafting, the NH_4^+ (1.6 mg g^{-1} DW, 0.6-fold higher) was replaced by SO_4^{2-} (8.8 mg g^{-1} DW, 1.1-fold higher) and Na^+ (14.9 mg g^{-1} DW, 2.3-fold higher). Nongrafted and self-grafted plant stems showed similar behaviours: NO_3^- (3.0 mg g–1 DW, 0.2-fold higher) and K^+ (26.0 mg g^{-1} DW, 0.5-fold higher) were replaced by Na^+ (39.5 mg g^{-1} DW, 6.8-fold higher), Cl^- (37.1 mg g^{-1} DW, 2.6-fold higher) and PO_4^{3-} (8.3 mg g^{-1} DW, 1.6-fold higher). In the interspecific grafting, there was a greater increase in Cl^- (35.4 mg g^{-1} DW, 2.8-fold higher) and PO_4^{3-} (11.5 mg g–1 DW, 2.3-fold higher). In leaves belonging to non- and self-grafted plants undergoing 80mMsalt stress, the increased concentrations of Cl^- (27.9 mg g^{-1} DW, 4.2-fold higher), Na^+ (11.0 mg g^{-1} DW, 5.8-fold higher), PO_4^{3-} (7.1 mg g^{-1} DW, 2.0-fold higher) and $NH4^+$ (5.7 mg g^{-1} DW, 1.4-fold higher) were associated with a decrease in K^+ (23.4 mg g^{-1} DW, 0.6-fold). In the interspecific graftings, changes in Na^+ concentration (1.7 mg g^{-1}DW,1.4-fold higher) were negligible, whereas K^+ appeared to be the most common cation (23.0 mg g^{-1} DW).

4. DISCUSSION

4.1. Grafting improves plant response to salinity

The grafting-related effect on the development and growth of the scion was probably the result of physiological interactions between the scion and rootstock genotypes (Colla *et al*. 2010), but also a consequence of grafting *per se*. Grafting directly affects plant growth, either

by an increase inof water and nutrient uptake due to the rootstock's vigorous root system (Romero *et al*. 1997), the enhanced production of endogenous hormones (Zijlstra *et al*. 1994) and enhancement of scion vigour (Davis *et al*. 2008). Similarly, for both plant biomass (Fig. 1*a*) and leaf area (Fig. 1*b*), a general reduction was observed under salinity (Badr and Abou Hussein 2008). Indeed, canopy size reduction was greater in nongrafted than in grafted plants, thus confirming greater stress sensitivity by the former. It is commonly accepted that an interdependent relationship exists between roost and shoots: active shoots that ensure a sufficient supply of carbohydrates to the roots may stimulate and maintain active root functions; the activation of root functions can, in turn, improve shoot growth and physiology by supplying a sufficient amount of nutrients, water and phytohormones, thus ensuring increased biomass productivity (Orsini *et al*. 2012). The R : S ratio was always increased by salinity (Fig. 1*c*), although under control conditions, it was highest in the interspecific graftings. This was caused both by a lower shoot biomass (Fig. 1*a*, *b*) and the greater size of the squash root system compared with that of the melon.

4.2. Grafted plants acclimate to salinity through efficient stomatal adaptation

In response to salinity, plants combine strategies in order to both preserve tissue hydration and maintain growth (Fernández-García *et al*. 2004). Measurements of water potential and RWC (Fig. 2) suggest that all plants under study efficiently recovered from the stress without impairing the overall water status. Nevertheless, the observed differences in plant biomass indicate that the preservation of plant growth was achieved in different ways among grafting combinations. Self-grafted plants showed constitutively lower stomatal size, a critical trait involved in salt stress adaptation (Orsini *et al*. 2010*b*). The observed reduction in SA concurrent to the increase in SI (Fig. 3*a*, *b*) represents a functional response to salinity, enabling plants to modulate transpirational fluxes more precisely, alleviate salinity symptoms and preserve plant performance (Orsini *et al*. 2011). Although this appeared to be a common strategy pursued by all plants under study, self-grafted plants showed the highest increase in SI, a strategy that has been claimed to be crucial for rapid acclimation to salinity (Barbieri *et al*. 2012). Stomatal closure under salt stress acts as a defence against desiccation but, at the same time, limits CO2 diffusion into the leaf. In other words, the beneficial effects due to a reduced transpiration under stress (e.g. re-establishment of tissue turgor or delayed ion accumulation) would also limit photosynthesis and consequently yield. Nongrafted plants responded strongly to the stress through stomatal closure, but leaf gas exchange was reduced to a lower extent in grafted plants. However, self-grafted plants were able to restore transpiration more efficiently (Fig. 3*c*) than nongrafted ones, even under severe salinity, thus

confirming the efficiency of the observed stomatal regulation (Yoo *et al*. 2010). Interestingly, although stomatal adaptations to salinity were less evident in interspecific graftings, their transpiration scarcely suffered even when salt was applied at the highest concentrations, thus preserving WUE (Fig. 4) (He *et al*. 2009). It should be noted, though, than when transpiration and photosynthetic fluxes are preserved upon salinity, ionic accumulation in epigeous plant organs may occur (Orsini *et al*. 2012). However, regardless of the increased water fluxes, a reduced Na^+ load was observed in interspecific graftings, where the K^+ concentration and the K^+: Na^+ ratio were better preserved under salinity (Fig. 5). Apparently, other mechanisms were present that Prevented these plants from experiencing excessive salt accumulation.

4.3. Interspecific graftings may prevent nutritional imbalances upon salinity

The reduction in plant performances under saline conditions is generally associated with osmotic, toxic and nutritional factors. Nonetheless, although the former two have been extensively explored in the last 50 years of saline agriculture research, nutritional imbalances were hardly considered to be relevant to the whole picture (Maas and Grieve 1987; Vieira Santos *et al*. 2001). Recent indications (De Pascale *et al*. 2012) associate the detrimental effects of salinity to phosphate and potassium imbalances in tomato (*Lycopersicon esculentum* Mill.), thus suggesting that the ability of the plants to cope with nutritional deficiency would be a determinant in conferring salt tolerance. The PCA analysis presented here (Fig. 6) may link with further considerations in the subject: interspecific graftings responded to salinity mainly through Na^+ loading in the root system, whereas in the aerial parts of the plant, Na^+ and Cl^- accumulation was negligible, counterbalanced by a functional build-up of K^+. On the other hand, independent of the grafting method, Na^+ and Cl^- were found abundantly in nongrafted and self-grafted melon plants, indicating that no filtering of these salts operated at rootstock level (Edelstein *et al*. 2011), with concurrent depletion in the concentration of important elements such as potassium and nitrate in the stems and phosphate and ammonium in the leaves. The capability of a plant to exclude Na^+ at the root level may vary substantially among species (Orsini *et al*. 2010*b*). Consistently, squash and melon root systems behaved differently under salinity, the former being an excluder and the latter an accumulator. Differential compartmentalisation was thereafter responsible for the ion partitioning, as recently suggested in a study of melon plants grafted on squash (Edelstein *et al*. 2011). K^+ plays an essential role in the growth of all plants. However, due to its similar physicochemical structure to Na^+, when this ion is abundant at the transport sites for K^+, it enters predominantly into the symplast and might cause K^+ deficiency. Salinity dominated by Na^+ and Cl^- not only reduces K^+ availability, but also its transport and mobility to growing

regions of the plant, thus affecting the quality of both vegetative and reproductive organs. Moreover, many studies have shown that high concentrations of Na^+ and Cl^- in the nutrient soil solution may depress nutrient ion activities and deplete the K^+: Na^+ ratio, causing the plants to be susceptible to osmotic and specific ion injury as well as to nutritional disorders that result in reduced yield and quality. In the experiments described here, under elevated NaCl, plant K^+ content decreased similarly in both grafted and nongrafted plants (Fig. 5), although K^+ concentration under salinity was always higher in interspecific graftings (Fig. 5). Consequently, there could be a grafting effect on the K^+ movement to shoots, as previously indicated by Zhu *et al*. (2008) on cucumber (*Cucumis sativus* L.), where grafting facilitated the transport of K^+ to the leaves. In the leaves of interspecific graftings, an increase in the concentration of K^+ (Fig. 6) occurred under salinity; in stems, this element decreased, together with small changes in other ions, e.g. NH_4^+ or PO_4^{3-}(Fig. 6), meaning that these plants would efficiently contrast osmotic stress and nutritional imbalance, giving priority to leaves (Colla *et al*. 2006*b*). The similar behaviour of nongrafted and self-grafted plants (Figs 5 and 6) suggests that grafting *per se* does not affect ion accumulation and partitioning. Indeed, the RS841 rootstock was able to withhold high Na^+ content in its tissue, thus alleviating Na^+ toxicity and raising the overall salt tolerance by both Na^+ accumulation in roots and facilitating K^+ transport to the leaves in order to maintain the K^+ : Na^+ ratio (Orsini *et al*. 2012).

5. CONCLUSIONS

In this study, grafting significantly improved plant performances under salt stress, with similar responses, regardless of whether the plant was self-grafted or grafted on interspecific rootstock. Interspecific graftings presented reduced stress symptoms as a consequence of the efficient salt accumulation at the rootstock level, resulting in lower toxic and osmotic effects in the aerial parts. On the other hand, melon plants (either self-grafted or nongrafted) were not able to limit ionic fluxes throughout the plant. This resulted in salt accumulation in the stems and leaves, leading to marked nutritional imbalances in the same organs. In non-grafted plants, the osmotic component of salinity was counteracted by downregulation of the physiological functions and plant growth. Self-grafted plants, instead, made functional changes at the stomatal level that enabled them to maintain leaf gas exchanges under elevated salinity and to restart growth.

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Table 1. Results of the ANOVA on selected features of plant stress response in four experiments (Experiments 1–4)

ns, non-significant differences; *, significant differences at $P \leq 0.05$; **, significant differences at $P \leq 0.01$; ***, significant differences at $P \leq 0.001$;LA, leaf area; R: S ratio, root-to-shoot ratio;Ψw, leaf water potential, Ψp, osmotic potential; SI, stomatal index (Eqn 3); SA, stomatal area (Eqn 2); *g*s, stomatal conductance; E, leaf transpiration rate; A, net photosynthesis; WUE, water use efficiency; Cv, cultivar; Graft, grafting; S, salt; Exp, experiment

		Cv (*Cv*)	Graft. (*G*)	Salt (*S*)	Exp. (*E*)	*Cv*x*G*	*Cv*x*S*	*Cv*x*E*	*G*x*S*	*G*x*E*	*S*x*E*	*Cv*x*G*x*S*	*Cv*xGxE	*CvxSxE*	*CvxGxSxE*
FW	(g plant^{-1})	ns	ns	***	*	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
FW	(%)	ns	ns	***	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
LA	(cm^2 plant^{-1})	ns	*	**	*	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
LA	(%)	ns	*	**	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
R:S ratio	(%DW)	*	*	**	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
Ψ_w	(MPa)	ns	ns	***	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
Ψ_π	(MPa)	ns	*	***	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
RWC	(%)	ns	ns	**	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
SI	(%)	ns	*	***	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns
SA	(μm^2)	ns	ns	**	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
g_s	(mM m^{-2} s^{-1})	ns	*	***	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns
E	(mM m^{-2} s^{-1})	ns	*	***	ns	ns	ns	ns	***	ns	ns	ns	ns	ns	ns
A	(μM m^{-2} s^{-1})	ns	*	***	ns	ns	ns	ns	***	ns	ns	ns	ns	ns	ns
WUE	(μM CO_2 mM^{-1} H_2O)	ns	*	*	ns	ns	ns	ns	***	ns	ns	ns	ns	ns	ns

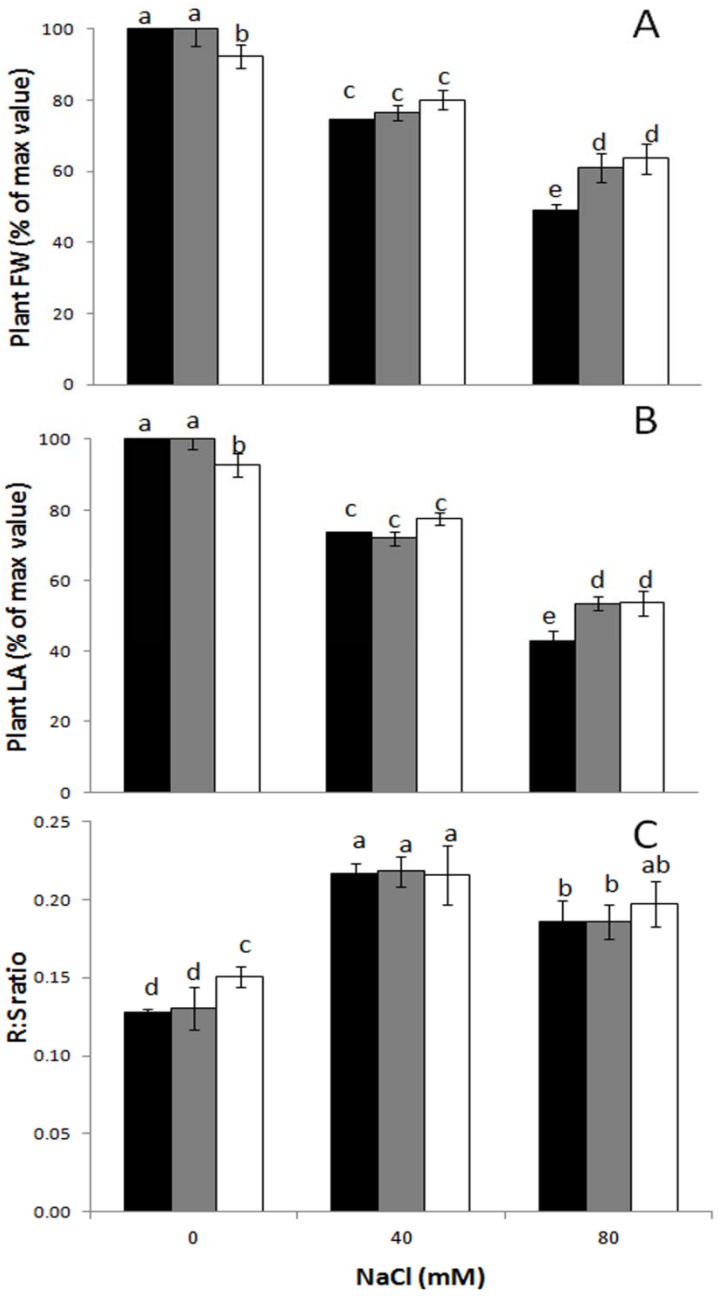

Fig. 1. Effect of salt stress (0, 40, and 80mM NaCl) on (*a*) relative FW, (*b*) relative leaf area (LA) (c) and root : shoot ratio (R : S ratio) in melon plants: nongrafted (black), self-grafted (grey) and grafted onto the RS841 rootstock (white). FW and LA are presented as relative values of their best performance for each grafting combination. Mean values of two cultivars, four experiments and three replicates ($n = 24$). Bars indicate s.e. and different letters indicate significant differences at $P \leq 0.05$.

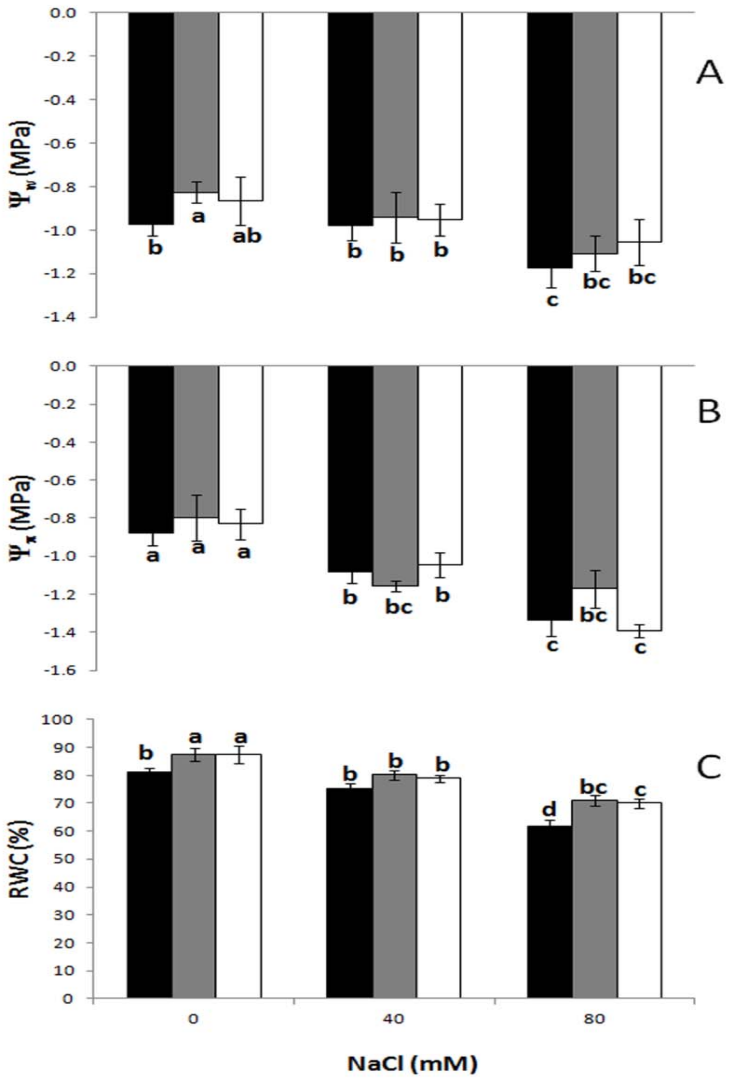

Fig. 2. Effect of 15 days of salt stress (0, 40 and 80mM NaCl) on (*a*) water potential (Ψw), (*b*) osmotic potential (Ψp) and (*c*) relative water content (RWC) in melon plants: nongrafted (black), self-grafted (grey) and grafted onto the RS841 rootstock (white). Mean values of two cultivars, two experiments (Experiments 1 and 3) and three replicates (n = 12). Bars indicate s.e. and different letters indicate significant differences at $P \leq 0.05$.

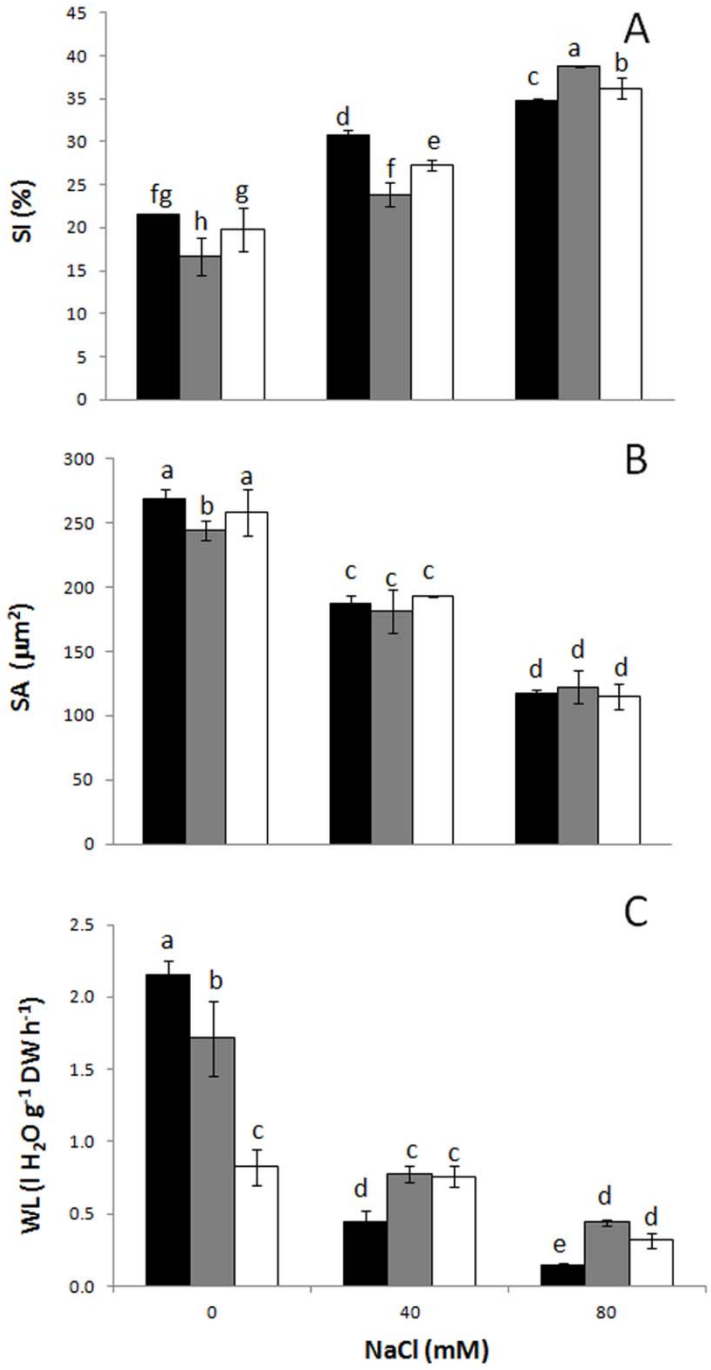

Fig. 3. Effect of 15 days of salt stress (0, 40 and 80mMNaCl) on (*a*) stomatal index (SI), (*b*) stomatal area (SA) and (*c*) overnight water loss (WL) in melon plants: nongrafted (black), self-grafted (grey) and grafted onto the RS841 rootstock (white). Mean values of two cultivars, four experiments and three replicates (n = 24). Bars indicate s.e. and different letters indicate significant differences at $P \leq 0.05$.

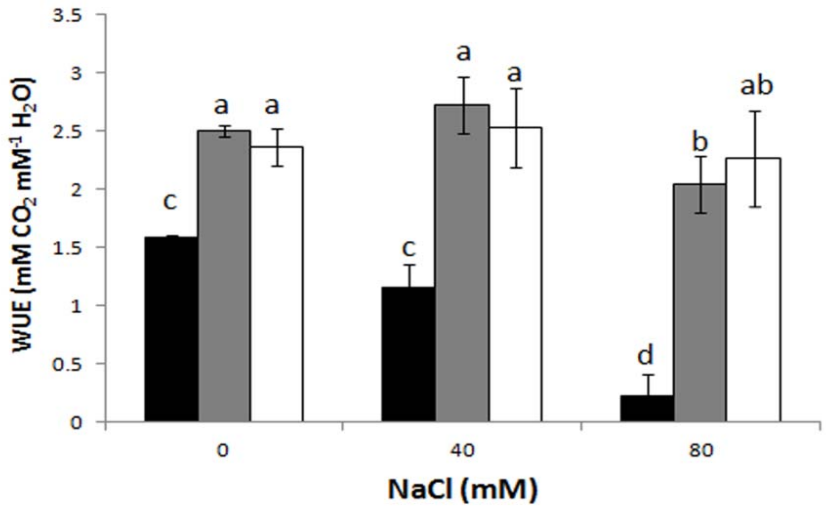

Fig. 4. Effect of 15 days of salt stress (0, 40 and 80mMNaCl) on water use efficiency (WUE) in melon plants: nongrafted (black), self-grafted (grey) and grafted onto the RS841 rootstock (white). Mean values of two cultivars, two experiments (Experiments 1 and 3) and three replicates (n = 12). Bars indicate s.e. and different letters indicate significant differences at $P \leq 0.05$.

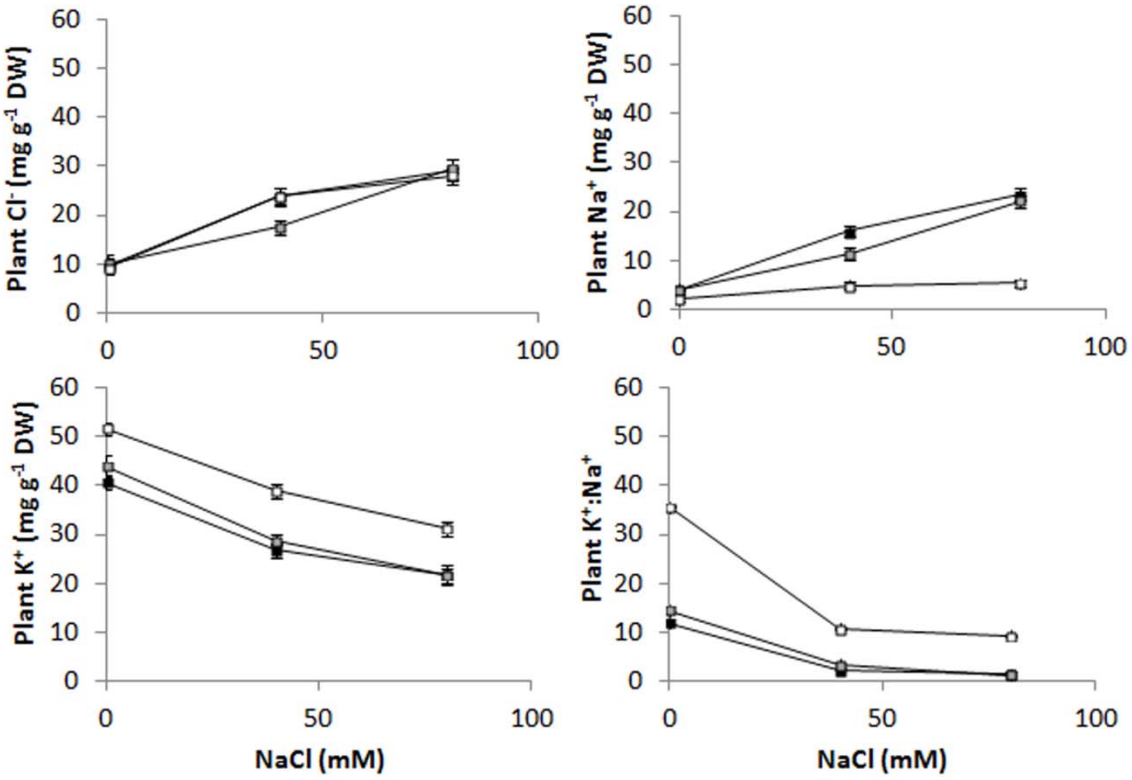

Fig. 5. Effect of salt stress (0, 40 and 80mMNaCl) in the ion concentration of (*a*) Cl^-, (*b*) Na^+ and (*c*) K^+, and (*d*) the K^+: Na^+ ratio) in melon plants: nongrafted (black), self-grafted (grey) and grafted onto the RS841 rootstock (white). Mean values of two cultivars, two experiments (Experiments 1 and 4) and three replicates (n = 12).

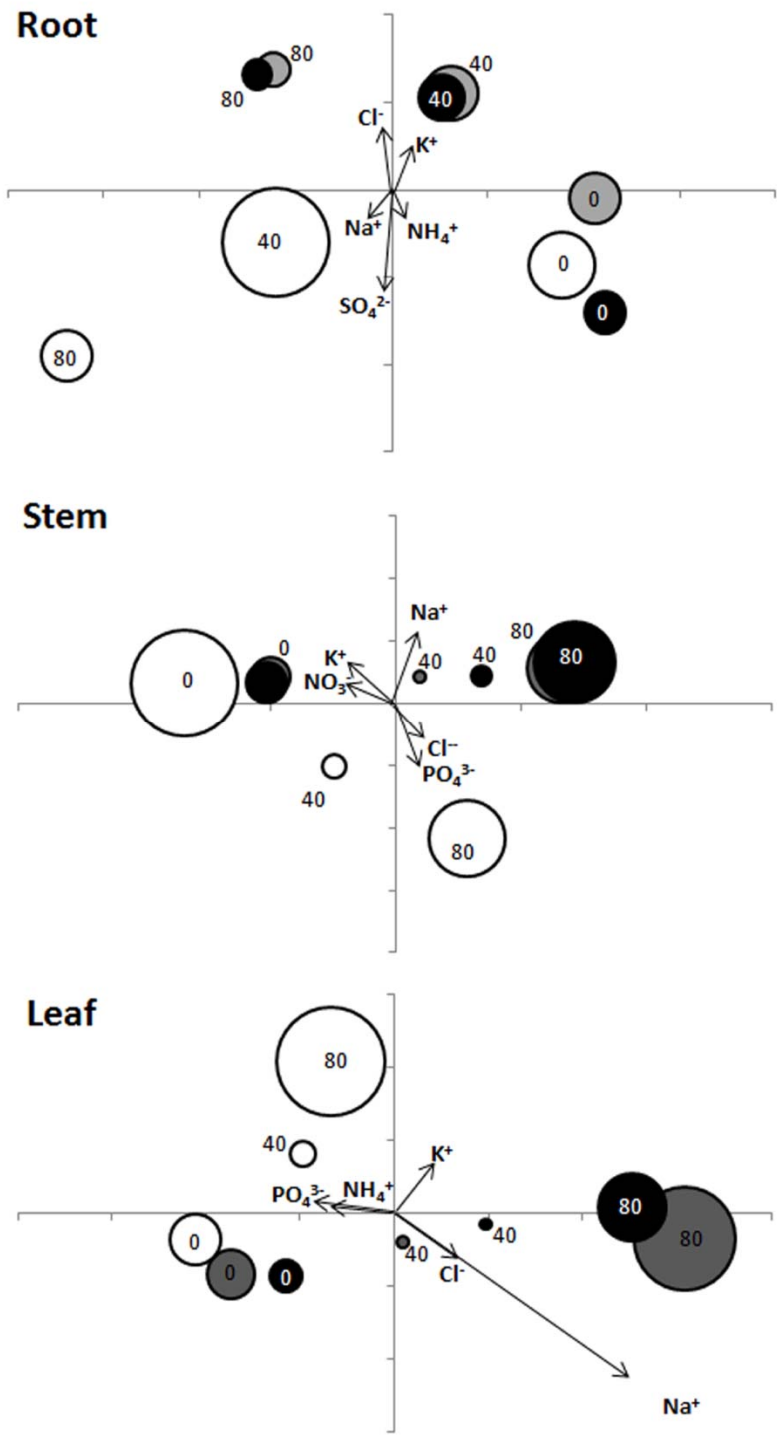

Fig. 6. Principal component analysis (PCA) of the ion distribution in (*a*) roots, (*b*) stems and (*c*) leaves upon salt stress (0, 40 and 80mM NaCl), in melon plants: nongrafted (black bubbles), self-grafted (grey bubbles, and with interspecific grafting (white bubbles). The analysis was performed on results from Experiments 1 and 4. The position of the bubbles was determined by the centroid of the sample distribution, and their size was determined by the variability within the sample replications. Vectors of the most explanatory ions were determined by the functions defined by PCA.

CHAPTER 2

II. Ionic Partitioning And Stomatal Regulation Dissecting Functional Elements Of The Genotypic Basis Of Salt Stress Adaptation In Grafted Melon

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ABSTRACT

Vegetable grafting is commonly claimed to improve crop's tolerance to biotic and abiotic stresses, including salinity. Although the use of inter-specific graftings is relatively common, whether the improved salt tolerance should be attributed to the genotypic background rather than the grafting per se is a matter of discussion among scientists. It is clear that most of published research has to date overlooked the issue, with the mutual presence of self-grafted and non-grafted controls resulting to be quite rare within experimental evidences. It was recently demonstrated that the genotype of the rootstock and grafting per se are responsible respectively for the differential ion accumulation and partitioning as well as to the stomatal adaptation to the stress. The present paper contributes to the ongoing discussion with further data on the differences associated to salinity response in a range of grafted melon combinations.

Keywords: vegetable grafting, *Cucumis melo* L., salt stress, NaCl, ion partitioning, stomata

1. INTRODUCTION

Salinity results in plant downregulation of physiological functions, accumulation of ions in vegetative tissues up to toxic concentrations and, more generally, to the overall depletion of the crop performances.[1] Several researches have confirmed the beneficial role played by the adoption of grafting in counteracting the detrimental effect of salinity.[2,3,4] To date, grafting is a common practice in melon (*Cucumis melo* L.) cultivation, mainly through the adoption of interspecific rootstocks, which are generally selected genotypes of squash (*Cucurbita maxima* Duch x *Cucurbita Moschata* Duch.). Although these rootstocks have proven to improve crop's performances in presence of biotic[5,6] and abiotic[4] stresses, whether the beneficial effects should be attributed to the rootstock genotype rather than grafting per se is still a controversial matter among scientists.

2. MATERIAL AND METHOD

In a recent publication,[7] the comprehension of the role of grafting in improving the response to salinity in melon was addressed. The manuscript reported on the effects associated to cultivar, grafting, and salinity over 4 experiments in a range of different environmental conditions. As only grafting and salinity presented significant interactions, results from all experiments were jointly discussed, thus offering innovative elements for the comprehension of the effect of grafting on the plant physiological response to the stress. In the present manuscript, additional results obtained from one of the experiments conducted at Bologna University, Italy (experiment 1#) [7] are discussed, with the aim of further elucidating how the differential stomatal and ionic response observed in self-grafted vs. interspecific grafting may lead to similar performances upon salt stress (0, 40, and 80 mM NaCl, starting from 10 Days After Transplanting, DAT, and lasting 30 d). Similarly to other experiments presented in the manuscript, 2 melon cultivars (namely Brennus, ZKI, Hungary, and London, Nunhems, The Netherlands) were used, altogether with a squash rootstock (Rs841, Monsanto, USA). Every genotype was used either non-grafted, self-grafted, or grafted on the interspecific squash rootstock. However, the peculiarity of this experiment was that also non-grafted and self-grafted Rs841 plants were included in the trial.

3. RESULT AND DISCUSSION

The ANOVA analysis highlighted that the melon scion genotype did not actually affect the plant's response to salinity, with significant differences observed only between plants either non-grafted, self-grafted, and interspecific graftings. Fresh biomass production was depleted as a consequence to salinity in all grafting combinations, although to a greater extent [7] in non-grafted plants undergoing 80 mM NaCl. Upon salinization, an increase in Na^+:K^+ ratio in

roots was observed in all grafting combinations under study (Fig. 1), confirming that, in both grafted and non-grafted plants, an increase of Na^+ was to be experienced independently from the genotype of the root system.[4] An explanation of the highest Na^+:K^+ values observed in all salinized root tissue of Rs841 (either non-grafted, self-grafted, or grafted with a melon scion) may be found in the consistent lower Na^+ loading in the epigeous organs: filtration of Na^+ at the grafting union level was observed (Fig. 1), although to a limited extent, whereas major differences were observed in stems and leaves. In these organs, although negligible changes could be detected upon salinity in any of the plants with a Rs841 root system, a general increase of the Na^+:K^+ ratio was recorded in all self- and non-grafted melons. Despite the observed changes in Na^+ concentrations, it has been suggested that functional response to salinity in grafted plants may include K^+ accumulation in aboveground organs.[8] Nonetheless, while salinity imbalanced the Na^+:K^+ ratio in epigeous tissue of all melon plants (either non- or self-grafted), the adaptation to the stress in self-grafted plants was mediated by the tight transpirational regulation operated at stomatal level. This is clearly represented by the response of the Water Use Efficiency (WUE) to salinity, which was scarcely (Brennus) or not (London) affected in self-grafted plants (Fig. 2). Although a similar behavior was observed also in interspecific-graftings, it should be noted that the rootstock (Rs841) had very little to do with it: WUE was extremely responsive to salinity in both self-grafted and non-grafted Rs841 plants (Fig. 2).

Plant preservation of WUE under stressful environments is generally achieved through tight stomatal control, which mainly consists in the reduction of water luxury consumption observable under non-limiting growing conditions. [9,10] The increased CO_2 assimilation per unit water used was a consequence of the changes in the stomatal morphology (mainly through increase in the stomatal index, SI),[11] rather than stomatal closure, which, on the other hand, turned out to be the main response to salinity in non-grafted plants.[7] Clearly, the improved response of grafted plants to salinity followed a binary pathway: signaling (possibly mediated by wound-related hormones, i.e., ABA and ethylene) resulted in a physiological pre-adaptation to the stress, most efficient when rootstock and scion belonged to the same species. On the other hand, the interspecific rootstock efficiently operated as a toxic-ions filter, thus resulting in lower accumulation in the epigeous plant organs.

4. ACKNOWLEDGMENTS

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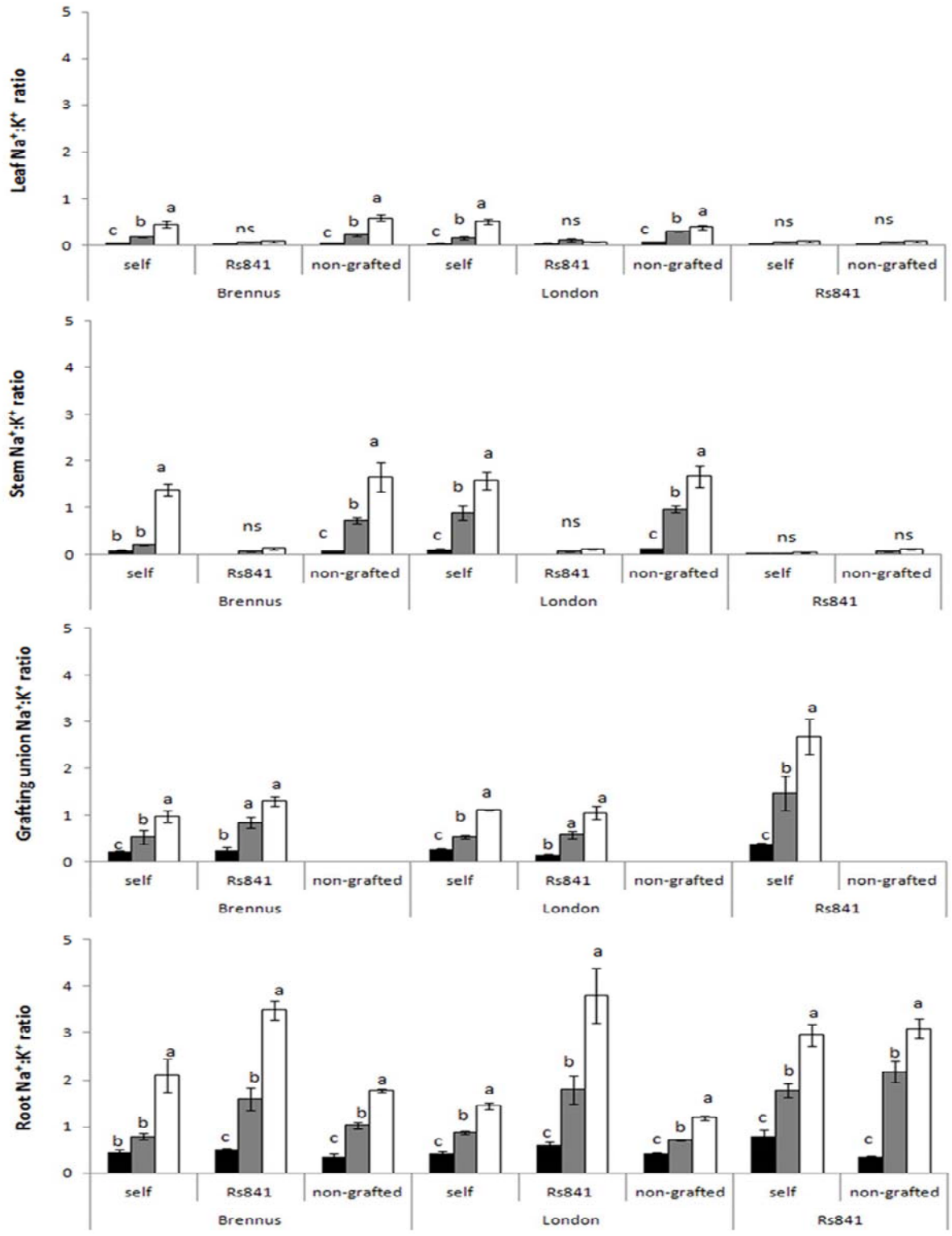

Figure 1. Na^+:K^+ ratio in plant organs (root, grafting union, stems, and leaves) from grafted and non-grafted plants of melon (cv Brennus and London) and squash (rootstock Rs841), as affected by 0 (black), 40 (gray), and 80 (white) mM NaCl. Mean values ± SE (n = 9). Different letters indicate significant differences within grafting combination at $P \leq 0.05$.

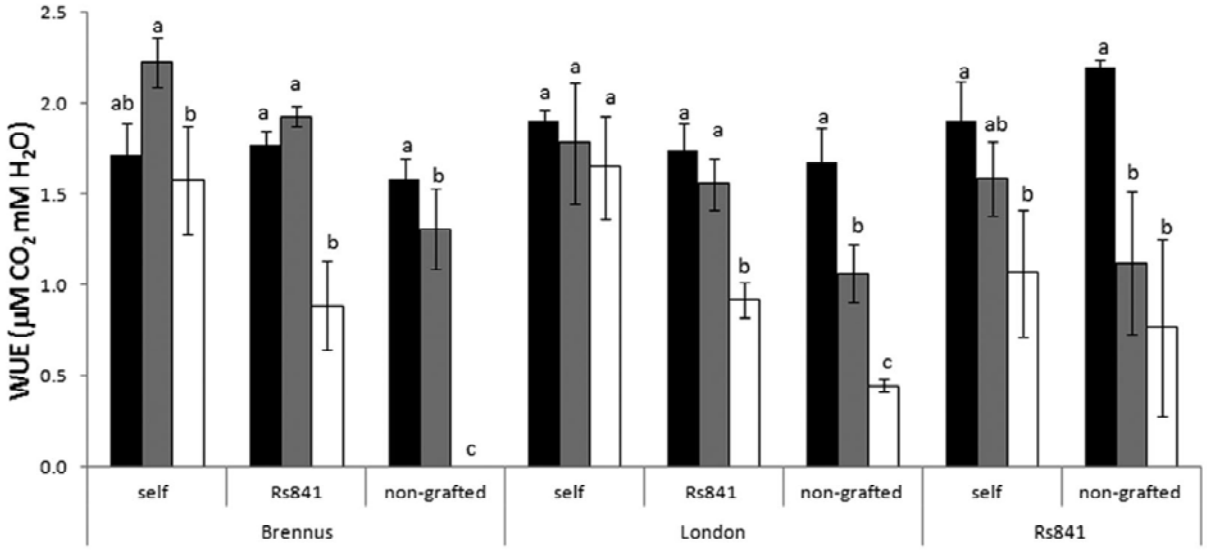

Figure 2. Water Use Efficiency (WUE) in grafted and non-grafted plants of melon (cv Brennus and London) and squash (rootstock Rs841), as affected by 0 (black), 40 (gray), and 80 (white) mM NaCl. Mean values ± SE (n = 9). Different letters indicate significant differences within grafting combination at $P \leq 0.05$.

CHAPTER 2

III. Physiological And Biochemical Alteration Modulated By Different Rootstock And Scion Genotypes In Tomato Plant Under Salinized Condition

4.1.2.1. Vegetative growth and physiological responses of Haruki scion against different rootstocks

4.1.2.2. The pattern of ion accumulations of Haruki scion against different rootstocks

4.1.2.3. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of Haruki scion against different rootstocks

4.1.3. The graft combinations of Kamonium scion against different rootstocks

4.1.3.1. Vegetative growth and physiological responses of Kamonium scion against different rootstocks

4.1.3.2. The pattern of ion accumulations of Kamonium scion against different rootstocks

4.1.3.3. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of Kamonium scion against different rootstocks

5. DISCUSSION

5.1. SALT TOLERANT INDUCED MECHANISMS BY DIFFERENT ROOTSTOCKS AGAINST ONE SCION GENOTYPE

5.1.1. Salt inclusion mechanism and leaf ion compartmentation of Nerina F1 scion

5.1.1.1. Vegetative growth response, water status and gas exchange parameters of Nerina F1scion

5.1.1.2. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of Nerina F1scion

5.1.2 Salt exclusion/inclusion mechanisms and leaf ion compartmentation of Haruki scion

5.1.2.1. Vegetative growth response, water status and gas exchange parameters of Haruki scion

5.1.2.2. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of Haruki scion

5.1.3. Salt exclusion mechanism and leaf ion compartmentmentation of Kamonium scion

5.1.3.1. Vegetative growth response, water status and gas exchange parameters of Kamonium scion

5.1.3.2. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of Kamonium scion

4. RESULT

4.2. THE PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF DIFFERENT SCION GENOTYPES AGAINST ONE ROOTSTOCK

4.2.1. The graft combinations of different scion genotypes against Maxifort F1 rootstock

5.2.3. Leaf compartmentaion and root extrude mechanisms of graft combinations against Arnold rootstock

5.2.3.1. Vegetative growth response, water status and gas exchange parameters of graft combinations against Arnold rootstock

5.2.3.2. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of graft combinations against Arnold rootstock

6. CONCLUSION

7. REFERENCES

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III. Physiological And Biochemical Alteration Modulated By Different Rootstock And Scion Genotypes In Tomato Plant Under Salinized Condition

ABSTRACT

A range of contradictory results have been reported in ascription to the positive effect of grafting in alleviating the deleterious effect of salt, claiming the role of either rootstock characteristics or scion genotypic and/or to the scion-rootstock interaction. Thus, this research was carried out in order to assess whether the primary factor that conferring the salt tolerance of grafted tomato plants actually belong to rootstock characteristics, scion genotypic differences regardless the rootstock character, and/or belong to the scion-rootstock interaction. In this research, two main approaches have been used to identify the relative abilities of different rootstock genotypes and scion genotypes to modulate the fruit yield and plant growth, transpiration rate (*E*), net assimilation rate (*A*), leaf water status, accumulation of ions in different plant organs, accumulation of fruit organic solutes, and the enzymatic antioxidant defence system of tomato fruit. The first approach was depending on identifying the main salt defence mechanisms that were induced in the shoot by different rootstock genotypes based on rootstock potential to regulate the absorption of ions from saline solution and regulate their transportation into shoot in long-term salt stress. The second approach was to determine the contribution of rootstock in inducing useful salt tolerance to the shoot growth and fruit productivity depending on salt tolerance mechanisms of the shoot genotype. Six tomato (*Lycopersicon esculentum* Mill) genotypes were used: cv. Haruki (HAR), cv. Kamonium (KAM), and cv. Nerina F1 (NER) were used as scions and grafted against three different tomato rootstock genotypes cv. Maxifort F1 (MAX), cv. Arnold (ARN), and cv. R1 (R1). Nine scion/rootstock combinations were obtained utilizing the three scion genotypes as follows: with Haruki genotype scion (HAR/MAX, HAR/ARN, and HAR/R1); with Kamonium scion (KAM/MAX, KAM/ARN, and KAM/R1) and with Nerina F1 scion (NER/MAX, NER/ARN, and NER/R1). The grafted plants were grown in environmentally controlled conditions and were irrigated by a hydroponic closed system. Three salt concentrations were applied in a single step by adding 100 mM NaCl (moderated salinity),

200 mM NaCl (high salinity), and 0 salt (control). The result showed that the plant growth and root development, the pattern of ions accumulation, and stomatal performance and net assimilation rate were significantly affected by scion genotypes and rootstock characters as well as the great physiological complexity that was associated by the specific interaction between scion and rootstock genotypes. Different rootstocks that have been grafted against one scion genotype showed different capacity to extrude and regulate the ion transportation into the shoot; and the differential effects induced by different rootstocks in term of transpiration rate, net assimilation rate and water use efficiency was closely related to the salt ion accumulation by different rootstocks. Additionally, this study pointed out that those rootstocks are not relatively salt tolerant and they are not able to donate the resistant to their shoots. Besides, the used rootstocks were not able to enhance effectively the activities of antioxidant enzymes in the tomato fruit, as in general lower fruit enzymatic activities were recorded and high level of H_2O_2 was accumulated. Thus, all rootstocks failed to achieve high productivity and fruit fresh weight was highly reduced even at mild salt. On the other hand, it is important to note that the differential patterns of salt ion accumulation that were induced by one rootstock genotype against different scions was actually closely associated with scion genotype characters. Furhtermore, different scion genotypes exhibited different mechanisms of salt resistance. The different responses of shoot in term of transpiration and net assimilation rate, WUE, leaf water relation, and fruit antioxidant defence system could be attributed to shoot genotypic variation. Additionally, there was low contribution of scion genotypes in term of enhancing the fruit enzymatic antioxidant system upon salinized condition.

1. INTRODUCTION

Most of the vegetable crops are glycophyte and salt sensitive plant (Shannon and Grieve 1998). The response of horticultural crop toward salinity varies depending on several factors like the surrounding environment, plant development stage, salt concentration and time of exposure (Munns 2002). Soils are classified as saline when the electric conductivity (EC) is above 4 ds m^{-1} which is equivalent to approximately 40 mM NaCl and generates an osmotic pressure of approximately 0.2 MPa (Munns and Tester 2008). The hyperosmolarity decreases the osmotic potential of the soil solution and restrict water uptake by the plant root and causes a significant increase in the stomatal resistance and reduction of CO_2 photosynthesis assimilation rate (Meloni et al. 2003).

Plant growth responds to salinity in two phases: a rapid osmotic phase that inhibits growth of young leaves, and a slower ionic phase that accelerates senescence of mature leaves (Munns and Tester 2008). Osmotic effect resulting from salinity may cause disturbances in the water balance of the plant, including a reduction of turgor and an inhibition of growth, as well as stomatal closure and reduction of photosynthesis. As a result, plants become susceptible to osmotic and specific-ion injury as well as to nutritional disorders that may result in reduced yield and quality. However, the plant response depends on the salinity level, composition of salt, exposed period to salinity, the crop species and cultivars, the growth stage of plants and a number of environmental factors (Grattan and Grieve 1999).

Frequent attempts have been made to raise the salt tolerance of crops either by traditional breeding programme or genetic transformation (Cuartero et al. 2006). However, the commercial success has been very limited due to the nature of the genetically and physiologically complex mechanisms of abiotic stress tolerance, and potential detrimental side effect of gene transferring (Wang et al. 2003; Flowers 2004). Even when halophytic species exist in a gene pool, as is the case of tomato, the development of salt resistant cultivars has been slow (Cuartero and Fernandez-Muňoz 1999). Thus, solving a problem as complex as the profitable use of saline water in irrigation require more than one strategy, each contributing to small extent to make the plant withstand better the deleterious effects of salt.

One environment friendly technique for reducing losses caused by salinity in fruit crop plant of *Solanaceae* and *Cucurbitaceae* families would be graft them onto salt tolerant rootstock which enable plant breeder to combine desired shoot characteristic with good root features (Zijlstra et al. 1994) and capable of ameliorating salt induce damage to the shoot of tomato (Santa-Cruz et al. 2002; Fernández-García et al. 2002, 2004; Estaň et al. 2005; Martinez-

Rodriguez et al. 2008, He et al. 2009), watermelon (Colla et al. 2006a; Goreta et al. 2008; Zhu et al. 2008b; Uygur and Yetisir 2009; Yetisir and Uygur 2010), melon (Colla et al. 2006b), eggplant (Wei et al. 2007), cucumber (Zhu et al. 2008a; Huang et al. 2009a,b,c, 2010; Zhen et al. 2010). Grafting is considered as a rapid alternative to the relatively-slow breeding methodology aimed at increasing fruit quality and as a way to overcome the problems caused by irrigation with saline water, grafting of commercial cultivars onto selected rootstocks could be a promising tool (Colla et al. 2008; Proietti et al. 2008). Grafting can raise the plant salt tolerance by inducing higher accumulation of proline and sugar in the leaves (Ruiz et al. 2005), higher antioxidant capacity in the leaves (López-Gómez et al. 2007), and lower transportation and accumulation of Na and /or Cl in the leaves (Fernández-García et al. 2004; Estaň et al. 2005; Goreta et al. 2008; Zhu et al. 2008a,b).

Salt stress reduces stomatal conductance, thereby limiting CO_2 supply to the leaf (Apel and Hirt 2004). This in turn causes the over-reduction of the photosynthetic electron transport chain, resulting in the production of reactive oxygen species (ROS) (Pinheiro et al. 2004) such as superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Mittler 2002). Wang et al. (2007) reported that generation of ROS is a common feature of plants subject to abiotic stresses. It has been described that under salt stress condition ROS production is stimulated increasing the risk of oxidative damage (Hernández et al. 2003). These ROS can injure the plant cell when not eliminated in time (Zhang et al. 2005) as they are highly reactive and can seriously disrupt normal metabolism through oxidative damage to lipids, proteins, and nucleic acids (Apel and Hirt 2004). It is well documented that the resistance to oxidative stress may be involved in salt stress tolerance (Ashraf 2009; Badawi et al. 2004). To prevent or alleviate these damages, plants possess a complex antioxidant system to detoxify ROS, including low-molecular mass antioxidants as well as antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and enzymes involved in the ascorbate–glutathione cycle (Halliwell–Asada cycle) such as ascorbate peroxidase (APX), and glutathione reductase (GR) (Foyer and Halliwell 1976). The activities of these antioxidant enzymes are increased in response to several abiotic stresses such as drought (Jaleel et al. 2007) and salinity (Manivannan et al. 2008). It is believed that a simultaneous increase in several components of the antioxidative defence system would be necessary in order to obtain an increase in the plant protective mechanisms (Jaleel et al. 2009). Ruiz-Lozano. (2003) mentioned that antioxidant enzymes play an important role in removing ROS when the plants are subject to osmotic stress. Antioxidants can be used as markers of salinity tolerance in grafted vegetable such as *Cucumis sativus* L. (Huang et al.

2010; Zhen et al. 2010) and *Solanum lycopersicum* L. (He et al. 2009; Zhang et al. 2008). SOD constitutes the first cellular defence line as it catalyses the conversion of superoxide anion radical ($O_2^{\bullet -}$) and give raise to H_2O_2 and O_2. The activity of CAT enzyme plays a vital role in eliminating H_2O_2 by catalysing H_2O_2 to H_2O and O_2 in the mitochondrion and microbody (Shi and Zhu 2008). It is known that APX and GR are considered the main enzymes involved in ascorbate-glutathione cycle. In this cycle, APX reduces H_2O_2 to H_2O and O_2 using AsA as a reducing substrate, while GR is required for the generation of AsA which is an important antioxidant scavenging ROS directly or indirectly (Asada 1999).

Tomato (*Lycopersicon esculentum* Mill.) is considered one of the most prominent horticultural crops in the world (Flores et al. 2010). The cultivated area has increased about 25% during the last 10 years and greenhouse hydroponic production of tomato has become economically important (He et al. 2007; FAO 2009). Rus et al. (2001) mentioned that at 50 mM NaCl, yield reduction of tomato plant was 50% or more in the dry condition of the Mediterranean region. The main sources of salinity in hydroponic cultivation are salted irrigation water and the long-term circulation of drain solution (Carmassi et al. 2005). However, tomato production is today concentrated in semi-arid regions where the saline water is frequently used for irrigation. Thus, searching for new strategies to enhance salinity tolerance is a critical task to overcome salinity stress on tomato (Asins et al. 2010). Indeed, lots of studies directed towards using grafting as a valid strategy for improving tomato yield under saline condition (Fernández-García et al. 2004; Estaň et al. 2005). The practical and horticultural aspects of grafting technology have been described in several reviews (Lee and Oda 2003), but less has been compiled about the physiological implications of the rootstock-scion interaction as a barriers for the translocation of water and nutrients or the effect of the rootstock-scion connection on the morphology, growth, biomass and photosynthesis of the grafted plant (Martínez-Ballesta et al. 2010).

Beyond the productive advantage offered by grafting as a useful tool to enhance plant tolerance toward abiotic stress, important contradictory issues have been addressed, which on many occasion remained overlooked or conflicted, whether the positive effect of grafting in alleviating the deleterious effect of salt and increasing plant tolerance was attributed to rootstock characteristics or scion genotypic differences, and/or belong to the scion-rootstock interaction. To date, to our knowledge most of the published data for graft tomato growing under saline condition have assessed the effect of rootstock on fruit yield and quality and few works have been done to show the exchangeable effects of rootstock and/or scion on salinity response depending on root system/and or shoot genotype. However, different contradictory

results have been obtained in relation to the root system role in the tolerance. Many reports mentioned that the use of tolerant rootstocks demonstrated to be a valid strategy in increasing salt tolerance of tomato (Fernández-García et al. 2004; Cuartero et al. 2006; Albacete et al. 2009) and watermelon (Goreta et al. 2008), and cucumber (Huang et al. 2010; Zhen et al., 2010). Zhu et al. (2008) recommended that using salt tolerant rootstock in grafting cucumber inhibited K^+ leakage and Na^+ uptake, consequently, a high K^+/Na^+ ratio remained. Romero et al. (1997) reported that root characteristics are the primary element for determining salt tolerance of melon plants. Also, many studies mentioned that the alleviated effects of stressed grafted plants belonged to the rootstock characteristics (Martinez-Rodriguez et al. 2008; He et al. 2009; Zhen et al. 2010). Tomato tolerant rootstock endows the grafted plant with an enhanced capacity for potassium homeostasis under salinity, which depends on the characteristics of the genotype used as a rootstock (Estaň et al. 2005). Santa-Cruz et al. (2001) found an increase in growth and fruit yield when a salt-sensitive tomato cultivar 'Moneymaker' was grafted onto a tolerant rootstock 'Pera' as compared to self-grafted plants under 50 mM NaCl. Furthermore, Shaterian et al. (2005) reported about the importance of the root system in the regulation of salt tolerance in salt-sensitive and tolerant potato genotypes. Additionally, Zhu et al. (2006a, b) suggested that the use of rootstock could increase the fruit yield of cucumber. A recent work from the same authors on cucumber suggested that the salt tolerance of grafted cucumber seedlings is related to the shoot genotype (Zhu et al. 2008a, b). Estaň et al. (2005) mentioned that grafting provides an alternative way to improve fruit yield in a commercial tomato hybrid ('Jaguar') and the differential fruit yield response among several graft combinations were mainly related to the different potentials of the tomato rootstocks ('Radja', 'Volgogradskij', 'Pera', and 'Volgogradskij'×' Pera') to exclude saline ions and regulate its transportation. More recently, Martinez-Rodriguez et al. (2008) questioning whether shoot genotype with an 'excluder' character ('Moneymaker') is able to increase its salt tolerance when grafted onto rootstocks ('Radja' and 'Pera') with different ability to regulate the transport of saline ions to the shoot over time. They concluded that grafting onto either 'Radja' or 'Pera' improved tomato fruit yield compared to self-grafted plants of 'Moneymaker' when plants were grown at 50 mM NaCl, whereas there was no effect of either rootstocks or grafting per se on fruit yield in the absence of salinity or at 25 mM NaCl. However, Rahman et al. (2002) and Yoshida et al. (2004) reported that the wild species *Solanum sisymbriifolium* Lam. and *Solanum integrifolium* Poir. (=*Solanum aethiopicum* L. Aculeatum group) have been tested as rootstocks for grafting of eggplant, the results were not very promising due to poor performance. On contrary, Gisbert et al. (2011)

investigated rootstock effects on fruit yield, apparent quality and the mineral composition of *S. melongena* 'Black Beauty' (BB) scions grafted on interspecific hybrid rootstocks developed from crosses of *S. melongena* with *Solanum incanum* L. (SI×SM) and *Solanum aethiopicum* L. (SM×SA). They mentioned that grafting eggplant onto interspecific hybrids rootstock, particularly (SI×SM), conferred the highest vigour to the scion, which resulted in the highest values for fruit earliness and early and total yield. Similarly, the growth performance of the eggplant cultivar 'Suqiqie' (*Solanum melongena* L.) was improved under saline stress conditions when the 'Torvum Vigor' (*Solanum torvum* Swartz) was used as rootstock (Liu et al. 2007; Wei et al. 2007). Chen et al. (2003) concluded that scion genotypes play an important role in the growth of grafted tomato plants, whereas rootstock has little influence. Also, Santa-Cruz et al. (2002) suggested that the characteristics of the tomato rootstock conferring salt tolerance on the shoot depend on the salt tolerance of the shoot genotype. Finally, Etehadnia et al. (2008), in a study on potato, indicated a significant influence of the scion on the rootstock, in addition to the effect of the rootstock on the scion, since the use of a resistant genotype as a scion has a positive impact, increasing the root biomass of the ABA (-) mutant rootstock. This might have been due to a higher rate of photosynthesis of the more vigorous scions leading to greater potential for partitioning of assimilates to the rootstock (Chen et al. 2003). To date, the major efforts have being directed towards the importance of grafting to enhance plant performance under salinized condition in related to none or self-grafted plant. The capacity of some rootstocks is essential for salt resistance in some species, while for other species the salt resistance conferred by rootstock depends on complex physiological interactions which are not well understood yet, that involve the type of scion genotype or the complexity of specific interaction between the scion genotype and rootstock (Santa-Cruz et al. 2002). Although the inclusion capacity of some tomato includer rootstock (Pera) that have the ability to accumulate pattern of Na in leaves quite similar to that observed in halophyte, was associated with certain physiological characteristics favourable to salinity resistance (Perez-Alfocea et al. 1996), biochemical and physiological mechanisms involved with the interaction between scion genotypes and rootstock are still poorly characterized. Therefore, more studies are necessary to investigate the primary factor that confer salt tolerance in grafted plants (Colla et al. 2010). Consistently, in this research we address the attention for comprehending whether the conferred salt tolerance of tomato graft plants was depends on the rootstock character or is affected by scion genotypic differences and/or exchangeable effects of scion-rootstock interaction.

2. OBJECTIVE

The main objectives of this research were determining the contribution of rootstock in inducing useful salt tolerance to the shoot growth and fruit productivity depending on salt tolerance mechanisms of the shoot genotype; identifying the main salt defence mechanisms that were induced in the shoot by different rootstock genotypes based on rootstock potential to regulate the absorption of ions from saline solution and regulate their transportation into shoot in long-term salt stress; and identifying the conditioned significant effect of the scion genotypes on physiological and biochemical shoot performance that involved in plant salt tolerance.

3. MATERIAL AND METHODS

3.1. Plant material, experimental conditions and saline treatments

To evaluate the contribution of root system characters and shoot genotypic differences in conferring the salt tolerance of grafted tomato plants, two main approaches have been used. The first approach was was to identify the main salt defence mechanisms that induced in the shoot by different rootstock genotypes based on rootstock potential to regulate the absorption of ions from saline solution and regulate their transportation into shoot in long-term salt stress. The second approach was determining the contribution of rootstock in inducing useful salt tolerance to the shoot growth and fruit productivity depending on salt tolerance mechanisms of the shoot genotype. However, this work has not been performed to evaluate the performance of control mechanisms of non-grafted or self-grafted plant in related to graft plants since lots of studies have been exclusively directed the appreciative positive effects of grafting to increase plant salt tolerance with respect to non-grafted or self-grafted plants in different plant species of *Solanaceae* and C*ucurbitaceas* families. Consistently, control in the hereby presented experiments will be provided by non-stressed tomato plants.

The experiment was conducted in environmentally controlled conditions (T° max 23°C; T°min 13°C; RH: 60%) in the experimental glasshouse at the University of Bologna, Italy. Three commercial tomato (*Lycopersicon esculentum* Mill) cultivars: cv. Haruki (HAR), cv. Kamonium (KAM), and cv. Nerina F1 (NER) were used as scions and grafted against three different tomato rootstock genotypes cv. Maxifort F1 (MAX), cv. Arnold (ARN), and cv. R1 (R1). Nine scion/rootstock combinations were obtained among the three scion genotypes; with Haruki scion: HAR/MAX, HAR/ARN, and HAR/R1; regarding Kamonium scion: KAM/MAX, KAM/ARN, and KAM/R1; and finally with Nerina F1 scion: NER/MAX, NER/ARN, and NER/R1.

Seeds of nine tomato genotypes were sown in polyethylene trays filled with peat moss till germination. Grafting was performed when seedling developed 3-4 true leaves by applying cleft grafting method. A V-shaped cut was made in the stem of the scion and then the scion inserted into the rootstock, which had a vertical slice cut down the centre of the stem. The rootstock and scion are then held together by a spring clip while the graft union forms (Oda 1999). After grafting, six plants per treatments were covered with a transparent plastic lid to maintain a high humidity level and facilitate the graft formation and were left in the shade for one week. The covering plastic lid was opened slightly every day to allow reduction in

relative humidity and was finally removed seven days after grafting. After the graft had established, each plant was transferred onto 5 l-pots filled with a mixture of perlite and vermiculite (1:1, v:v). Plants were grown on a hydroponic system and fed with nutrient solution having the following composition: NO_3^-: 16.5 mM; NH_4^+: 1 mM; $H_2PO_4^-$: 1.50 mM; SO_4^{2-}: 1.50 mM; K^+: 7.0 mM; Ca^{2+}: 5.0 mM; Mg^{2+}: 1.5 mM; Fe^{2+}: 15 μM; Mn^{2+}: 10 μM; B^+: 25 μM; Zn^+: 5.0 μM; Cu^+: 0.5 μM; Mb^{2+}: 0.5 μM. Salt treatment started at 30 days after plant grafting and lasted till the harvest time. Three salt concentrations was applied in a single step by adding 100 mM NaCl (moderated salinity), 200 mM NaCl (high salinity), and 0 salt (control) to 250 l-container.

3.2. Yield and plant growth determination

Harvest of ripen fruits was conducted weekly starting from 30 DAS for 2 months. 120 days after grafting tomato plants were harvested (90 DAS). Shoot and root biomass were measured at harvest time. Dry weight of whole plant and different plant organ were measured after oven drying at 65 ° C for three days with three replicates per salt treatment. For each plant, total fruit fresh weight was recorded every harvest.

3.3. Leaf gas exchange measurements

Three plants of 60 days after salinization were randomly selected from each salt treatment for leaf gas exchange measurements. The most recently fully expanded leaf of the 5-7th floor from the top was selected to measure leaf transpiration (*E*), stomatal conductance (g_s) and net photosynthesis (*A*) using a CIRAS-2 (PPSystem, Hitchin, UK) infrared gas analyser (closed system) with a Parkinson's Automatic Universal Leaf Cuvette (PAR 1000 mmol m^{-2} s^{-1}, 26°C, CO_2 13.63 mmol l^{-1} and 300 cm^3 min^{-1} flow rate) equipped with 18-mm diameter, 2.5-cm^2 area cuvette inserts. Water Use Efficiency (WUE) was determined as the ratio between *A* and *E*.

3.4. Leaf water relation

Three plants of 60 days after salinization were randomly selected from each salt treatment for water status determination. Leaf water potential (Ψ_w) was measured on fresh young and fully expanded leaves using a dewpoint potentiometer (WP4, Decagon Devices, Pullman, WA, USA). The same leaves were then frozen at – 20 °C for at least 24 h and then thawed out to measure the osmotic potential ($\Psi\pi$). Relative water content (*RWC*) was 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. 2010a). Osmotic adjustment was determined as OA = $\Psi_{\pi 0}$ V_0 - Ψ_π V , where $\Psi_{\pi 0}$ V_0 is the product of

(osmotic potential) × (osmotic volume) of unstressed plants and Ψ_π V is the product of (osmotic potential) × (osmotic volume) of leaves from salinized plants.

3.5. Ion analysis

Three plants of 90 days after salinization were randomly selected from each salt treatment for ion accumulation analysis. Samples of roots, stems, and leaves were collected from the dried material for mineral analysis. Sodium, potassium, and calcium concentration were determining by using Inductive Coupled Plasma Optical Emission Spectrometry (ICP-OES) after acidification of 0.1 g dry material with 65% nitric acid HNO_3 (1:100 ml, v: v), while chloride determination was done by ion chromatography (IC).

3.6. Biochemical analysis

3.6.1. Enzymes extraction and assays

For protein and antioxidant enzyme extraction, 10 g of fresh fruit were homogenized in 10 ml of 200 mM chilled potassium-phosphate buffer (pH 7.5) containing 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) and 0.1% (v/v) Triton X-100 placed in an ice bath. The homogenate was filtered through a layer of muslin cloth and centrifuged at 10000 × *g* for 20 minutes at 4 °C. The supernatant was collected and eluted through Sephadex G-25 gel column (NAP-25, Amersham Biosciences, Piscataway, NJ, USA) then re-suspended in 10 mM sodium-potassium phosphate buffer (pH 7.0) and used for the determination of the antioxidant enzymes. All enzymatic activities were assayed spectrophotometrically, the analysis was performed in triplicate and the results were normalized by plant fresh weight.

3.6.2. Determination of total soluble organic solute, lipid peroxidation, and enzymatic activities

The fruit soluble proteins concentration of the extract was estimated according to Bradford's method using bovine serum albumin as a standard (Bradford 1976). Fruit free proline content was determined according to Bates et al. (1973). The level of fruit lipid peroxidation was determined by measuring malondialdehyde (MDA) formation using the thiobarbituric acid-reactive substance (TBA) method as described by Heat and Packer. (1968). Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined using the method of Chen and Asada. (1990). Catalase activity (CAT, EC 1.11.1.6) was assayed by measuring the initial rate of disappearance of H_2O_2 and determined using the method of Havir and McHale. (1987). Glutathione reductase (GR, EC 1.6.4.2) activity was determined using the method of Foyer et al. (1991). Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined using

the method of Masia. (1998) by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) to blue formazan by flavins under illumination.

3.7. Experimental design and statics

The experiment was performed with a completely randomized design with nine combinations of scion/rootstock (HAR/MAX, HAR/ARN, and HAR/R1; KAM/MAX, KAM/ARN, and KAM/R1; and NER/MAX, NER/ARN, and NER/R1) and three NaCl levels (0, 100, and 200 mM) with three replicates. Data were subjected to Co-Stat-ANOVA and treatment means were compared using Student-Newman-keuls at significant level of 0.05 ($P<0.05$).

4. RESULTS

We have studied the physiological and biochemical responses of nine scion/rootstock graft combinations that have been generated by grafting three scion cultivars of tomato against three tomato rootstock genotypes. The plant developments were estimated under control and two salt levels (mild and high). All results and discussion of physiological and biochemical plant performances were organized in two main frames: studying the effect of one scion genotype against different rootstock, and viceversa studying the effect of different scion genotypes against one rootstock.

4.1. THE PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF ONE SCION GENOTYPE AGAINST DIFFERENT ROOTSTOCKS

Three tomato cultivars: cv. Haruki (HAR), cv. Kamonium (KAM), and cv. Nerina F1 (NER) have been grafted onto three different tomato rootstock genotypes cv. Maxifort F1 (MAX), cv. Arnold (ARN), and cv. R1 (R1) and resulted in nine scion/rootstock combinations. We investigated the main salt defence mechanisms that induced in the shoot by different rootstock genotypes based on rootstock potential to regulate the absorption of ions from saline solution and regulate their transportation into shoot in long-term salt stress.

4.1.1. The graft combinations of Nerina F1 scion against different rootstocks

4.1.1.1. Vegetative growth and physiological responses of Nerina F1 scion against different rootstocks

This experiment was carried out with the aim of studying the effect of grafting of one shoot genotype against different rootstock genotypes in term of shoot growth, fruit yield and physiological plant performance under both control and saline conditions. The plant development was restricted upon salinization and this reduction was significantly affected by rootstock, scion, and rootstock × scion interaction (Table 2). Three rootstock genotypes: Maxifort F1, Arnold, and R1 have been used against Nerina F1 cultivar scion and generated three graft combinations: NER/MAX, NER/ARN, and NER/R1 (Table 1). The positive effect

of the used rootstocks to alleviate the shoot fresh and dry weight diminishing under stressed condition was presented when Arnold genotype was used as a rootstock in NER/ARN plant, where this plant showed less reduction in shoot FW and DW as (-47% and -21%) at mild salt and (-77% and -65%) at high salt level in related to control plant (Table 1). However, the other rootstocks Maxifort F1 and R1 showed higher reduction in shoot FW and DW as (-65% and -47%) in NER/MAX plant and (-65% and -53%) in NER/R1 plant at moderated salt stress. Upon high salt stress, Maxifort F1 rootstock in NER/MAX plant induced the highest reduction in shoot FW and DW in related to control plant (-96% and -84%), while the respective values of these parameters were (-84% and -74%) in presence of Nerina F1 scion in NER/R1 (Table 1).

Indeed, root growth showed different behaviours under salt stress and high significant effect was related to rootstock and rootstock × scion (Table 2). In the presence of Nerina F1 genotype as a scion, the rootstock Arnold in NER/ARN plant presented a going up value in root DW by +62% in related to control plant under moderated salt, whereas the Maxifort F1 rootstock in graft plant NER/MAX showed a noteworthy reduction by -41% comparing with control plant, and the this reduction was less in presence of R1 rootstock and reached to -7% (Table 1). However, at high salt stress, the root DW reduction was higher in NER/MAX plant and accounted for -76%, while NER/ARN and NER/R1 plants showed less reduction as -31% in related to their control plants (Table 1).

The yield of all graft combinations was significantly reduced by the ionic composition and this reduction varied significantly according to the characteristic of the shoot genotype where significant effects were found between scion, rootstock × scion, and salt × rootstock ×scion interaction (Table 2). However, different rootstocks did not affected the fruit yield as the ANOVA analysis showed no interaction between rootstock and the ionic composition × rootstock were observed (Table 2). Accordingly, the three graft combination presented high reduction in FW fruit even at moderated salt stress by -74%, -82% and -58%, respectively in NER/MAX, NER/ARN, and NER/R1 (Figure 1). Nonetheless, this reduction was higher under high salt stress and accounted averagely for -96% for the three graft plants (Figure 1).

To study the plant physiological response, some parameters related to osmotic stress (relative water content, water and osmotic potential, and osmotic adjustment) were determined. Relative water content (RWC) showed slight insignificant reduction upon salinization in all

graft combinations, regardless the scion or rootstock genotypes, in related to control plant (data not shown). The effect of salinity stress on leaf water relations was again marked differently from control plants, as the applied salt induced sharp decreases in leaf water potential (Ψ_w) and the medium redaction among the three graft plants: NER/MAX, NER/ARN, and NER/R1 was as 1.9 and 3 times as their control plants upon 100 and 200 mM NaCl (Figure 2). Similarly, osmotic potential ($\Psi\pi$) showed high reduction at both salt stresses and the most important reduction was recorded when Maxifort F1 was used as a rootstock in NER/MAX plant and reached -3.87 MPa, while the medium reduction in presence of other rootstocks Arnold and R1 was -2.94 MPa at high salt stress (Figure 2). Accordingly, Maxifort F1 rootstock in NER/MAX plant achieved the highest significant value of osmotic adjustment 1.60 MPa while the medium OA of NER/ARN and NER/R1 plants were 0.6 MPa at high salt stress (Figure 2).

Regarding the leaf gas exchange, the results obtained in the current study showed that not only the rootstock genotypes but also the scion genotypes affect the stomatal performance and net assimilation rate, in which (*E*) and (*A*) values were expressively affected by salt, scion, rootstock, and their interactions (Table 4). However, in the three graft plants of NER/MAX, NER/ARN, and NER/R the transpiration rate (*E*) and net CO_2 assimilation rate (*A*) declined significantly with increasing level of salt stress (Table 3). This decrease was accompanied by substantial decreases in stomatal conductance (G_s) and intercellular CO_2 concentration (C_i) (data not shown). However, the differential response of different rootstocks was clear in term of net assimilation rate in the leaves of high salinity treated plants, as Maxifort F1 rootstock in NER/MAX plant showed the highest significant reduction in net assimilation rate (-91%), while Arnold and R1 rootstocks in graft plants NER/ARN and NER/R1 showed less reduction in (*A*) value by 66% in respect to their control plant at 200 mM NaCl (Table 3). However, the three rootstocks showed no significant effects with Nerina F1 scion in term of WUE as the three graft plants showed similar responses to that of their control plants (Table 3).

4.1.1.2. The pattern of ion accumulations of Nerina F1 scion against different rootstocks

The physical characteristics of the rootstock such as lateral and vertical development of roots influence significantly the nutrient and water uptake and pattern of ion accumulation. In this study, plants of the tomato (*Solanum Lycopersicum*) cultivars Haruki, Kamonium, and Nerina F1 were grafted onto three different rootstock genotypes Maxifort F1, Arnold, and R1 to

assess the ability of these rootstocks to extrude and regulate the transport of different ions into the shoot. In the present study, the concentration of Na^+ ion in the leaves of different grafting combinations increased progressively as NaCl concentration increased (Figure 3). However, the pattern of increasing leaf Na^+ concentration in salt treated plants was depending on scion, rootstock genotypes and the interaction between them (Table 5). However, different rootstocks showed significantly different capacity to take up and transport Na^+ ion from the root medium into the shoot. Among the three graft combinations of Nerina F1 scion (NER/MAX, NER/ARN, and NER/R1), the Maxifort F1 rootstock in NER/MAX plant accumulated more leaf and stem Na^+ ion (150 and 156 $mg.g^{-1}$ DW) in respect to its root (113 $mg.g^{-1}$ DW) upon 200 mM NaCl (Figure3). However, R1 rootstock in NER/R1 plant showed similar accumulation of Na^+ ion in the leaf and root (104 versus 112 $mg.g^{-1}$ DW), while the root of Arnold rootstock in NER/ARN plant showed high ability to regulate the Na^+ transportation into its aerial part and accumulate less sodium in its leaf (97 versus 77 $mg.g^{-1}$ DW). On other hand, the comparison of the different rootstocks performance revealed that Maxifort F1 rootstock in NER/MAX plant accumulated significantly higher leaf Na^+ ion by +95% and +45% than the other rootstocks in NER/ARN and NER/R1, respectively at 200 mM NaCl. (Figure3). The pattern of stem Na^+ accumulation was similar to that observed for leaf Na and showed significant increasing in the three graft plants depending on different abilities of rootstocks to transport the sodium ions into plant shoot. The rootstock maxifort F1 in NER/MAX showed significant increasing by +201% and +134% with respect to Arnold and R1 rootstocks in NER/ARN and NER/R1 plants at 200 mM NaCl (Figure 3). Alike Na^+ content of leaves and stems, root Na^+ concentration of the three graft plants exhibited high significantly increasing by 7 times as control plant at both salt levels (Figure 3). However, the root Na^+ ion in NER/MAX and NER/R1 plants showed similar and higher root Na content in respect to NER/ARN at severe salt stress (113 versus 97 $mg.g^{-1}$ DW) (Figure 3). Furthermore, the result of Na^+/K^+ ratio was consistent to that observed with Na content. The leaf Na^+/K^+ value was higher than root Na/K value by three times in NER/MAX and NER/R1 plants and by two times in NER/ARN plant (Figure 4). Also, the Maxifort F1 rootstock in NER/MAX graft plant showed an increasing of leaf Na^+/K^+ ratio by +105% and +29% in related to NER/ARN and NER/R1, respectively at 200 mM NaCl. This increasing was also associated with higher stem Na^+/K^+ ratio in NER/MAX plant by +209% and +128%, respectively in related to NER/ARN and NER/R1plants. In addition root Na^+/K^+ ratio of NER/MAX plant was higher by +50% than other grafted plants (Figure 4).

What's more, the partitioning of total plant Na^+ data in term of total dry weight showed that the three rootstocks accumulate Na^+ ion in different percentage (Figure 8). The Maxifort F1 rootstock in NER/MAX plant accumulated half of total Na^+ concentration in its root (53%) and distributed the other half equally between stem and leaf (25% and 22%) at 200 mM NaCl, where Arnold and R1 rootstocks in NER/ARN and NER/R1 plants partitioned the highest percentage of total Na^+ ion in the root zone (67%), while 13% and 12% of total Na ion were located in its stem and leaf respectively.

With regard to Ca^{2+} concentration, the highest Ca^{2+} concentration was located in leaf of the three graft plants (NER/MAX, NER/ARN, and NER/R1) and showed medium accumulation as 19 $mg.g^{-1}$ DW at high salt stress (Figure 5). However, there was no effect of both salt and rootstock genotypes on leaf Ca^{2+} concentration (Table 5) and all graft plants accumulate similar amount as non-treated plant. However, the effect of rootstock was shown at stem and root levels only with Maxifort F1 rootstock in NER/MAX plant, where this plant accumulated more root Ca^{2+} as 1.5-fold as NER/ARN and NER/R1 plants and more stem Ca^{2+} as 2-fold and 3.4-fold as NER/ARN and NER/R1 plants at 200 mM NaCl (Figure 5).

Respecting the K^+ ion content, the distribution of this ion differs between plant organs as follow: 4, 14, 11 $mg.g^{-1}$ DW, respectively in leaf, stem, and root as an average of the three graft plants NER/MAX, NER/ARN, NER/R1 at 200 mM NaCl (Figure 6). However, at high salt stress, there was a significant reduction about 40% in leaf K content in the three graft plants in respect to control plant regardless the rootstock genotypes, while stem K^+ showed no effect either of rootstock or salt level (Table 5). Nonetheless, the root potassium increased significantly in Arnold and R1 rootstocks in NER/ARN and NER/R1 plants by 2.2-fold and 3.6-fold comparing with their control plant at 200 mM NaCl (Figure 6).

Concerning the Cl^- concentration, the three graft plants NER/MAX, NER/ARN, and NER/R1 showed significant increasing in leaf Cl^- as 5, 2 and 3 times as their control plants at 200 mM NaCl (Table 6 and Figure 7). Moreover, NER/MAX plant showed higher leaf Cl^- by +55% and +81% than NER/ARN and NER/R1, respectively upon high salt stress. Like Cl^- leaf, the stem Cl^- concentration was increased significantly in the three mentioned plants upon high salt stress; however, NER/MAX plant showed an increasing as two times as the other graft plants. Similarly, root Cl^- ion increased significantly in all those plants without any significant effect of the rootstock genotypes (Figure 7).

4.1.1.3. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of Nerina F1 scion against different rootstocks

The effect of different rootstocks on the activities of APX, CAT, GR, and SOD in the fruit of grafted plants was measured upon the excess of NaCl in the root medium (Figure 9).Under normal condition, no significant differences was observed in the activities of CAT and SOD enzymes among the three graft plants of Nerina scion: NER/MAX, NER/ARN, and NER/R1; however, the activity of APX enzyme of NER/ARN plant was significantly less than that of NER/MAX and NER/R1. Under high salt stress, the rootstock Arnold in NER/ARN plant showed high activation of APX enzyme by 17 times as control plant while the other rootstocks Maxifort F1 and R1 in NER/MAX and NER/R1 plants did not induce any changes in the activity of this enzyme. Regarding the CAT activity, Maxifort F1 and Arnold rootstock showed significant increasing in the activity of these enzymes upon both salt stresses, while R1 rootstock in NER/R1 plant did not provoke any change in related to control plant (Figure 9). However, Maxifort F1 rootstock in NER/MAX plant showed higher CAT activity than that of Arnold rootstock in NER/ARN plant by +135% at high salt stress. The different rootstock genotypes performed differently in regard to GR activity (Figure 9). R1 rootstock in NER/R1 plant did not affect GR activity upon salinization and showed similar response to that of control plant; however, Arnold rootstock caused an increasing in GR activity by +93% regarding the control plant at mild and high salt stress, while Maxifort F1 rootstock in NER/MAX plant showed a sharp reduction in the activity of this enzyme by -74% at high salt stress respecting the control plant (Figure 9). Nonetheless, there was no appreciative performance of any rootstocks used in term of SOD activity as the salt treated plant responses in the three grafting combinations were similar to that of relief plants (Figure 9).

The positive effect of different rootstocks was evident in the pattern of compatible solute accumulations (proline and protein) in the fruit of three graft plants upon salinization (Figure 10). Maxifort F1 and Arnold rootstocks in NER/MAX and NER/ARN plants showed similar significant increasing in fruit protein by +142% and +193% at 100 and 200 mM NaCl in related to control plants, while R1 showed negligible increasing in protein content upon salt treatments. With regard to fruit proline content, the three graft plants showed similar increasing in proline concentration at mild salt stress as 14 times as the control plant regardless the rootstock genotypes (Figure 10). However, the pronounced positive effect of different rootstocks was clear when Maxifort F1 was employed as a rootstock at high salt stress, where only NER/MAX plant showed an appreciative increasing in proline content as

49 times as control plant. Regarding the MDA content as a measure of lipid peroxidation, neither salt nor rootstocks affect the fruit MDA in three grafting combinations NER/MAX, NER/ARN, and NER/R1 (Figure 10).

4.1.2. The graft combinations of Haruki scion against different rootstocks

4.1.2.1. Vegetative growth and physiological responses of Haruki scion against different rootstocks

The genotype Haruki was used as a scion and grafted onto three different rootstocks: Maxifort F1, Arnold, and R1 and, accordingly, three graft combinations have been constituted: HAR/MAX, HAR/ARN, and HAR/R. The differential effect of different rootstocks was presented in morphological and physiological plant responses upon salinization. The rootstock Maxifort F1 in HAR/MAX plant exhibited the highest shoot DW redaction by (-54% and -72%) at mild and high salt stresses in respect to control plant, while the other two rootstocks Arnold and R1 presented less medium reduction as (-32% and -64%) respectively at 100 and 200 mM NaCl regarding their control plants (Table 1). Similarly, the sharpest root DW redaction was recorded in HAR/MAX plant as (-30% and -37%) respectively at both salt levels, while both rootstocks Arnold and R1 showed significant increasing in root DW as (+22% and +131%) in HAR/ARN and HAR/R1 plants respectively at mild salt stress regarding the control plants (Table 1). However, even at high salt stress, these latter two rootstocks showed continued medium increasing in root DW as +4% as the control plant (Table 1). Fruit yield was affected strongly with salt stress and showed similar reduction in HAR/ARN and HAR/R1 as -80%, while this reduction was -66% in HAR/MAX plant at moderated salt stress with relation to control plants (Figure 1). However, the three graft plants illustrated sever yield reduction at high salt stress as 95% regarding the control plant irrespectively the rootstock genotypes (Figure 1).

Water potential affected apparently upon salinization and showed significant reduction in the three graft plants regardless the rootstocks used (Figure 2). Similarly, the osmotic potential decreased significantly in all graft plants and especially in HAR/MAX plant that showed the lowest $\Psi\pi$ value at high salt stress; however, the $\Psi\pi$ reduction in HAR/MAX plant was associated with the highest OA value at 200 mM NaCl (Figure 2).

Regarding the gas exchange parameters, HAR/MAX and HAR/ARN plants showed similar higher reduction of *E* and *A* values as (-84% and -62%) than HAR/R1 (-46% and -34%),

respectively at moderated salt stress comparing with the control plants (Table 3). However, this reduction in HAR/MAX and HAR/ARN plants was associated with higher WUE by +153% comparing with control plant, while the WUE increasing was about +25% in HAR/R1 at 100 mM NaCl. On contrary, at high salt stress HAR/R1 plant showed less *E* and higher WUE values than HAR/MAX and HAR/ARN plants (Table 3).

4.1.2.2. The pattern of ion accumulations of Haruki scion against different rootstocks

The effect of the same rootstock genotypes (Maxifort F1, Arnold, and R1) on the Na ion accumulation pattern of Haruki scion has been studied on the three generated graft combinations (HAR/MAX, HAR/ARN, and HAR/R1) (Figure 3). All plant organs showed sharp increasing in Na^+ content upon salinization (Figure 3). The root of the Maxifort 1 and R1 rootstocks accumulated Na^+ ion as 8 times as their control plants, while Arnold rootstock accumulate 5 times more than control plant at 200 mM NCl (Figure 3). Similarly, stem Na^+ content of the three graft plants showed 7 times increasing in regarding the control plant, while the increasing of leaf Na^+ was 13 times as control plant at 200 mM NaCl (Figure 3). However, the finding of this experiment showed the effect of rootstock genotypes was pronounced only at root level, where Arnold rootstock in HAR/ARN plant showed significantly lower Na accumulation by (-48% and -69%) than Maxifort F1 and R1 rootstocks in HAR/MAX and HAR/R1 plants, respectively at 200 mM NaCl (Figure 3). However, the stem and leaf Na^+ concentration showed no significant differences between the rootstocks performance as the shoot of the three graft plants accumulated similar stem Na^+ ion (54 $mg.g^{-1}$ DW) and leaf ion (87 $mg.g^{-1}$ DW) (Figure 3). It was worthy to mention that the Na partitioning of different plant organs was affected by rootstock genotypes. At moderated salt stress, root Na^+ accumulation rate was higher than Na leaf ion in the three grafts mentioned above plants by 2-fold, 1.9-fold, and 1.3-fold, respectively in HAR/MAX, HAR/ARN and HAR/R1 (Figure 3). However, upon high salt stress, HAR/ARN plant showed higher Na^+ content in its leaf than the root (81 versus 60 $mg.g^{-1}$ DW), while HAR/MAX and HAR/R1 continued to accumulate higher Na^+ ion in the roots (114 and 101 $mg.g^{-1}$ DW) in respect to their leaves (87 and 93 $mg.g^{-1}$ DW) (Figure 3). Additionally, at mild salt stress, there were differences in the leaf Na^+/K^+ ratio between graft combinations depending on rootstocks, where the plant grafted onto R1 rootstock in HAR/R1 plant showing higher value of Na^+/K^+ by two times as HAR/MAX and HAR/ARN, while the respective value of this ratio was the same in the root of the all plants (Figure 4). Likewise, at 200 mM NaCl, the root Na^+/K^+ ratio showed similar value among the three graft plants, while leaf Na^+/K^+ ratio of the HAR/R1 plant presented slightly higher value by 1.6 times as the other graft plants (Figure 4).

Regarding Ca^{2+} ion, the most abundant Ca^{2+} ions of the three graft plants (HAR/MAX, HAR,ARN, and HAR/R1) was located in the leaves and averaged of 20 $mg.g^{-1}$ DW compared with stem Ca^{2+} (3 $mg.g^{-1}$ DW) and root Ca^{2+} (5 $mg.g^{-1}$ DW) upon high salt stress (Figure 5). However, the pattern of leaf and stem Ca^{2+} accumulation in all graft combinations was not affected by neither salinity nor rootstock genotypes and showed similar values as control plant, even at 200 mM NaCl and all the interactions between scion and rootstocks were not significant (Table 5) However, only the Maxifort F1 rootstock in HAR/MAX plant showed higher Ca^{2+} root content by 50% than Arnold rootstock in HAR/ARN plant at high salt stress (Figure 5).

With reference to K^+ ion concentration, leaf K^+ of the three graft combinations (HAR/MAX, HAR/ARN, and HAR/R1) showed a slight non-significant reduction upon 200 mM NaCl (Figure 6). However, stem K^+ ion showed a constant K^+ content (14 $mg.g^{-1}$ DW) either in salt treated or not treated plants. The significant effects of rootstocks was apparently clear at root level where Maxifort F1 and R1 rootstocks showed a significant increasing as two times as control plants and Arnold rootstock in HAR/ARN plant at both salt levels (Figure 6).

The pattern of Cl^- accumulation in the three graft plants HAR/MAX, HAR/ARN, and HAR/R1 was different depending on the rootstock genotypes. Maxifort F1 rootstock induce similar Cl^- ion accumulation in the leaf and root (1.5 and 1.8 $mg.g^{-1}$ DW) and less amount in the stem (0.8 $mg.g^{-1}$ DW) in HAR/MAX plant at 200 mM NaCl (Figure 7). Similarly, R1 rootstock persuades the highest Cl^- ion concentration in the root (1.5 $mg.g^{-1}$ DW) followed by leaf (1.1 $mg.g^{-1}$ DW), while the Cl^- stem was limited (0.6 $mg.g^{-1}$ DW). On contrary, Arnold rootstock in HAR/ARN plant accumulated higher Cl^- ion in the stem (1.4 $mg.g^{-1}$ DW) in regard to its root and leaf (0.9 and 1.1 $mg.g^{-1}$ DW) (Figure 7).

4.1.2.3. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of Haruki scion against different rootstocks

The differential effects of different rootstocks in the three graft plants: HAR/MAX, HAR/ARN, and HAR/R1 were recorded in term of APX enzyme, as the Rootstock R1 in HAR/R1 plant showed a critical increasing in APX activity as +233% as the non-treated plant at high salt stress, while there was no significant differences in the activity of this enzyme with other two rootstocks Maxifort F1 and Arnold in HAR/MAX and HAR/ARN plants (Table 7 and Figure 9). Moreover, the three salt treated graft plants showed no significant

changes in the activities of CAT and GR enzymes in their fruit at both salt stresses and showed similar responses to that of control plants (Figure 9). However, SOD activity showed significant increasing by 98%, 70%, and 163% respectively in HAR/MAX, HAR/ARN, and HAR/R1 in relation to control plant at high salt stress (Figure 9).

Regarding the protein concentration, the three rootstocks Maxifort F1, Arnold, and R1 attempt to induce protein accumulation upon salt application, however, these efforts were not enough to make any significant changes in protein accumulation respecting the control plants (Table 7 and Figure 10). On the other hand, the different rootstocks affected strongly the fruit proline accumulation and showed medium significant increasing by 13 and 16 times as control plant at moderated and high salt stress, respectively (Table 7 and Figure 10). In related to lipid peroxidation, both rootstocks Maxifort F1 and Arnold in HAR/MAX and HAR/ARN plants did not show any significant changes in MDA level upon salinization regarding the control plant (Table 7 and Figure 10). However, R1 rootstock in HAR/R1 plant exhibited noteworthy increasing in MDA level by +141% respecting the control plant at 200 mM NaCl.

4.1.3. The graft combinations of Kamonium scion against different rootstocks

4.1.3.1. Vegetative growth and physiological responses of Kamonium scion against different rootstocks

The kamonium cultivar was grafted onto three different rootstocks: Maxifort F1, Arnold, and R1 and three graft combinations have been generated: KAM/MAX, KAM/ARN, and KAM/R1. Among the three graft mentioned combinations, KAM/R1 plants exhibited the highest shoot DW reduction as (-62% and -84%) at both salt stresses in relation to control plant, while this reduction was less in KAM/MAX plant (-48% and -75%) and KAM/ARN (-56% and -78%), respectively at mild and high salt levels (Table 1). Upon salt exposure, the three graft plant responses in term of root dry weight differed depending on the rootstock genotypes. Arnold rootstock in KAM/ARN showed an increasing in root DW as +11% as the control plant, while Maxifort F1 and R1 rootstocks exhibited reduction by (-5% and -43%) at mild salt comparing their control plants (Table 1). The fruit yield reduced significantly in the three graft plants when salt was added to the root medium. However, KAM/ARN plant showed less fruit FW reduction as -62% as the control plant, while KAM/MAX and KAM/R1 showed similar higher reduction as -83% as the control plant at mild salt stress (Figure 1). Nonetheless, the fruit yield reduction was so high at 200 mM NaCl for all graft plants and averaged 96% regarding the control plants. Concerning the water status, water and osmotic

potential decreased significantly upon both salt stresses without specific performance of any used rootstock genotypes (Figure 2). The rootstock R1 in KAM/R1 plant has higher ability to adjust osmotically than Maxifort F1 and Arnold rootstocks in KAM/MAX and KAM/ARN (Figure 2). On the topic of the parameters of photosynthesis apparatus, the highest *E* value redaction was recorded in KAM/ARN plant as -82% that associated with high preserve value of WUE (16.51 mM CO_2 mM^{-1} H_2O), while this reduction was (-56% and -75%) in KAM/MAX and KAM/R1 respectively at mild salt in relation to control plants (Table 3). Similarly, the rootstock Maxifort F1 in high salt treated KAM/MAX plant illustrated the lower reduction in *E* value -60% in relation to control plant, while this reduction accounted for (-78% and -71%) in KAM/ARN and KAM/R1 plants (Table 3).

4.1.3.2. The pattern of ion accumulations of Kamonium scion against different rootstocks

The contribution of the root system into ion accumulation responses has been under investigation by grafting Kamonium scion genotype onto three rootstock genotypes (Maxifort F1, Arnold, and R1). The three rootstocks showed similar capacity to take up the sodium ion from the root medium (106 $mg.g^{-1}$ DW) (Figure 3). Regarding the aerial part, the stem Na^+ of KAM/R1 plant showed two times more Na^+ ion content than KAM/MAX and KAM/ARN plants; in addition, the leaf of the same plant accumulated more Na^+ ion by 60% and 25%, respectively than KAM/MAX and KAM/ARN at 200 mM NaCl (Figure 3). However, these rootstocks exhibited different ability to transport the saline ion into the shoot, where the graft combination of KAM/MAX and KAM/ARN plants showed higher accumulation rate of sodium ion in their roots by +56% and +27%, respectively than the leaf, whereas KAM/R1 plant showed similar accumulation of Na ion in its root and leaf (102 and 108 $mg.g^{-1}$ DW) at 200 mM NaCl (Figure 3). Like Na^+ distribution, root Na^+/K^+ ratio showed no different effects of any used rootstock genotypes as they accumulated similar value of Na^+/K^+ ratio that accounted for 10 (Figure 4). Similarly, leaf Na^+/K^+ ratio of the three plants showed no clear effect of the rootstocks although KAM/R1 plant showed a slight increased value by 1.4 time of other plants KAM/MAX and KAM/ARN (18 versus 12) (Figure 4). However, stem Na^+/K^+ ratio in R1 rootstock in KAM/R1 illustrated higher value by 1.9 and 1.6 times respectively in related to KAM/MAX and KAM/ARN plants (Figure 4).

Like Ca^{2+} concentration in Haruki and Nerina F1 scions, the Kamonium scion presented the highest Ca^{2+} content in leaf (21 $mg.g^{-1}$ DW) and the lowest value in the stem (3 $mg.g^{-1}$ DW) as an average of all graft plants (KAM/MAX, KAM/ARN, and KAM/R1) (Figure 5).

Nevertheless, there was no effect either of salt or rootstocks on leaf and stem accumulation of all plants, while these effects appeared with root Ca^{2+} accumulation of maxifort F1 rootstock in KAM/MAX plant, where it accumulated higher Ca^{2+} by (+72% and +27%) than KAM/ARN and KAM/R1, respectively at 200 mM NaCl (Figure 5).

On the subject of K^+ content, the three graft plants KAM/MAX, KAM/ARN, and KAM/R1 showed similar partitioning of this ion in the stems and roots (13 versus 11 $mg.g^{-1}$ DW, respectively, as an average), while the leaf presented the lowest amount (6 $mg.g^{-1}$ DW) at 200 mM NaCl (Figure 6). However, the root k^+ ion increased essentially when Arnold and R1 genotypes employed as rootstocks in KAM/ARN and KAM/R1 plants and showed increasing by two times as the control plants at both salt levels, while there was no effect of different rootstocks on the leaf and stem K^+ accumulation (Figure 6).

Regarding Cl^- content, the three rootstocks accumulated similar root Cl^- content that averaged 1.6 $mg.g^{-1}$ DW (Figure 7). Also, there were no significant differences in Cl^- accumulation at leaf level between the three rootstocks that presented averagely 1.4 $mg.g^{-1}$ DW. However, the Cl^- stem of Maxifort F1 rootstock in KAM/MAX plant showed higher stem Cl^- accumulation about 1.5-fold than the other grafted plants KAM/ARN and KAM/R1 plants.

4.1.3.3. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of Kamonium scion against different rootstocks

There was no effect either of rootstock genotypes or salt stress on the activities of APX and CAT enzymes as the three graft plants of Kamonium scion: KAM/MAX, KAM/ARN, and KAM/R1 showed similar responses of APX and CAT activities under high salt stress as that of their control plants (Table 7 and Figure 9). However, the positive effect of R1 rootstock was present in KAM/R1 plant that showed increasing in the activity of GR by +50% regarding the control plant at both salt levels, while both rootstocks Maxifort F1 and Arnold showed no significant increasing in GR enzyme (Figure 9). Furthermore, the three rootstocks prompted the increasing in SOD activity at high salt stress; however, the significant increasing was only recorded by Maxifort F1 rootstock in KAM/MAX plant that account for +56% as control plant (Figure 9).

Regarding the organic solute accumulation, R1 rootstock in KAM/R1 plant showed substantial increasing in protein concentration by +296% as control plant at high salt stress,

while the other rootstocks Maxifort and Arnold did not differ in protein accumulation from their control plants in KAM/MAX and KAM/ARN graft combinations (Figure 10). On contrary, the three mentioned rootstocks provoked a significant increasing in proline content at moderated salt stress by 15 times as control plant, while KAM/MAX and KAM/ARN plants showed continued increasing in proline level at 200 mM NaCl and showed increasing by 29 times as control plants (Figure 10). Likewise, the three rootstocks induced MDA accumulation particularly at high salt stress that accounted averagely for 16 μM g^{-1}FW in three plants (Figure 10).

5. Discussion

5.1. SALT TOLERANT INDUCED MECHANISMS BY DIFFERENT ROOTSTOCKS AGAINST ONE SCION GENOTYPE

Mineral nutrients are essential for plant growth and they are virtually involved in all metabolic and cellular function like energy metabolism, primary and secondary metabolism, cell protection, gene regulation, signal transduction and plant reproduction (Hansch and Mendel 2009). However, plants store the mineral in different organs like root, stem, and leaf and these organs have a considerable influence on mineral uptake and translocation (Flowers and Colmer 2008). The role of rootstock in regulating leaf Na^+ and Cl^- accumulation under salinized condition is of a great interest for preventing the toxic influence which is the main effect induced by salinity in long term (Santa-Cruz et al. 2002). It is supposed that useful rootstocks, termed as salt excluder, should be able to reduce the uptake and transport rates of saline ions to the shoot. This will slow or prevent the accumulation of toxic level of sodium and chloride in the leaves. Salt ion exclusion by roots may result in higher salt resistance of the plants due to lower ionic toxicity, contributing to a metabolic stability and protection of the leaf tissues (Martinez-Rodriguez et al. 2008; Paranychianakis and Angelakis 2008).

The tomato plant is moderately tolerant to salinity stress (Maas 1990), however, the long-term damage in cultivated tomato that are caused by salinity was essentially related to the excessive accumulation of Na^+ and Cl^- in leaves even when the plant are grown in moderated salt level (50 mM NaCl) (Cuartero and Fernandez-Muňoz 1999). The salt tolerance mechanisms differ essentially among plant species (Munns and Tester 2008). Grafted plants develop numerous physiological mechanisms to cope with salt stress. These strategies include (i) salt exclusion in the shoot and retention of salt ions in the root, (ii) better maintenance of potassium homeostasis, (iii) compartmentation of salt ions in the vacuole, accumulation of compatible solutes and osmolytes in the cytosol (Colla et al. 2010). The mechanism associated with the exclusion of Na^+ and Cl^- ions from leaves involve both the uptake selectivity in roots and resistance to transferring these ions to the shoot (Maathuis and Amtmann 1999). These mechanisms minimize ionic toxicity in leaf tissues and consider essential for developing resistance to salt stress in some rootstock of citrus (García-Sánchez et al. 2002), rose (Wahome et al. 2001), grape (Paranychianakis and Angelakis 2008) and tomato (Estaň et al. 2005). Furthermore, in grafted tomato plant, tomato rootstocks showed different mechanisms to cope with salt stress in relation to their ability to regulate the transport of Na^+ and Cl^- into the shoot. However, Estaň et al. (2005) observed that the salt

tolerant mechanism of cv. Pera was depended on the salt level and exposure time, as its includer character was observed at either low salinity or short exposure times; while when the salinity and time exposure increased, cv. Pera was able to regulate the saline ion transport to the shoot.

5.1.1. Salt inclusion mechanism and leaf ion compartmentation of Nerina F1 scion

In the presented experiment, plants of commercial tomato cultivars (Haruki, Kamonium, and Nerina F1) were grafted onto several different rootstocks (Maxifort F1, Arnold, and R1) to assess some physiological and biochemical changes that have been induced by different rootstock genotypes under mild and high salt stress. Generally in this study, the concentration of Na in the entire plant was essentially affected by the imposed salt regardless the rootstock genotypes and showed a significant increasing in this ion particularly at 200 mM NaCl (Figure 3). There was a quantitative variation in Na distribution throughout the plant organs in which the most absorbed Na ion was withhold in roots and leaf of all graft combinations (102 and 95 $mg.g^{-1}$ DW as an average) while the lowest content was allocated in stems (63 $mg.g^{-1}$ DW as an average) at 200 mM NaCl (Figure 3). This result may devote the idea that the plant was suffering from ionic stress.

When Nerina F1 was employed as a scion, the three rootstocks: Maxifort F1, Arnold, and R1 showed different capacity to take up and transport the Na^+ ion into shoot. The rootstock Maxifort F1in NER/MAX plant was unable to influence the distribution of Na^+ between the root and its aerial part and induce effectively higher Na flux toward the leaves and stem by (+33% and +38%), respectively than the root at 200 NaCl (Figure 3). In contrast, R1 rootstock in NER/R1 plant was able to compartment a nearly constant Na ion in the leaves and root (104 versus 112 $mg.g^{-1}$ DW), while Arnold rootstock in NER/ARN plant showed lower Na content in its leaves (77 $mg.g^{-1}$ DW) regarding the root (97 $mg.g^{-1}$ DW) at 200 NaCl (Figure 3). Interestingly, the comparison among these three rootstock genotypes revealed that Maxifort F1 showed continuing increasing of Na^+ and achieving significantly higher Na leaf by 1.9-fold and 1.5-fold than NER/ARN and NER/R1 plants, and higher Na^+ stem by 3-fold and 2.3-fold than NER/ARN and NER/R1 at 200 NaCl (Figure 3). This result confirms that the Maxifort F1 rootstock lost its ability to limit the Na^+ influx into aerial part and has a high salt includer character. This salt inclusion mechanism could be supported by mechanism of Ca^{2+} uptake selectivity in roots as well as Ca^{2+} transportation selectivity of this ion, where only NER/MAX graft plant accumulated significantly more root Ca^{2+} by +54%

than NER/ARN and NER/R1, and more stem Ca^{2+} by +97% and +235% than the other graft plants (Figure 5). The significant increasing in Ca^{2+} concentration of stem suggested that the ability of this rootstock to regulate the mobility of this ion to upper parts of plants and this could possibly be one of the factors involved in conferring salt tolerance (Sivritepe et al. 2005). However, it is worth mentioned that this inclusion mechanism was effectively operated under high salt stress.

Regarding, R1 rootstock in NER/R1, it has the ability of compartmentation excessive Na^+ in the leaves, where it presented similar content of Na^+ ion in its leaves as those in its root, suggesting that this rootstock utilize lower salt inclusion mechanism than Maxifort F1 rootstock to cope with stressed condition. The main long term damaged caused by salinity in the cultivated tomato is related to the excessive accumulation of Na^+ in leaves which provokes a wide variety of physiological and biochemical alteration that inhibit plant growth and production (Maggio et al. 2001; Munns 2005). The compartmentation of excessive Na^+ in the vacuole by the tonoplast Na^+/H^+ exchanger is considered to be one of the key mechanisms to NaCl tolerance (Zhang and Blumwald 2001), in turn, the increased vacuolar Na^+ concentrations would require a coordinated increase in the osmotic pressure of the cytoplasm, which can be achieved by an increase in the concentration of K^+ and compatible solutes (Munns and Tester 2008). Consequently, this might interpret, to some extent, the higher K^+ content in root of NER/R1 plant as 1.5-fold as NER/MAX plant at 200 mM NaCl (Figure 6). On contrary, Arnold rootstock in NER/ARN plant regulate more efficiently the transportation of Na ion into leaf and showed less Na^+ accumulation by 20% comparing with Na root. However, the Na^+ inclusion strategy of salt tolerant that used by Maxifort F1 and R1 rootstocks must be accompanied by an adequate compartementation of these ions between and within the cell that would avoid the plant to achieve the toxic level of saline ions in the cytoplasm and or the apoplast (Yeo 1983). Taken together, these results confirm that the rootstock genotypes play a significant role in ions entry at root level and in ion translocation into leaf level. These results agree with those of other studies that reported the importance of rootstock capacity to reduce the Na^+ and Cl^- accumulation in leaves of the grafted plants (Romero et al. 1997; Fernandez-Garcia et al. 2002; Estaň et al. 2005). Also, our results were consistent with other reports that mentioned the lower accumulation of Na^+ and Cl^- in the shoot of grafted plantlets of citrus (García-Sánchez et al. 2002), grape (Paranychianakis and Angelakis 2008) and tomato (Estaň et al. 2005) under salinity is also associated with the

exclusion capacity of the rootstock, which is attributed to mechanisms of selective transport and/or retention in the roots.

Moreover, maintaining a low ratio of Na^+/K^+ is considered the best overall indicator of plant ability to select and use K^+ under Na+ salinity (Perez-Alfocea ei al. 1993) and, consequently, reflects, the salt tolerance in plant (Santa-Cruz et al. 2002). It is known that the value of one of Na^+/K^+ ratio is considers critical for maintaining metabolic activity (Munns and Rawson 1999), however, NER/MAX plant was affected strongly by salinity whereas the leaf Na^+/K^+ ratio was 35-fold higher than the mentioned above value (Figure 4). Moreover, only Maxifort F1 rootstock in NER/MAX plant showed essentially higher leaf Na^+/K^+ ratio as 2.1 and 1.3 times as Arnold and R1 rootstocks in NER/ARN and NER/R1 plants (Figure 4); similarly, stem Na^+/K^+ ratio of NER/MAX plant showed also higher Na^+/K^+ ratio by 3 and 2 times as the other two plants. Moreover, Maxifort F1 rootstock showed higher Na^+/K^+ ratio at the root and leaf level of NER/MAX plant (Figure 4) that associated with lower K^+ content (Figure 6). This reduction in K^+ content could due to the similar physiochemical structures of Na^+ and K^+ ions which mean that Na at transport sites for K^+ will enter predominantly and might cause K^+ deficiency (Maathuis and Amtmann 1999).

K^+ ion plays an essential role in the growth of all plant. However, Moreover, high K^+ concentration is a key factor in salinity tolerance as NaCl negatively affects K^+ nutrition (Aleman et al. 2009). The root data of K^+ concentration showed that only the root of Arnold and R1 rootstocks increased significantly upon high salt level, while K^+ content in the root of Maxifort F1 showed similar value as non-treated plant at 200 mM NaCl (Figure 6). However, the leaf K^+ of the three graft plants reduced upon 200 mM NaCl. The reduction, particularly, in NER/MAX plant showed that the highest leaf Na content could be caused by the inhibition of K^+ influx into the cell by Na^+ or the stimulation of K^+ efflux from the cell by Na^+ (Figure) (Britto et al. 2010).

Cl^- ion is considered as inorganic osmotic anion that plays an important role in osmotic adjustment. However, the excessive accumulation of Cl^- results in ion toxicity and growth inhibition (Ashraf and Harris 2004). It is not easy to determine whether the toxic effects that induced by salt stress are due to Na^+ or Cl^- or the contribution of both. Nonetheless, Cl^- has been shown to be more toxic than Na^+ to citrus seedling (Fernandez-Ballester et al. 2003; Moya et al. 2003). However, for many plants, Na^+ is considered the primary cause of ion-

specific damage (Tester and Davenport 2003). Some authors observed that there were some rootstocks able to exclude more Cl^- than Na^+ or vice versa (Romero et al. 1997). According to our data, the Cl^- accumulation rate (Figure 7) was too much less than Na^+ ion (Figure 3) in the different plant organs, this indicate that the Na^+ uptake and transport to the leaves was faster than that of Cl^-, and might indicate that the toxic effect, even at salt long term, was due to mainly Na^+ ion. However, the fact that Maxifort F1 rootstock in NER/MAX plant induces significantly higher stem and leaf Cl^- content than other rootstocks (Figure 7), it seems that this rootstock lost the ability to regulate leaf Cl^- transportation and trigger the Cl^- salt inclusion mechanism altogether with Na^+ ion. However, contrast results were recorded with grafted fruit trees, where Chaplin and Westwood. (1980) stated that there was no effect of the rootstock on leaf mineral content and more influence of the scion was observed. While, Brown et al. (1994) reported that the change in rootstock had a significant influence on the leaf content of pistachio grafted plant. However, Tagliavani et al. (1993) suggested that the vigour of both scion and rootstock had a parallel role in the uptake and translocation of nutrient in grafted fruit trees. Kawaguchi et al. (2008) mentioned that the rootstock species was the main factor affecting the absorption and translocation of P in the graft combinations of *Solanaceous* plants. Ruiz et al. (1997) showed significant decreases of Na^+ and K^+ minerals in grafted plants, indicating that the Na^+ and K^+ concentrations were affected by the use of certain rootstocks.

5.1.1.1. Vegetative growth response, water status and gas exchange parameters of Nerina F1scion

The shoot and root genotype can be crucial for the plant development and the efficiency of rootstock-scion connection is fundamental for optimal growth. The harmful effects induced by salinity involve the excessive accumulation of salt in the leaves. It is often reported that the positive rootstock effects on shoot growth and fruit yield are related to its ability to reduce the transport of saline ions over time (Estaň et al. 2005; Martinez-Rodriguez et al. 2008; Huang et al. 2009). The identification of rootstocks on the basis of shoot growth and physiological traits would be a selective aid of useful rootstock (Martinez-Rodriguez et al. 2008). Munns and Tester. (2008) indicated that saline condition reduces rates of photosynthesis and leaf expansion, thus leading to the shoot growth reduction. In this study, we have investigated the effect of different rootstock genotypes in term of plant growth response, water status and photosynthesis apparatus parameters. This study demonstrated that the differential growth response of the three graft combinations of Nerina F1 scion (NER/MAX, NER/ARN, and NER/R1) was related to different capacity of the used

rootstocks (Maxifort F1, Arnold, and R1 rootstocks) to extrude the ions and regulate the ion transport to the shoot. Arnold rootstock in NER/ARN plant showed high efficiency to regulate the Na entry at root level and transported less Na ion into the stem by (-51% and –44%) in related to Maxifort F1 and R1 rootstocks in NER/MAX and NER/R1 plants at moderated salt stress (Figure 3). Thus, this plant showed less reduction in shoot FW and DW (-47% and -21%) in related to control plant at 100 mM NaCl (Table 1). On contrary, both Maxifort F1 and R1 rootstocks accumulated higher Na ion in their shoot and, accordingly, showed higher reduction in shoot FW and DW as (-65% and -47%) in NER/MAX plant and (-65% and -53%) in NER/R1 plant. Nonetheless, the shoot growth showed more depressing as long as higher salt content was in the root medium depending on rootstock genotypes. The reduction of shoot FW and DW accounted for (-77% and -65%) in NER/ARN plant and it was higher in NER/R1 plants (-84% and -74%) (Table 1). However, Maxifort F1 rootstock induce the highest reduction in these parameter in NER/MAX plant (-96% and -84%) in related to control plant at 200 mM NaCl (Table 1). Indeed, the slower growth is also adaptive mechanism of plant grow under stressed condition, in which some plants act responsively toward stress condition and tend to stop growing while others keep running the risk of dying by continuing to grow even in sever stressed condition (Fernández-García et al. 2004). Pervious report linked shoot growth variation under salt stressed condition in tomato grafted plant to the shoot genotype (Santa-Cruz et al. 2002), while Martínez-Rodríguez et al. (2008) mentioned that the salt tolerance of the shoot depends on the root system, independently of the genotype used as a scion, although the positive effect of rootstock may show to a different degree depending on the higher or lower exclusion ability of the shoot genotype. These results could explain the contradictory result obtained in relation to the role of the rootstock in the salt tolerance of the shoot (Abd-Alla et al. 1998).

Plants store minerals and other nutrition in different organs such as roots, stems, leaves, and/or fruits. These organs have a considerable influence on the uptake and translocation of mineral nutrients in plant and this play an essential role in physiological process such as growth, signal transduction and development (Wang et al. 2006; Flowers and Colmer 2008). Root characteristics: root length, density, number or root hair and its surface area play an active role in ions and water uptake (Krasilnikoff et al. 2003). Colla et al. (2010) indicated that root system is considered as the main reason for alleviation the deleterious effect of salt stress on shoot growth. Also, Oztekin and Tuzel. (2011) demonstrated that root growth in tomato appears to be less effected by salt than shoot growth. Accordingly, in this trial, the

positive effect of rootstock genotypes on root dry weight was strong at both salt stress, where the rootstock ARN showed a significant increasing in root DW at moderated salt stress as +62% as control plant, while the highest root DW reduction was presented by Maxifort F1 (-41%) (Table 1). However, the role of rootstocks were still presents even under 200 mM NaCl, where Maxifort F1 exhibited the highest root DW reduction -76% respecting the control plant, while both Arnold and R1 rootstocks in NER/ARN and NER/R1 plants displayed lower similar reduction -31%. The increase in the root dry weight support the hypothesis that the tomato allocated more dry weight to root to maximize capacities for nutrient and water absorption (Debouba et al. 2006). This result was homogenized with ion data where Arnold rootstock in NER/ARN plant showed lower Na^+ ion accumulation at all plant organ, while Maxifort F1 in NER/MAX revealed the highest (Figure 3). However, our result was not in consistent with other author (He et al. 2009) who mentioned that tomato root dry mass declined under salt stress (100 and 150 mM NaCl) in comparison with non-saline conditions but the decrease was similar in all grafted plants. While, Colla et al. (2006a) and Yetisir and Uygur. (2010) documented that the root dry weight reduction was significantly lower in grafted than non-grafted watermelon plant under salinized condition. Also, Zhu et al. (2008a, b) and Huang et al. (2009a) stated that grafted cucumber showed less root dry weight reduction in related to non-grafted plant in presence of NaCl. Accordingly, the lowest shoot FW and DW and root DW of NER/MAX plant (Table 1), the most entire Na^+, Na^+/K and Cl^- ions accumulation (Figure 3, 4 and 7). Moreover, many studies have shown that high concentrations of Na^+ and Cl^- in the soil solution may depress nutrient–ion activities and produced extreme ratios of Na^+/K^+ in the plants, causing the plants to be susceptible to osmotic and specific ion injury as well as to nutritional disorders that result in reduced yield and quality (Grattan and Grieve 1999; Sivritepe et al. 2003). Taken together, these results confirm that the rootstock effect on the shoot growth is based on the rootstock ability to reduce the transport of saline ion into the shoot over the time (Martinez-Rodriguez et al. 2008).

Salinity affects plant growth by imposing both osmotic stress and ionic stress. The osmotic stress takes place when the stress level or the time exposure are not enough, while the toxicity provoke by the excessive accumulation of ions in the leaves in presence of high salt concentration or elongation of salinized period. Munns. (2002) concluded that the plant growth in salinized condition was predominantly prevented by osmotic stress in species having a low salt uptake rate. It is known the improvement in fruit yield is a consequence of reduced ion transport into the shoot by the rootstock. In this regard, there was no appreciated

performance of any used rootstocks where there was no variation among them in term of fruit fresh weight and the ANOVA analysis showed neither rootstock nor salt × rootstock have significant effect on fruit yield (Table 2). Even at moderated salt stress, the three graft combination presented high reduction in FW fruit by -74%, -82% and -58%, respectively in NER/MAX, NER/ARN, and NER/R1 (Figure 1). Nonetheless, this reduction was higher under high salt stress and accounted for -96% for all used rootstocks. The high fruit FW reduction even at mild salt might indicate that the fruit yield redaction was related to osmotic stress, while the continued reduction in this parameter at high salt content might correlated to ionic stress as cause of excessive accumulation of Na^+ and Cl^- ions on long-term (Tester and Davenport 2003; Estaň et al. 2005). Accordingly, these data demonstrated that in spite of the three rootstocks showed different salt morphological and physiological adaptations to cope with salt condition, they actually lead to the same preservation of fruit yield and showed similar reduction in fruit FW by 96% at high salt stress (Figure 1). Thus, the three rootstocks failed to counteract the deleterious effect of salinity stress in term of yield. The highest deficient improvement of plant growth and productivity in NER/MAX plant regarding the Maxifort F1 rootstock can be ascribed to the ineffectiveness of reducing long-distance transport of Na^+ or could be related to excessive accumulation of Na^+ ion in the shoot since although the availability of Na^+ as a cheap osmotic adjustment is generally beneficial (Figure 2) (Maathuis and Amtmann 1999), excessive accumulation of Na^+ resulted in toxicity and growth inhibition (Saqib et al, 2005; Tester and davenport 2003). However, our results corroborated previous data obtained by (Huang et al. 2009) who stated that rootstock had no significant effect on mean fruit weight of tomato but had a significant effect on fruit number. Also, our result was in agreement with (Fan et al. 2011) who mentioned that there was no significant difference in tomato yield and all growth associated characteristics between the tolerant (Edkawi) and sensitive (zs-5) rootstocks. However, other contradictory reports showed that the rootstock can also increase salinity tolerance in tomato that evaluated in term of fruit yield (Estaň et al. 2005; Martinez-Rodriguez et al. 2008). Colla et al. (2006a, b) reported that the use of rootstock raises fruit yield of melon and watermelon due to both the higher mean fruit weight and fruit number under saline condition. While Flores et al. (2010) stated that depending on the scion–rootstock combination, either a decrease or an increase in fruit quality seemed to occur.

Water status response

A wide range of morphological and physiological characteristics are affected by rootstocks, scions and their interactions (Santa-Cruz et al. 2002; Fernández-García et al. 2002, 2004;

Estaň et al. 2005; Colla et al. 2006b; Yang et al. 2006; Martinez-Rodriguez et al. 2008; Huang et al. 2009b; He et al. 2009). Some of these characteristics have the potential for improvement of plant water relations, growth and plant development during salt stress. The mechanisms of resistance against salinity in grafted plants display a great complexity, such complexity may be associated with rootstock structure, scion genotype or specific interaction between the scion genotypes and rootstock characters.

Tomato plants have some tolerance avoidance mechanisms such as decreases in water and osmotic potential to maintain their water status during the soil water deficit (Monneveux and Belhassen 1996). Osmotic adjustment helps the plant cells to withstand salt stress and water deficit by maintaining sufficient turgor for growth. It involves the transport, accumulation, and compartmentation of inorganic ions and organic solutes (Mustard and Renault 2004). This processing allows increasing the water potential gradient between the soil and plant and improving the water absorption under soil water deficit (De Herralde 2000). In this research, the three graft plants NER/MAX, NER/ARN, and NER/R1 showed medium significant reduction in water potential as 1.9 times in related to control plants upon 100 mM NaCl (Figure 2). The Ψ_w reduction was associated with Na^+ ion accumulation in leaves (67 $mg.g^{-1}$ DW, as an average) (Figure 3). However, under higher salt stress (200 mM NaCl), the plant showed sharper significant diminishing in water potential in which the medium reduction among the three plants was higher by 1.6 times in respect to moderated salt treated plants, and this reduction was associated with higher averaged of leaf Na^+ ion accumulation by 1.6 times in related to moderated salt plants. Thus, this result confirm that the main effect induced by salinity at 100 mM NaCl was osmotic effect; while at higher salt exposure, the plant accumulate more excessive Na^+ ion and suffering from ionic toxicity. However, one explanation of this extreme diminishing in water and osmotic potential might be indicative of hardening process and may present fundamental mechanism of adaptation to salinity (Richardson and McCree 1985). These results were inconsistent with other authors who mentioned that the tomato plant react predominantly to the osmotic effect induced by salinity and not to ion toxicity at low or moderated salt levels (Neumann 1997), while other researcher documented that the response could be differing depending on salt stress level and salt time exposure (Perez-Alfocea et al. 1993).

Indeed, it is interesting to emphasize that the induced high inclusion tolerance strategy of rootstock Maxifort F1 in NER/MAX plant upon high external salt stress has been associated with highest accumulation rate of Na^+ and Cl^- ions in its leaves and stem (Figure 3 and 7) (Santa-Cruz et al. 2001), and accordingly the plant maintain the lowest significant osmotic potential (-3.87 MPa) and the highest osmotic adjustment values (1.60 MPa) at 200 mM NaCl (Figure 2). This result suggested that the plant has used these high concentrations of inorganic solutes from the substrate in order to adjust osmotically, which is considered as an osmotically adaptive strategy to cope with salt stress since the energetic cost of inorganic solute accumulation is less expensive process than the use of organic solutes (Yeo 1983). However, the reduction of water and osmotic potential in NER/MAX plant was also associated with significant reduction in stomatal conductance (data not shown), while the relative water content has not been affected and showed similar value as non-treated plant (data not shown). This might indicate that the osmotic adjustment improved the water status of the plant under salinity as the dehydration did not appear under salt stress since. This result was in agreement with the finding of Weng. (2000) who stated that the leaves of grafted tomato plants under water deficit maintained higher leaf water potential in spite of higher water loss through transportation, indicating a greater ability to promote water uptake. While Fernández-García. (2004) reported that grafted tomato plant failed to adjust osmotically as the leaf water potential was not altered.

Gas exchange parameters response

He et al. (2009) revealed that the alleviation effects of the tolerant rootstock may be related to an improvement of the photosynthetic process. Other authors mentioned that not only the rootstock but also the scion affect the stomatal performance, producing a higher CO_2 assimilation rate and less stomatal resistance than in non-grafted or self-grafted plant (Yetisir et al. 2007; Rouphael et al. 2008; He et al. 2009; Zheng et al. 2009). Also, Ferreira-Silva et al. (2009) suggested that the type of cashew rootstock plays an essential role in transpiration intensity either under control or mild and high salt condition. In this work, under control condition, the three graft combinations showed high transpiration rate (Table 3) that associated with high values of stomatal conductance (data not shown) which are considered the favourable conditions to photosynthesis and net assimilation rate (Ferreira-Silva et al. 2010; Souza et al. 2005). Nevertheless, the differential response of different rootstocks was clear in term of net assimilation rate in the leaves of high salinity treated plants, where Arnold and R1 rootstocks in graft plants NER/ARN and NER/R1 showed less reduction in *A* value by 66% in respect to their control plant, while the Maxifort F1 rootstock in NER/MAX

plant showed the highest significant reduction in net assimilation rate (-91%) at 200 mM NaCl (Table 3). However, this reduction in net assimilation rate in NER/MAX plant was associated with significant reduction in stomatal conductance and intercellular CO_2 (data not shown). This reduction of stomatal conductance that accompanying with reduction in *Ci* concentration implying that stomatal limitation of CO_2 diffusion could be more important in *A* CO_2 limitation than non-stomatal factors upon salt treatment (He et al. 2009; Zgallaï et al. 2006). Also, this tendency of redaction of (G_s) under salt stressed NER/MAX plant could be a mechanism of water conservation, limiting the loss of water in order to maintain leaf turgor and osmotic adjustment (Figure 2) (De Herralde et al. 1998). On other hand, the highest *A* value in NER/ARN and NER/R1 plant at high salt stress could interpret according to Meloni et al. (2003) and Munns. (2002) who stated that the salt excess in the root medium decreases the osmotic potential of soil solution and restrict water uptake by plant root which result in significant increasing in stomatal resistance and net assimilation rate. Furthermore, the lower reduction in *A* value in NER/ARN and NER/R1 plant could be because these plants presented less root biomass reduction under stress. It is commonly accepted that an interdependent relationship exists between root and shoot: i.e., active shoots that ensure a sufficient supply of carbohydrates to roots can stimulate and maintain active root functions; the activation of root functions can, in turn, improve shoot characteristics by supplying a sufficient amount of nutrients, water, thus ensuring increased biomass productivity (Yang et al. 2004).

Although the both mentioned above rootstocks Arnlod and R1 displayed the least reduction in root DW at 200 mM NaCl (Table 1), the yield reduction was high because the water absorption by these roots was suppressed, as the presence of salt, and accordingly scion growth decreased (Oda et al. 2005).

5.1.1.2. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of Nerina F1scion

It was documented that stress tolerance of plant is associated with their ability to remove ROS (Senaratna et al. 1985). Activities of many antioxidant enzymes are enhanced to scavenge ROS when plants are subject to osmotic stress (Ruiz-Lozano. 2003). Grafted cucumbers have a higher relative expression of SOD and CAT mRNA and higher activities of SOD and CAT thereby a higher salt tolerance under NaCl stress than self-rooted plants (Gao et al. 2008). The increased salt tolerance of cucumber plants grafted onto *C.ficifolia* was linked to the increased SOD and POD activities under saline condition (Huang et al. 2010). Higher antioxidant capacities in grafted plants under salt stress have been observed in fruit

bearing vegetable. The activities of SOD, POD, and CAT in the leaves of grafted watermelon seedling were significantly higher than those of self-rooted seedling under NaCl stress (Zhu et al. 2008b). The alleviation of oxidative damage in grafted tomato plants under NaCl initiated from the increase in activities of CAT and enzyme involved in ascorbate-glutathione cycle such as APX and GR (He et al. 2009). Studies have shown that the alleviation of oxidative damage and increased resistance to salinity is correlated to the efficient of antoxidative defence system (Bor et al. 2003; Alscher et al. 2002). Rout and Shaw. (2001) mentioned that salt tolerance capacity of salt tolerant species is closely related with the maintenance of antioxidant enzymes for the effective removal of ROS. Azevedo Neto et al. (2006) reported that the complex defence antioxidative system included low-molecular mass antioxidant as well as antioxidative enzymes such as SOD, CAT, APX and GR.

The plant need to maintain internal water potential below that of soil in order to maintain turgor and water uptake and growth. This requires an increase in osmotica either through uptake of inorganic ions or synthesis of metabolically compatible solutes such as proline and protein. The organic solutes are often referred to as compatible osmolytes and they are not harmful to enzymes and other cellular structure even at high concentration (Zhu 2001). The high concentrations of compatible osmolytes accumulate in the cytosol and organelles to balance the osmotic pressure of the ions in the vacuole (Munns and Tester 2008). In addition, Zhu. (2001) and Huang et al. (2009c) suggested that the increased amount of compatible osmolytes may protect plants by scavenging oxygen free radicals caused by salt stress and alleviates free-radical damage induced by salt stress (Wang et al. 2007). In many plant species, the increase in proline level under stressed condition is considered as a typical mechanism of the biochemical adaptation, and it has been suggested that proline may function as osmoticum, a sink of energy and reducing power, a ROS-scavenger and a compatible solute that protect enzyme (Sánchez E et al. 2002).

Although a great deal of work has been done to improve plant salt tolerance, only few studies have examined the effect of grafting with different scion genotype onto one rootstock and vice versa one scion genotype against different rootstock. In the present study, the effect of different rootstock genotypes in term of enhancing the activity of fruit enzymatic antoxidative system was studied. Our result showed that different graft combinations that have been generated from grafting one scion genotype onto different rootstock characters differ in their abilities to activate the enzymatic antoxidative defence system in the fruit depending on the

rootstock genotypes. Under control condition, the APX activity in the fruit was different depending on the used genotypes, where NER/ARN plant showed less APX activity by -92% and -86% respectively than NER/MAX and NER/R1 plants (Figure 9). This result implies that the APX fruit activity could be affected by rootstock even under control condition. This result was in agreement with Zhen et al. (2010) who stated that SOD and POD activities in the cucumber leaves were different depending on rootstock genotypes even under zero stressed condition. Also, Zhu et al. (2008a) mentioned that SOD and APX activities in the cucumber leaves were different according to rootstock and irrespectively of the saline condition. Under high salt stress, the scion Nerina F1 in presence of Arnold rootstock was able to increase significantly the activities of APX, CAT, and GR enzymes as 17, 3, and 2-fold as the control plant (Figure 9). On contrary, when Nerina F1 scion was grafted onto R1 rootstock, there was no activation of any enzymes even at high salt stress (Figure 9). However, this scion showed activation of CAT enzyme and deactivation of GR enzyme when Maxifort F1 was used as a rootstock at high salt stress (Figure 9). As we have seen before that there were significant differential responses of rootstock genotypes in term of ions accumulation and, accordingly, in plant growth and productivity. At moderated salt stress, Arnold rootstock in NER/ARN plant showed higher efficiency to regulate the Na entry and transport less Na ion into the aerial part (Figure 3) that associated with less reduction in shoot FW and DW than Maxifort F1 and R1 rootstocks in related to control plant (Table 1). However, the increased salt tolerance of Arnold rootstock in NER/ARN plant, expressed as less shoot growth reduction, was found to be linked to the higher fruit enzymatic antioxidants activity, where this plant showed a significant increasing in the activity of APX, CAT, and GR enzymes under saline conditions (Figure 9). In the processing of removal H_2O_2 under NaCl stress, CAT, APX and GR play vital roles (Shi and Zhu 2008), as the alleviation of oxidative damage in grafted tomato plants originated from the increase in activities of CAT and the enzymes involved in the ascorbate-glutathione cycle (He et al. 2009). Thus, we could suggest that the NER/ARN plant had higher ability to remove ROS than other graft plants.

Grafted tomato plant of NER/MAX induced the highest proline accumulation as two times as Arnold and R1 rootstocks of NER/ARN and NER/R1 plants under salt stress (Figure 10) (Chen et al. 2005, 2006), suggesting that the increase in proline content in the fruit was influenced strongly by rootstock genotypes (Ferreira-Silva et al. 2009). However, the proline level increasing NER/MAX plant was associated also with higher Na^+ ion accumulation in the leaves (Figure 3), indicated that the enhanced salt tolerance is related to the change in

osmotic component (Figure 2) (Yang et al. 2006; Chen and Wang 2008; Huang et al. 2009a). Though compatible solutes have a higher energy requirement, grafted plants can still benefit from the higher accumulation of compatible solutes. The over accumulation of Na in the leaf tissue can cause premature leaf senescence or even death, while a higher soluble proline content can prevent to some extent the detrimental effect induced by salinity (Tester and Davenport 2003).

Although Maxifort F1 rootstock has the capacity to induce the highest proline accumulation in fruit of NER/MAX plant at 200 mM NaCl (Figure 10), the plant showed sharp reduction in shoot growth and fruit yield (Table 1and Figure 1). However, under saline condition for some species, proline is associated with stress resistance as it plays a metabolic function in osmotic adjustment and cell protection (Silva-Ortega et al. 2008); while for other plant species, the changes in content of this organic solute under salinity could be related to metabolic disturbances in the proline biosynthesis pathway than with cell protection or osmotic adjustment (Silveira et al. 2003). Additionally, the proline level reduction (Figure 10) was associated with high significant reduction in fruit GR activity (Figure 9). The higher reduction in GR activity may contribute to lower AsA content, indicating that the ascorbate-glutathione cycle did not function in a proper way and this plant lost its ability to detoxifying a great amount of H_2O_2 that reaches high toxicity level and thereby the oxidative damage occurred. With regard to R1 rootstock, it seems that R1 rootstock in NER/R1 plant was unable to activate any enzymes even at high salt stress, suggesting that this rootstock is a relatively salt sensitive (Figure 9). Zhen et al. (2010) stated that the higher antioxidant capacity of grafted cucumber be existent when the plants grafted onto relative salt tolerant rootstock. MDA is the direct production of lipid peroxidation and its content is often used as indicator of the extent of lipid peroxidation. However, although the tomato grafted plants of Nerina F1 scion was affected hardly by salt imposing into root medium, unexpectedly we could not link the salt stress injury with MDA level as the salt treated plants showed similar accumulation as the control plant irrespectively of the rootstock genotypes (Figure 10). To conclude, the result of this work showed that the scavenging system of fruit free radicals was non-effective and different rootstocks showed less power of protective mechanisms of antioxidant enzymes under salt stress condition. Accordingly, the three graft combinations were not able to sustain the plant performance and a huge redaction in shoot growth and fruit yield were registered upon saline condition.

To summarize, in this study we have investigated the effect of different rootstock genotypes in term of plant growth response, water status and photosynthesis apparatus parameters, and enhancing the activity of fruit enzymatic antoxidative system. The main finding of this study was the differential responses of graft combinations were related to different capacity of the used rootstocks to extrude the ions and regulate the ion transport to the shoot. The Maxifort F1 rootstock lost its ability to limit the Na^+ influx into aerial part and showed salt inclusion mechanism that associated with excessive accumulation of Na^+ in stem and leaf, while the R1 rootstock showed high ability of compartmentation excessive Na^+ ion in the leaves and presented similar content of Na^+ ion in its leaves as those in its root. On contrary, Arnold rootstock in NER/ARN plant regulate more efficiently the transportation of Na ion into leaf and showed less leaf Na^+ accumulation comparing with Na^+ root. However, the three rootstocks induced different morphological, physiological, and biochemical mechanisms to cope with salt stressed conditions. Arnold rootstock showed high efficiency to regulate the Na entry at root level and transported less Na^+ ion into shoot which was associated with less reduction in shoot growth. Moreover, this plant showed increased in root biomass productivity upon salinization which helps the plant to maintain higher *A* value. On other hand, the Maxifort F1 rootstock lost its ability to limit the Na^+ influx into aerial part and showed salt inclusion mechanism that associated with excessive accumulation of Na^+ in the shoot, and accordingly this rootstock restrict the plant growth as an adaptive mechanism to cope with stressed condition. However, the salt inclusion mechanism in Maxifort F1 help the plant to achieve lower values of water and osmotic potential, and accordingly, maintaining higher value of OA by using high concentrations of inorganic solutes from the substrate which is considered as an osmotically adaptive strategy. Moreover, Maxifort F1 rootstock induced reduction in net assimilation rate and stomatal conductance which could be a mechanism of water conservation in order to maintain leaf turgor and osmotic adjustment under salt stressed. Additionally, the Maxifort F1 rootstock induced higher proline accumulation in the fruit as an attempt to prevent to some extent the detrimental effect induced by salinity. However, though the different rootstocks induced different morphological, physiological, and biochemical mechanisms deal with the stressed surrounding environment, the three rootstocks failed to counteract the deleterious effect of salinity stress in term of yield and lead to the same preservation of fruit yield.

5.1.2 Salt exclusion/inclusion mechanisms and leaf ion compartmentation of Haruki scion

In this study, we investigated the role of different rootstock genotypes in regulation the physiological and biochemical plant responses under salt stressed condition based on different ability of rootstocks to regulate the transport of saline ions into the shoot genotype. The results confirm that different mechanisms of salt tolerance were operated in the three graft plants (HAR/MAX, HAR/ARN, and HAR/R1) depending on the rootstock characters. Besides, one used rootstock was able to trigger an inclusion or exclusion strategies to cope with salinized condition depending on external stress level. The comparison between the two rootstocks Maxifort F1 and R1 rootstocks in HAR/MAX and HAR/R1plants presented similar capacity of the roots to take up Na^+ ion from the root medium either at mild (97 $mg.g^{-1}$ DW) or high salt stress (108 $mg.g^{-1}$ DW) (Figure 3). However, the graft plant against Maxifort F1 rootstock in HAR/MAX plant showed high capacity to extrude the sodium ion from the shoot and retention this ion into root, where the root kept back higher Na^+ ion by 106% and 32% than the leaf at 100 and 200 mM NaCl, respectively (Figure 3), while the root of HAR/R1 plant accumulated more Na^+ root by 33% and 9% (Figure 3). Accordingly, HAR/R1 plant tend to compartment the Na^+ ion in the leaves and exhibited similar Na^+ leaf ion as the Na^+ root content (93 versus 101 $mg.g^{-1}$ DW) at external high salt, while it showed higher leaf Na^+ concentration by +51% than HAR/MAX at moderated salt stress (Figure 3). Additionally, the leaf Na^+/K^+ ratio was higher in HAR/R1 by 2-fold and 1.4-fold than HAR/MAX plant at mild and high sat stress (Figure 4). Taken together, it seems that R1 rootstock in HAR/R1 plant utilize the mechanism of leaf ion compartmentation as the high Na^+ ion was located in the leaves, while the higher accumulation of Na^+ in the root of Maxifort F1 rootstock in HAR/MAX plant pointed out that this rootstock is considered as salt excluder and displayed high capacity to extrude the salt ion from the shoot and retention it in its root (Estaň et al. 2005). These results corroborate the effect of rootstock genotypes on the intensity of salt ion transport from the root towards the leaves and confirm that the salt tolerance of the shoot depends on the root system performance (Ferreira-Silva et al. 209).

The interesting finding of this study was when Arnold genotype has been employed as a rootstock in HAR/ARN plant as this rootstock showed more efficient regulation of the entry Na^+ ion at root level than HAR/MAX and HAR/R1 and showed less significant root Na^+ ion accumulation by 44% in respect to other graft plants (HAR/MAX and HAR/R1) at 200 mM NaCl (Figure 3). Attractively, the Na^+ accumulation pattern in the HAR/ARN plant differs depending on salt level. Under moderated salt, the root of HAR/ARN plant accumulated Na^+ ion as two times as its leaf Na^+ (77 versus 41 $mg.g^{-1}$ DW), while upon severe salt this plant

accumulated contrary more leaf Na^+ by 36% than its root Na^+ (Figure 3). Accordingly, Arnold rootstock would be able to induce two salt tolerant mechanisms into shoot depending on salt level either as an includer character (as showed by high saline ion accumulation in the leaves) at high salt stress or as an excluder character (as presented by high saline ion accumulation in the root) at mild salt stress.

With reference to K^+ ion, In spite of that Arnold rootstock in HAR/ARN plant showed about 45% less ability to take up this ion from the root medium than Maxifort F1 and R1 rootstocks in HAR/MAX and HAR/R1 plants, the K^+ leaves and stems of the three graft plants showed that there was no significant difference in K^+ accumulation in respect to their control plants (Figure 6). Accordingly, this result might suggest that grafting itself facilitate the transport of K to the leaves and alleviate K^+ deficiency even under high salt stress.

As regards to Cl^- ion, both Maxifort F1 and R1 rootstocks in HAR/MAX and HAR/R1 plants showed almost similar Cl^- content in root and leaf and lower Cl^- value in the stem, suggesting that there was no limitation to the Cl^- transportation to the leaves under 200 mM NaCl (Figure 7). Thus, we might indicate that these both rootstocks have Cl^- includer character. Similarly, the rootstock R1faild to regulate the Cl^- transportation and accumulate significantly higher stem Cl^- anion by 1.8-fold and 2.3-fold than HAR/MAX and HAR/R1 plants, respectively at 200 mM NaCl (Figure 7).

5.1.2.1. Vegetative growth response, water status and gas exchange parameters of Haruki scion

The three graft plants of Haruki scion: HAR/MAX, HAR/ARN, and HAR/R1showed differences in term of transpiration rate and Water use efficiency (WUE) under both salt stresses depending on the rootstock genotypes (Table 3). Water use efficiency (WUE), calculated as the ratio of *A* (net assimilation) to *E* (transpiration), is considered as an important indicator of plant salt tolerance, since high WUE may reduce the uptake of salt and alleviate the water deficiency induced by salinity (Moya et al. 1999; Karaba et al. 2007). At moderated salt stress, HAR/MAX and HAR/ARN plants showed similar higher reduction of *E* and *A* values as (-84% and -62%) than HAR/R1 (-46% and -34%), respectively comparing the control plant. This reduction in both plants was associated with higher WUE by +153% comparing with control plant, while the WUE increasing was about +25% in HAR/R1 at 100

mM NaCl. However, we might ascribe this increasing in WUE upon moderated salt stress to the high decreasing in the transpiration rate altogether with moderated redaction in net assimilation rate. These results were consistent with He et al. (2009) who mentioned that the increased value of WUE of rootstock-grafted tomato plant under salt was resulted from fast reduction in transpiration rate and milder reduction of photosynthetic performance. It is important to note that the deferential effect induced by different rootstocks on transpiration was closely related to the salt ion accumulation. At moderated salt stress, the plant grafted on R1 rootstock of HAR/R1 plant showed significantly higher transpiration rate than HAR/MAX and HAR/ARN plants (1.47 versus 0.49 and 0.38 mM m^{-2} s^{-1}) (Table 3) that associated with higher leaf Na ion (72 versus 48 and 41 $mg.g^{-1}$ DW). This result indicate that R1 rootstock was more effective in term of inducing higher water and salt flux towards the leaf of HAR/R1 plant and accordingly accumulated more Na ion (Ferreira-Silva et al. 2009). On contrary, under 200 mM NaCl, contrast result was recorded in HAR/R1 plant as it showed higher reduction in *E* value -87% and increasing in WUE +169% comparing with control plant, while the other graft plants showed less redaction in *E* value -72% as well as less increasing in WUE as 35% (Table 3). These result demonstrated that the type of rootstock employed in grafting influenced the leaf responses in term of transpiration (Ferreira-Silva et al. 2010). Nonetheless, all these plant combinations failed to achieve high productivity where the fruit fresh weight reduction was high even at mild salt; however, this reduction with Arnold and R1 rootstocks was higher (80%) than Maxifort F1 (66%) at moderated salt stress (Figure 1). The lower yield reduction showing in graft plant onto Maxifort F1, to some extent comparing with other two rootstocks, may due to a high efficient salt exclusion mechanism induced in this graft (Figure 3) as it is known that the salt ion exclusion by roots may result in higher salt resistance of the plant because of lower ionic toxicity that contribute to a metabolic stability and protection of the leaf tissues (Figure 2) (Martinez-Rodriguez et al. 2008; Paranychianakis and Angelakis 2008). However, the high reduction in fruit yield at high salt stress in HAR/R1 plant (-80% and -94% in related to control plant) indicated to the fact that the induced tolerant mechanisms by R1 rootstock were not enough to counteract the deleterious effect of Na^+ ion on the plant, and it might be possible that the compartmentation breaks down, and accordingly the toxic effect of excessive Na^+ in the shoot may predominate over any benefit from better osmotic adjustment (Martinez-Rodriguez et al. 2008). However, the lower Cl^- concentration at both salt stresses in all graft plants indicated that the plant growth inhibition was not related to Cl^- ion.

5.1.2.2. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of Haruki scion

A common damage of plants under saline condition is the accumulation of excessive ROS (Asada 2006); and it is supposed that the salt tolerant rootstock should be able to alleviate the oxidative damage caused by NaCl by enhancing the antioxidant capacity for the effective removal of ROS in cucumber (Zhen et al. 2010), in eggplant (Wei et al. 2009), and in tobacco (Ruiz et al. 2006). However, the three graft plants of Haruki scion: HAR/MAX, HAR/ARN, and HAR/R1 were not able to enhance CAT and GR activities even under high salt stress and showed similar activities to their control plant regardless the rootstock genotypes (Figure 9). Accordingly, high level of salt stress may trigger phenomena of massive release of ROS, resulting in cell and tissue damages. This result might interpret, at least partially, the observed reduction in fruit yield in the three graft plants either at mild or high salt stresses. SOD enzyme is considered as a main scavenger enzyme by detoxifying superoxide radical into H_2O_2 and the higher activity of this enzyme is usually associated with higher capacity to eliminate superoxide radicals and higher tolerance to oxidative stress (Bowler et al. 1991). Some studies indicated that stressed condition can induce SOD activity by an overproduction of ROS (Hameed et al. 2011). The result of this research showed that at high salt level, the fruit SOD enzyme increased in the three graft plants, but more considerably in HAR/R1 plant that exhibited the highest SOD activity as 163% as control plant while this increasing was restricted to 98% and 70% in presence of Maxifort F1 and Arnold rootstocks (Figure 9). Moreover, HAR/R1plant exhibited also a significant increasing in MDA level by +141% in HAR/R1 plant regarding the control plant at high salt stress (Figure 10). Thus, the increasing of MDA level that associated with SOD activity might indicate that there was an attempt to deactivate the ROS and increase the plant ability to dismutate ($O_2^{\bullet -}$) in this cultivar (Zhang et al. 2008; Wei et al. 2009). Halliwell-Asada cycle constitutes an important pathway for dissipation of H_2O_2 and other reactive oxygen radicals in chloroplast (Sgherri et al. 2003). It is assumed that the increased activities of the enzymes of ascorbate-glutathione pathway, especially that of APX confer general resistant to array of environmental stresses (Gara et al. 2000). Our data showed that the graft plant (HAR/R1) presented the highest increasing in APX activity as +134% as the other rootstocks at high salt stress. Taken together, we deduce that in HAR/R1 plant the detoxification of H_2O_2 by APX and SOD enzymes is somewhat more efficient than other plants (Sánchez-Rodríguez et al. 2012). However, although of the enhancement of APX and SOD activity enzymes in HAR/R1 plant, there was still remarkable increasing in lipid peroxidation that expressed by rising of MDA level as +141% as the

control plant and we might link this fruit MDA increasing to the reduction of fruit fresh weight that accounted for -94% in this cultivar (Table 1).

All in all, different mechanisms of salt tolerance were operated in the three graft plants of Haruki scion depending on the rootstock characters. R1 rootstock utilize the mechanism of leaf ion compartmentation as the high Na^+ ion was located in the leaves, while the higher accumulation of Na^+ in the root of Maxifort F1 rootstock pointed out that this rootstock is considered as salt excluder and displayed high capacity to extrude the salt ion from the shoot and retention it in its root. However, Arnold rootstock would be able to induce two salt tolerant mechanisms into shoot depending on salt level either as an includer character at high salt stress or as an excluder character at mild salt stress. It is important to note that the deferential effect induced by different rootstocks in term of transpiration rate, net assimilation rate and water use efficiency was closely related to the salt ion accumulation. At moderated salt stress, Maxifort F1 and Arnold rootstocks showed higher reduction of *E* and *A* values that associated with higher WUE comparing with R1. The high WUE may reduce the uptake of salt and alleviate the water deficiency induced by salinity. R1 rootstock showed significantly higher transpiration rate than other two rootstocks that associated with higher leaf Na ion. On contrary, under 200 mM NaCl, R1 plant showed conversely higher reduction in *E* value and increasing in WUE, while the other graft plants showed less redaction in *E* value as well as less increasing in WUE. It is supposed that the salt tolerant rootstock should be able to alleviate the oxidative damage caused by NaCl by enhancing the antioxidant capacity for the effective removal of ROS. In this study, different rootstocks showed different ability to enhance the enzyme activities upon salinization. R1 rootstock exert efforts to induce APX activity at high salt stress, while Maxifort F1 and Arnold rootstocks lost their capacity to trigger APX, CAT, and GR enzyme activities. Thus, we might suggest that R1rootstock was more efficient in detoxification of fruit H_2O_2 by enhancing the activity of APX enzyme. However, the different tolerant mechanisms that induced by the three used rootstocks were not enough to counteract the deleterious effect of Na ion and the toxic effect of excessive Na in the shoot may predominate over any benefit from better osmotic adjustment. Moreover, there was still remarkable increasing in fruit lipid peroxidation that expressed by rising of MDA level in the graft plants. Thus, we could indicate that the three plant combinations failed to achieve high productivity where the fruit fresh weight reduction was so high even at mild salt, these result could pointed out that those rootstocks are not relatively salt tolerant and they are not able to donate the resistant to their shoots.

5.1.3. Salt exclusion mechanism and leaf ion compartmentmentation of Kamonium scion

Three graft combinations (KAM/MAX, KAM/ARN and KAM/R1) have been generated by grafting the cultivar tomato Kamonium onto three rootstock genotypes Maxifort F1, Arnold and R1 to assess the plant physiological and biochemical responses that induced as the effect of different rootstocks under NaCl stress condition. The data of this experiment showed that the pattern of ion accumulation was varied according to the characteristic of rootstock genotypes. At root level, there was no variation in Na^+ accumulation between the three rootstocks and showed medium accumulation as 106 $mg.g^{-1}$ DW at 200 mM NaCl (Figure 3). However, at stem level, only R1 rootstock in KAM/R1 plant showed higher Na^+ accumulation rate by +50% than the other rootstocks in KAM/MAX and KAM/ARN plants at high salt level (Figure 3). Similarly, Arnold and R1 rootstocks in KAM/ARN and KAM/R1 plants exhibited higher significant leaf Na^+ ion as 1.6 and 1.3 times as leaf Na^+ in KAM/MAX plant (Figure 3). Nonetheless, the rootstocks Maxifort F1 and Arnold in KAM/MAX and KAM/ARN graft plants showed higher ability to restrict more Na^+ ion in their roots by (+56% and +27%) than their leaves (Figure 3) while they accumulate similar ratio of Na/K between their root and leaf that accounted 10 (Figure 4). However, the both rootstocks Maxifort F1 and Arnold have the ability to extrude more sodium ion from the shoot and preserve it in their roots by operating a salt exclusion mechanism; While the rootstock R1 in KAM/R1 plant showed less efficient in regulation the Na^+ ion transport into leaf level and positioned similar ion concentration between root and leaf (102 versus 108 $mg.g^{-1}$ DW) at 200 mM NaCl (Figure 3). Besides, KAM/R1 plant accumulated more leaf Na^+/K^+ ratio value as 2 times of its root (Figure 4). Taken together, this result suggests that the R1 rootstock high ability to translocate Na^+ ion and compartment it at leaf level by operating leaf compatmentation strategy. This result was consistent with Tester and Davenport. (2003) and Møller et al. (2009) who mentioned that salt tolerance mechanisms can occur in a wide range of organizational levels from the cellular level (e.g., compartmentation of Na^+ within cells) to the whole plant (e.g., exclusion of Na^+ from the plant and exclusion of Na^+ from the shoot).

However, although both rootstocks Maxifort F1 and Arnold utilized the same salt exclusion strategy to deal with high salt stress, Maxifort F1 showed higher exclusion capacity from the shoot than Arnold rootstock as it accumulated less leaf Na^+ ion by 22% at high salt stress. The lower accumulation of Na^+ in the shoot that associated with exclusion capacity of the rootstock might be attributed to mechanisms of retention ion in the root or/and uptake

selectivity in the root as Maxifort F1 rootstock in KAM/MAX plant accumulates more Ca^{2+} by 1.7 and 1.3 times as KAM/ARN and KAM/R1 plants at 200 mM NaCl (Figure 5) (García-Sánchez et al. 2002; Estaň et al. 2005). However, stem and leaf Ca^{2+} of the three graft plants maintain the same ions content as the control plant at high salt stress (Figure 5).

5.1.3.1. Vegetative growth response, water status and gas exchange parameters of Kamonium scion

The positive effect of different rootstocks was evaluated in term of parameter of photosynthesis and yield productivity in the three graft combination of Kamonum scion KAM/MAX, KAM/ARN, and KAM/R1. At mild salt stress, KAM/ARN plant presented slightly lowest *E* value that associated with high preserve value of WUE (16.51) (Table 3), which possibly resulted in lower fruit fresh weight redaction (-62%) at 100 mM NaCl in related to control plant (Table 1), while the fruit fresh weight reduction was higher in KAM/MAX and KAM/R1 plants (-84%). Additionally, it seems that the leaf compartmentation mechanism that presented in KAM/R1plant at high salt stress supported the plant to accumulate more stem Na (Figure 3) and Na^+/K^+ (Figure 4) and accordingly the plant adjusted osmotically (Figure 2). However, the fruit and shoot fresh weights were still so high (-96% and -89%) regarding the control plant at high salt stress (Table 1). It is accepted that during osmotic adjustment the cells try to compartmentalize most of absorbed ions in vacuoles in order to maintain the osmotic adjustment (Hasegawa et al. 2000), however, in some cases the accumulation of solutes is so high that it goes beyond the limits of regulation of cytoplasmic content with associated impairment of growth (Pitman 1984). As we have mentioned above, the salt ion exclusion mechanism that associated with high root retention ability to the ion may result in higher salt resistant plant as lower Na^+ accumulation will present in the shoot which protect the leaf tissue and improve plant performance under salt condition (Martinez-Rodriguez et al. 2008). Unexpectedly, the exclusion strategy of maxifort F1 and Arnold rootstocks in KAM/MAX plant was accompanying with highest fruit weight reduction -97% comparing to control plant upon high salt stress. Thus, the exerted strategy of Maxifort F1 was not enough to relieve the plant under high salt condition. Moreover, the three rootstocks exhibited different reduction in transpiration responses upon salinization regarding their control plants (Table 3). The rootstock Maxifort F1 in high salt treated KAM/MAX plant illustrated the lower reduction in *E* value -60% in relation to control plant, while this reduction accounted for (-78% and -71%) in KAM/ARN and KAM/R1 plants (Table 3). This result was in line with (García-Sánchez et al. 2002) who demonstrated that the

exclusion strategy of toxic Na ion from the shoot was associated with the retention of this ion into the root and lower transpiration rate.

5.1.3.2. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of Kamonium scion

ROS are highly toxic and can damage many important cellular components such as lipid, protein, DNA, and RNA (Sainju et al. 2001). The salt tolerant of plants is linked to their abilities to enhance the enzymatic antioxidant capacity in order to eliminate ROS and alleviate the oxidative damage caused by NaCl. In this experiment, the three salt stressed plants KAM/MAX, KAM/ARN, and KAM/R1 showed similar activities of APX and CAT enzymes either under control or saline conditions (Figure 9). Moreover, the idleness activities of these enzymes in the three graft plants were associated with high significant increasing in the quantity of MDA concentration (Figure 10), and possibly with high level of H_2O_2. Thus, the detoxification of ROS by these enzymes is not efficient and might be enhanced the H_2O_2 accumulation and accordingly these plants are subject to oxidative stress. Willenkens et al. 1997) demonstrated that the accumulation of H_2O_2 in different tissue of plant could reduce the plant biomass which gives sense for interpretation the reasons behind the reduction in plant biomass in our data. Consequently, the rootstocks used were not in charge of conferring enough resistances to the shoot. However, the differential effect of different rootstocks was clear with GR enzyme, where the rootstock R1 in KAM/R1 plant presented a higher GR activity by (+55% and +45%) than KAM/MAX and KAM/ARN plants, respectively at high salt stress (Figure 9). Expectedly, the higher GR activity might be associated with higher AsA content (He et al. 2009; Sánchez-Rodríguez et al. 2012), and accordingly, this indicate that in this combination the ascorbate-glutathione detoxification system functions better than other two graft plants of KAM/MAX and KAM/ARN.

Free polyamines have been reported to be involved in the plant responses to osmotic stress by playing a role in the ROS-mediated damage caused by osmotic condition (Zhu 2002). Under salt stress, polyamines could increase the activities of key enzymes involved in oxidative stress such as GR and decrease lipid peroxidation in virginia pine and improved plant development (Tang and Newton 2005). In this work, the plant KAM/R1 that grafted onto R1 rootstock showed the higher fruit protein accumulation than the plant grafted onto Maxifort F1 or Arnold rootstocks in KAM/MAX and KAM/ARN plants at high salt stress (Figure 10). The remarkable protein increasing in KAM/R1 plant might be responsible to enhance activity of GR enzyme in this plant. Unexpectedly, the same rootstock R1 in KAM/R1 plant showed more reduction in proline accumulation at high salt stress. However, it was stated that salt

induced changes in the content of organic solutes such as amino acids and proline, which might represent metabolic alteration that are associated with resistance and/or sensitivity to salt (Ashraf and Harris 2004). All things considered, the enzyme activities have not been induced when Maxifort F1 and Arnold genotypes were used as rootstocks which might indicate that these rootstocks had a lower constitutive antioxidant enzyme levels under salt condition and accordingly had a lower capacity to scavenge ROS under both salt stress and this could be partly related to its lower salt tolerance (Hernández et al. 2003).

To conclude, the rootstocks Maxifort F1 and Arnold operated the mechanism of salt exclusion that extrude sodium ion from the shoot and preserve it back in their roots, while the rootstock R1 showed high ability to translocate Na^+ ion and compartment it at leaf level by operating leaf compatmentation strategy. It was expected that the salt ion exclusion mechanism that operated by maxifort F1 rootstock may result in higher salt resistant plant as lower Na accumulation will present in the shoot which improve plant performance under salt condition. Unexpectedly, this exclusion strategy was accompanying with high fruit weight reduction upon high salt stress. Moreover, the salt tolerant of plants is linked to their abilities to enhance the enzymatic antioxidant capacity in order to eliminate ROS and alleviate the oxidative damage caused by NaCl. In this study, the rootstocks used were not in charge of conferring enough resistances to the shoots as the three salt stressed plants showed similar activities of APX and CAT enzymes as their control plants. This lower enzymes activity was associated with high MDA concentration and possibly with high level of H_2O_2. The accumulation of H_2O_2 in different tissue of plant could be the reason behind the plant biomass reduction.

4. RESULT

4.2. THE PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF DIFFERENT SCION GENOTYPES AGAINST ONE ROOTSTOCK

Nine different graft combinations have been generated by grafting three tomato cultivars: Haruki, Kamonium, and Nerina F1 onto three different tomato rootstocks: Maxifort F1, Arnold, and R1. In this research, we assessed the contribution of rootstock in inducing useful salt tolerance to the shoot growth and fruit productivity depending on salt tolerance mechanisms of the shoot genotype; and we tried to identify the main differential physiological and biochemical responses of different scion genotypes that involved in plant salt tolerance.

4.2.1. The graft combinations of different scion genotypes against Maxifort F1 rootstock

4.2.1.1. Vegetative growth and physiological responses of different scion genotypes against Maxifort F1 rootstock

Three scion genotypes: Haruki and Kamnium, and Nerina F1 were grafted onto one rootstock cultivar Maxifort F1 in order to determine to which extent the scion genotype characters have an effect on conferring the salt tolerance into grafted plants under salt stressed condition. There were differential responses of the three shoot genotypes in term of plant growth and shoot biomass upon salinization (Table 1). The three graft plants HAR/MAX, KAM/MAX, and NER/MAX showed similar medium shoot fresh weight reduction at mild salt stress as -68% regarding the control plants; however, this reduction was higher at 200 mM NaCl, particularly, in NER/MAX plant that accounted -96%, while HAR/MAX and KAM/MAX presented less reduction as -84% in relation to control plant (Table 1). Alike shoot FW, the three scions showed similar reduction in shoot DW as -50% at moderated salt stress in related to non-stressed plant; at higher salt stress the highest reduction was recorded in NER/MAX plant -84%, whereas HAR/MAX and KAM/MAX plants recorded less reduction -73% (Table 1). Fruit yield was affected strongly upon salt exposure, and the severest reduction was recorded when Kamnium genotype was used as a scion in KAM/MAX plant that accounted for -86%, while Haruki and Nerina F1 scions showed lower fruit FW reduction by -66% in HAR/MAX and -74% in NER/MAX at mild salt stress in respect to control plants (Figure 1). Leaf water potential (Ψ_w) reduced significantly upon two salt stress levels regardless the scion genotypes (Figure 2). Similarly, the osmotic potential showed a strong decreasing upon salt stress, however, the lowest value of osmotic potential redaction was recorded in HAR/MAX and NER/MAX plants at 200 mM NaCl (-3.62 and -3.87 MPa) that associated with higher OA values (Figure 2). Transpiration rate and Net assimilation rate were affected strongly upon salinization and showed significant dropping in the three graft plants (Table 3). However, the highest reduction was recorded when Nerina F1 genotype was employed as a scion in NER/MAX plant and showed reduction as -88% and -91% respectively for *E* and *A* values at 200 mM NaCl in respect to control plant.

4.2.1.2. The pattern of ion accumulations of different scion genotypes against Maxifort F1 rootstock

In this experiment, three tomato cultivars, Haruki, Kamonum, and Nerina F1 were used as scions against Maxifort F1 rootstock and three graft combinations have developed: HAR/MAX, KAM/MAX and NER/MAX. The physiological and biochemical performances

of different scion genotypes have been investigated in order to determine to which extent the salt tolerance of the shoot depends on scion genotypes. Na^+ accumulation was fundamentally different in the three graft combinations depending on the shoot characteristics as different scion showed different pattern of leaf Na^+ accumulation (Figure 3). Nerina F1scion of plant (NER/MAX) showed significantly higher leaf sodium accumulation rate than Haruki (HAR/MAX) and Kamonium (KAM/MAX) scion genotypes by 73% and 123%, respectively at overstated salt condition (Figure 3). Similarly, the pattern of stem sodium accumulation in NER/MAX plant was fundamentally higher than those of HAR/MAX and KAM/MAX graft plants by 152% and 379%, respectively at 200 mM NaCl (Figure 3). However, root data showed also the influence of scion genotypes on sodium profile accumulation where HAR/MAX and NER/MAX accumulate more Na^+ root than KAM/MAX at 200 mM NaCl (Figure 3). Furthermore, the partitioning of Na^+ ion into different plant organs was also affected by the scion genotypes as Nerina F1 scion in NER/MAX plant accumulated higher Na ion in its leaf (150 $mg.g^{-1}$ DW) than its root (113 $mg.g^{-1}$ DW) (Figure 3). In contrary, both other scions Haruki and Kamonium in HAR/MAX and KAM/MAX plants partitioned more Na ion in their roots (114 and 105 $mg.g^{-1}$ DW) in related to their leaves (87 and 68 $mg.g^{-1}$ DW) (Figure 3). Interestingly, the finding of total sodium ion partitioning that expressed as a ratio of total plant Na^+ content on the basis of total plant dry weight support the same idea that shoot genotypes play an important role in orientation the ion transportation in grafting plant (Figure 8), where Nerina F1 scion in NER/MAX plant positioned 53% of total Na^+ ion in the root and 22% in the leaf, while HAR/MAX and KAM/MAX located the higher Na percentage in the root (73% as an average) and lower Na^+ percentage in the leaf (13% as an average) at severe salt stress (Figure 8).

Like Na^+ accumulation rate, Na^+/K^+ ratio showed different response depending on the scion genotypes (Figure 4). When Nerina F1 genotype was employed as a scion, NER/MAX plant exhibited higher significant Na^+/K^+ ratio than the other scions Haruki and Kamonium in HAR/MAX and KAM/MAX plants by (165% and 215%) of leaf Na^+/K^+, (163% and 388%) of stem Na^+/K^+, and (32% and 22%) of root under 200mM NaCl (Figure 4). Besides, Haruki and Kamonium scions in HAR/MAX and KAM/MAX plants showed similar value of Na^+/K^+ ratio in their roots and leave that accounted as average 11, while Nerina F1 scion showed higher leaf Na^+/K^+ ratio by 170% than root Na^+/K^+ ratio (Figure 4)

Regarding Ca^{2+} accumulation, in general the three graft plants HAR/MAX, KAM/MAX, and NER/MAX accumulated more leaf Ca^{2+} by 3.4 times than Ca^{2+} stem and 2.7 than Ca^{2+} root (Figure 5). Moreover, the three graft plant showed increasing in Ca^{2+} content upon high salt particularly with Nerina F1 scion that showed higher significant accumulation of root and stem Ca^{2+} by 29% and 195% at high salt stress than other scions. On the subject of K^+ ion, the three graft plants showed no fundamental effect either of salt or scion genotypes and showed similar K^+ accumulation pattern as control plant with two exceptions; the leaf of Nerina F1 scion showed significant reduction by -39% regarding its control plant, and K^+ root of HAR/MAX plant that showed significant increasing by 85% in related to control plant at high salt stress (Figure 6). In relation to Cl^- content, the three graft plants showed significant increasing in Cl^- content upon both salt stresses (Figure 7). Additionally, there was significant effect of the scion genotypes on leaf and stem Cl^- accumulation, where leaf and stem Cl^- increased essentially by +40% and +95%, respectively when Nerina F1 was used as a scion in NER/MAX plant in respect to other graft plants HAR/MAX and KAM/MAX upon high salt stress (Figure 7).

4.2.1.3. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of different scion genotypes against Maxifort F1 rootstock

Not too much data are available about the combined effects of salt stress on antioxidant mechanism that examined the effect of grafting with different scion genotypes against one rootstock or the grafting of one scion genotype onto different rootstock characters, and the information on how salt stress and ROS metabolism in the fruit are influenced by rootstock-scion combinations is still scare. In the present study, the effect of scion genotypes have been investigated in term of enzymatic antioxidant capacity in the fruit of salt stressed plants. Three scion genotypes: Haruki, Kamonium, and Nerina were grafted against Maxifort F1 rootstock and generated the following combinations: HAR/MAX, KAM/MAX, and NER/MAX. Our data illustrated that there were no significant changes in APX activity in three graft plants upon both salt stresses in relation to their control plants regardless the scion genotypes (Table 7 and Figure 9). Similarly, there were no differential responses in regard to CAT activity between Haruki and Kamonium scions as both of them showed similar responses as that of control ones (Figure 9). Controversially, Nerina scion showed a significant increasing in CAT activity by +217% and +391% respectively at both salt stresses in related to control plant (Figure 9). Similarly to CAT enzyme, there was no effect of Haruki and Kamonium scions on GR activity upon salinization while Nerina F1 showed sharp reduction in the activity of this enzyme by -74% regarding the control plant at high salt stress

(Figure 9). However, SOD activity of the three graft plants showed averaged increasing by +69% at high salt stress in related to control plants irrespectively to the scion genotypes (Figure 9).

Although Haruki and Kamonium scions exert efforts to increase amino acids accumulation upon salinization, this increasing was not significantly pronounced in related to that of control plants (Figure 10). However, there was remarkable increasing in protein content when Nerina F1 was used as a scion in NER/MAX plant that showed an important raising by +114% and +165% in fruit protein at mild and high salt stresses comparing the non-treated plant (Figure 10). Moreover, free proline level was provoked similarly among the three graft plant at moderated salt stress and showed averaged accumulation as 3.2 μM g^{-1}FW (Figure 10). However, Nerina F1 genotype showed continued increasing in proline level at high salt application as +158% as the other scions Haruki and Kamonium. Lipid peroxidation level in the fruit of three graft plants, measured as the content of MDA, showed significant increasing when Kamonium genotype was employed as a scion in KAM/MAX plant by +22% and +357% at both salt levels regarding the non-stressed plant, while the other graft plants of HAR/MAX and NER/MAX showed slight increasing (Figure 10).

4.2.2. The graft combinations of different scion genotypes against R1 rootstock

4.2.2.1. Vegetative growth and physiological responses of different scion genotypes against R1 rootstock

Three different scion cultivars were grafted against R1 rootstock in order to establish three graft combinations: HAR/R1, KAM/R1, and NER/R1. All growth parameters reduced significantly upon salinization. Shoot FW of the three graft plants showed similar reduction at mild salt stress as -65% regarding the control plants (Table 1). Upon 200 mM NaCl, Kamonium scion in KAM/R1 plant showed the highest shoot FW reduction as -89% in related to control plant (Table 1). Similarly, Kamonium scion in KAM/R1 plant presented the lowest shoot DW reduction as (-62% and -84%) respectively at mild and high salt stresses (Table 1). However, the effect of scion genotype even on root system was presented, where Haruki scion in HAR/R1 plant showed a noteworthy increasing in root dry weight by +131% at moderated salt stress, while KAM/R1 and NER/R1 showed reduction by (-43% and -7%), respectively in related to control plant (Table 1). Nevertheless, at high salt stress, root DW of Haruki scion in HAR/R1 plant was not affected and showed the same biomass as the control plant; however, Kamonium scion in KAM/R1 plant represented the lowest root DW reduction as -67%, while NER/R1 showed reduction by -31% with comparison to control

plant (Table 1). The fruit yield reduced significantly upon salinization in the three graft plants. However, the reduction was less with Nerina F1 scion and accounted -58% in NER/R1 plant, while this reduction gets hold of -80% in both HAR/R1 and KAM/R1 plants at mild salt stress in relation to control plant (Figure 1).There were no appreciative responses of scion genotypes in term of water relation as the three graft plants showed similar reduction in water and osmotic potential upon both salt stresses (Figure 2). The transpiration rate and net assimilation rate reduced sharply upon salt stress in all graft plants irrespectively to the shoot genotypes (Table 3). The Haruki scion in HAR/R1 plant illustrated the lowest significant reduction in *E* and *A* values as (-46% and -34%) respectively at moderated salt stress in relation to control plant. Besides, the reduction in this plant was associated with higher WUE value (14.04 versus mM CO_2 mM^{-1} H_2O) (Table 3). However, the other scion genotypes Kamonium and Nerina F1 in KAM/R1 and NER/R1 plants showed similar reduction as -73% and -52% in *E* and *A* values respectively at mild salt stress (Table 3). Under higher salt stress, the reduction in *E* and *A* value was harder and accounted for -87%, -71%, and -80% respectively in HAR/R1, KAM/R1, and NER/R1 plants regarding their control plants, while the three graft plants showed similar reduction in net assimilation rate as -66% at high salt stress (Table 3).

4.2.2.2. The pattern of ion accumulations of different scion genotypes against R1 rootstock

Unlike Maxifort F1 rootstock, the comparison of the three graft combinations that have been generated against R1 rootstock (HAR/R1, KAM/R1, and NER/R1) showed similar accumulation of leaf Na^+ among them (93, 108, 104 mg.g-1 DW, respectively) (Figure 3). Similarly, the root Na^+ data also revealed similar responses of Na^+ accumulation among the three above graft plants (101,102, and 112 mg.g-1 DW, respectively) (Figure 3). However, the Na^+ stem showed the lowest Na^+ ion content in all above mentioned graft plants (49, 60, 67 mg.g-1 DW, respectively). Furthermore, the partitioning of Na^+ ion was distributed almost equally between the leaves and root of the three graft plants (93 versus 101 mg.g-1 DW in HAR/R1; 108 versus 102 mg.g-1 DW in KAM/R1; and, 104 versus 112 in NER/R1 mg.g-1 DW at staid salt stress (Figure 3). Regarding Na^+/k^+ ratio, it showed significant increasing in the three graft plants HAR/R1, KAM/R1, and NER/R1upon both salt stress but without any pronounced effect of scion genotypes as all scion shoots illustrated similar value at organ levels (Figure 4). On the topic of Ca^{2+} accumulation, neither scion nor salt level affect Ca^{2+} ion in the three graft plants and they showed similar Ca^{2+} accumulation as control plants in each plant organ (Figure 5). The root potassium concentration of the three graft combinations

showed similar significant increasing by +97% in regard to their control plants upon both salt stress (Figure 6), while no effect of the scion genotype has been recorded. Stem K^+ was not affected by scion or salt stress and accumulate K^+ ion as control plant, while leaf K reduced slightly in stressed plants regardless the scion genotypes. In relation to Cl^- content, the three graft plants accumulate more root Cl^- ion as 10 times as their control plants upon high salt stress unrelatedly to the scion genotypes (Figure 7). Similarly, leaf Cl^- showed averaged significant increasing by 2.8 times regarding the control plant upon high salt stress without specific effect of the scion genotypes; however, the Cl^- stem of Nerina F1scion in NER/R1 plant showed higher increasing in Cl^- content as 1.3 time as the other graft plants HAR/R1 and KAM/R1at 200 mM NaCl.

4.2.2.3. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of different scion genotypes against R1 rootstock

The genotype Haruki in HAR/R1 plant expressed the highest APX activity as +233% regarding the control plant upon 200 mM NaCl, while the other scions of Kamonium and Nerina F1 showed negligible increasing regarding their control plant (Figure 9). In regard to CAT activity, there was no positive effect of any scions as the three graft plants showed similar CAT activity as the non-treated plants (Figure 9). The activity of GR was activated when Kamonium scion was used in KAM/R1 plant as it showed increasing by 51% at both salt levels in relation to control plant, while HAR/R1 and NER/R1 did not show any significant increasing in GR activity upon salt stress (Figure 9). In related to SOD enzyme activity, only Haruki scion in HAR/R1 plant showed significant increasing in this enzyme as +163% as the control plant at high salt stress (Figure 9).

The amino acids accumulated significantly with Kamonium scion in KAM/R1 plant and showed increasing by +296% regarding the control plant, while the other scions showed unimportant increasing in the fruit protein level (Figure 10). However, the proline content was expressively increased in the three graft plants upon salinization taking in our consideration that Haruki and Nerina scions showed higher averaged proline content by +103% and +117% than Kamonium scion respectively at mild and overstress salt levels (Figure 10). The lipid peroxidation measurement showed that Kamonium scion in KAM/R1 plant trigger dramatically MDA accumulation upon high salt stress and showed increasing by +269% as zero salt treated plant, while the other scion genotypes in HAR/R1 and NER/R1 plants showed less important increasing in this parameter (Figure 10).

4.2.3. The graft combinations of different scion genotypes against Arnold rootstock

4.2.3.1. Vegetative growth and physiological of different scion genotypes against Arnold rootstock

Three tomato cultivars: Haruki , Kamonium, and Nerina F1have been grafted onto rootstock Arnold in order to generate three graft combinations: HAR/ARN, KAM/ARN, and NER/ARN. There were differential responses in plant growth and up-down physiological regulation orders in the three graft plants depending on the scion characters. Shoot fresh and dry weight affected drastically upon adding the salt into root medium in all graft plants (Table 1). At moderated salt stress, in the existence of Nerina F1 scion, the plant NER/ARN showed less shoot FW and DW reduction as (-47% and -21%) comparing with control plant, while the highest reduction in shoot FW and DW (-67% and -56%) were recorded when Kamonium genotype was employed as a scion in KAM/ARN plant (Table 1). Under 200 mM NaCl, Kamonium scion in KAM/ARN plant exhibited the highest reduction in shoot FW and DW as (-85% and -78%) comparing the control plant, while Haruki and Nerina F1 scions in HAR/ARN and NER/ARN plants showed similar shoot FW and DW redaction as (-78% and -66%) in related to control plants (Table 1). Interestingly, the moderated salt stress induced significant increasing in root dry weight in the graft plants; however, there were differential responses among the three scion genotypes in which the Nerina F1 scion genotype in NER/ARN plant induced a substantial increasing in root DW as +62%, while this increasing was about (+22% and +11%) when Haruki and Kamonium scions used in HAR/ARN and KAM/ARN plants, respectively in related to control plants. Upon high sat stress, root DW was not affected by salt stress only when Haruki was used as a scion in HAR/ARN plant and shoot root DW value as the control plant, while KAM/ARN and NER/ARN affected negatively and showed reduction in root DW as (-62% and -32%) respecting their control plants (Table 1). Fruit yield affected strongly even at moderated salt stress and showed reduction by -62% in KAM/ARN plant, while this reduction was higher -82% in presence of Haruki and Nerina scions in HAR/ARN and NER/ARN plant regarding their control plants (Figure 1).The positive effect of scion genotypes in was evident in term of water use efficiency (WUE) where the both scion genotypes Haruki and Kamonium revealed a significant increasing in WUE upon moderated salt stress in HAR/ARN and KAM/ARN that averaged (15.8 mM CO_2 mM^{-1} H_2O), whereas the graft plant NER/ARN showed a slight increasing in WUE regarding the control plant (Table 3). The water status indicators such as $\Psi\pi$ and Ψ_w were affected strongly by salt application and showed significant reduction irrespectively of the scion genotypes (Figure 2). However, HAR/ARN and KAM/ARN plants

showed high ability to maintain higher OA value in respect to NER/ARN plant at both salt stresses (Figure 2). Photosynthesis parameters reduced significantly at salt application in the three graft plants. Unexpectedly, there was no variation among the performance of the three scion genotypes as all graft plants showed similar reduction in *E* value that averaged as (-79% and -78%) and *A* value (-59% and -66%) at 100 and 200 mM NaCl, respectively (Table 3).

4.2.3.2. The pattern of ion accumulations of different scion genotypes against Arnold rootstock

When Arnold genotype has employed as a rootstock, the three graft combinations HAR/ARN, KAM/ARN, and NER/ARN performed differently upon salinization depending on the scion genotypes at root level (Figure 3). The root sodium of Haruki scion in HAR/ARN plant accumulated less significant sodium by (-46% and -38%) compared to KAM/ARN and NER/ARN plants at 200 mM NaCl. However, the leaf Na^+ of the three plants showed similar increasing by 19 times as control plant at high salt stress. Moreover, the partitioning of the Na^+ ion showed that leaves of HAR/ARN plant accumulated higher Na ion than the root (81 versus 60 $mg.g^{-1}$ DW), while the leave of other two graft plants KAM/ARN and NER/ARN presented higher collective Na^+ ion in their roots (110 and 97 $mg.g^{-1}$ DW) with respect to their leave (87 and 77 $mg.g^{-1}$ DW), respectively at high salt stress (Figure 3). In relation to Na^+/K^+ ratio, root, stem and leaf Na/K value increased significantly in all plant organs upon salt stress regardless the scion genotypes (Figure 4). With regard to Ca^{2+} content, both salt and scion genotypes did not show any effect either on root or leaf Ca^{2+} of the three graft plants and accumulate Ca^{2+} ion similarly to their control plants at high salt stress (Figure 5). Pertaining to K^+ ion, root K^+ content was affected significantly by scion genotypes, where NER/ARN and KAM/ARN graft plants revealed higher K^+ ion accumulation rate than HAR/ARN by 72% at 200 mM NaCl, respectively (Figure 6). With respect to stem K^+ content, there was no significant effect of scion genotypes and the plants accumulated K ion as the control plant. With reference to Cl^- ion, Kamonim scion in KAM/ARN illustrated slightly higher root Cl^- ion than HAR/ARN and NER/ARN plants (Figure 7). However, the Cl^- stem of Haruki scion in HAR/ARN plant showed higher significant accumulation rate of Cl^- ion by 2.9 and 1.8 times than KAM/ARN and NER/ARN plants at 200 mM NaCl, while the Cl^- leaf was similar among the three graft plants (Figure 7).

4.2.3.3. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of different scion genotypes against Arnold rootstock

The comparison of scion genotypes performance showed that Nerina F1 scion in NER/ARN plant showed significant increasing in APX activity as 9 and 17 times as control plant at 100 and 200 mM NaCl, while Haruki and Kamonium scions in HAR/ARN and KAM/ARN plants showed no difference in the this enzyme at both salt levels (Figure 9). Similar to APX enzyme, CAT activity increased significantly only in NER/ARN graft plant by +226% and +179% at both salt levels regarding the control plant, while salt treated HAR/ARN and KAM/ARN plants presented similar activity in CAT enzyme as their control plants (Figure 9). Likewise, the activity of GR enzyme was increased significantly only with Nerina F1 scion in NER/ARN plant that showed increasing by +101% at both salt levels concerning the control plant (Figure 9). Furthermore, the amino acids accumulation was induced significantly upon salinization when Nerina F1 genotype was used as a scion in NER/ARN plant that showed increasing by (+175% and +226%) at mild and high salt stresses in relation to control plant (Figure 10). However, the other scion genotypes of Haruki and Kamonium showed a little increasing in protein level. On contrary to protein content, the accumulation of free proline in the fruit of the three graft plants increased significantly nevertheless the scion genotypes and the medium increasing of the three plants was as 16 and 28 times as control plants respectively at 100 and 200 mM NaCl (Figure 10). The MDA level was increased significantly only with Kamonium scion in KAM/ARN plant that showed increasing by +79% regarding the control plant at high salt stress, while Haruki and Nerina F1 scions did not differ of their control plant (Figure 10).

5. DISCUSSION

5.2. SALT TOLERANT INDUCED MECHANISMS OF DIFFERENT SCION GENOTYPES AGAINST ONE ROOTSTOCK

Lots of contradictory results have been reported in ascription the positive effect of grafting, in alleviating the deleterious effect of salt, into rootstock characteristics or scion genotypic and/or to the scion-rootstock interaction. Thus, in this study, we have examined the conditioned significant effect of the scion genotypes on shoot performance in term of ions accumulation in the different organs of salt treated plant.

5.2.1. Halophytic inclusion mechanism of graft combinations against Maxifort F1 rootstock

The most tolerant genotypes of many species are those better able to prevent excessive ion accumulation in their leaves. However, salt tolerance is not always associated with low ion concentration in leaves. The salt tolerance in halophytes plant is associated with high ion concentrations in leaves. Also, the higher salt tolerance of wild tomato species over cultivated

forms has generally been associated with the halophytic character of Na^+ accumulation in the wild relatives (Cuartero and Fernandez-Muñoz 1999). This high salt content is necessary to adjust the leaf water relations to low external potential as the plant use the cheapest solution from the energetic point of view (Raven 1985). Indeed, the higher salt tolerance of wild tomato species has been associated with halophytic character of Na^+ accumulation (Cuartero and Fernandez-Muñoz 1999), and many halophytes show growth stimulation upon addition of NaCl to a growth medium where NaCl is rapidly accumulated and employed preferentially as an osmoticum (Ramos et al. 2004).

Three different scion genotypes: Haruki, Kamonium, and Nerina F1 have been grafted onto Maxifort F1 rootstock and generated three graft combinations HAR/MAX, KAM/MAX and NER/MAX. The HAR/MAX and KAM/MAX plants showed less leaf Na^+ accumulation by (-42% and -55%) and stem Na^+ ion (-60% and -79%, respectively) than NER/MAX plant at 200 mM NaCl (Figure 3). Additionally, Maxifort F1 rootstocks in NER/MAX plant accumulated more leaf and stem Na by +33% and +38% than its root, while the other two combinations HAR/MAX and KAM/MAX exhibited less leaf Na accumulation (-24% and -36%) as well leas stem Na^+ content (-46% and -69%), respectively, in related to their Na root rate at 200 mM NaCl (Figure 3). All these observations demonstrate that the differential effects of the same rootstock, in term of ion accumulation, could be attributed to scion genotype characters, where Nerina F1 scion shows a halophytic character that accumulate the highest salt ions in the stem and leaves (156 and 150 $mg.g^{-1}$ DW) in respect to its root (113 $mg.g^{-1}$ DW) (Figure 3). On contrary, an exclusion mechanism presented when Haruki and Kamonium genotypes used as a scion and they show high retention capacity of the salt into their roots (114 and105 $mg.g^{-1}$ DW) in order to avoid the excessive accumulation of salt ions in the leaves (87 and 68 $mg.g^{-1}$ DW).

Furthermore, the partitioning of total plant Na^+ ion, in term of total plant dry weight, showed a similar scenario to that mentioned above, where NER/MAX plant showed that 53% of total Na^+ ion was located in the root and 47% in the shoot, while 78% of total Na^+ was positioned in root versus 22% in the shoot of HAR/MAX plant; and 67% of leaf Na^+ was detected in KAM/MAX plants versus 33% of root Na^+ upon 200 mM NaCl (Figure 8). Besides, the NER/MAX plant showed higher aggregation of leaf Na^+/K^+ ratio as 2.6-fold and 3-fold as HAR/MAX and KAM/MAX plants; and also higher stem Na^+/K^+ ratio by 2.6-fold and 4.9-fold was recorded in NER/MAX plant in related to HAR/MAX and KAM/MAX plants at 200

mM NaCl (Figure 4). Moreover, the halophytic character of Nerina F1 scion was associated with both uptake selectivity of Ca^{2+} ion at the root and transport selectivity of this ion into the shoot where NER/MAX plant showed higher root and stem Ca^{2+} accumulation than HAR/MAX and KAM/MAX at high salt stress (Figure 5). In spite of the fact that Cl^- content was less important to cause toxic effects on tomato regarding the Na accumulation in all plant organs, it is worth to mention that the Cl^- accumulation was affected by scion genotype as Nerina F1 accumulated more leaf and stem Cl^- by (+40% and +95%), respectively than HAR/MAX and KAM/MAX plants at 200 mM NaCl (Figure 7). Taken together, we could confirm that the Maxifort F1 rootstock has the ability to operate two different defence mechanisms upon high salt level depending on the character of the scion genotypes used; an inclusion strategy when Nerina F1 scion was used and an exclusion mechanism presented when Haruki and Kamonium genotypes used. Similar results have been reported by Perez-Alfocea et al. (1996) who mentioned that tomato cultivar Radja shows a typical Na excluder character when used as a rootstock in saline media, while cv. Pera is a salt tolerant tomato ecotype with a semi-halophytic inclusion mechanism similar to that found in salt tolerant wild relatives of the tomato as the accumulation pattern of Na^+ in the leaves of plant grafted onto this rootstock is quite similar to that observed in halophytes (Perez-Alfocea et al. 1993); both genotypes are considered as salt tolerant within cultivated tomato. While Estaň et al. (2005) stated that low rate of Na^+ and Cl^- accumulation were found in plant grafted on Radja independently of the stress level and period of salt exposure, whereas Pera rootstock was able to use a strategy of include/excluder depending on salt level and salinization period.

5.2.1.1. Vegetative growth response, water status and gas exchange parameters of graft combinations against Maxifort F1

It is known that the plant could combine more than one strategy in order to preserve tissue dehydration and maintain growth (Fernández-García et al. 2004). The stomatal clouser is considered to be an efficient indicator of plant performance under stressful conditions where the resistant plant down-regulate efficiently the transpiration rate to alleviate salinity symptoms and preserve plant performances in both halophytes (Orsini et al. 2011) and glicophytes (Turhan and Eris 2007). This processing reduced ion-accumulation or desiccation in plant tissue (Masle et al. 2005; Orsini et al. 2010b). Ferreira-Silva et al. (2009) documented that the cashew plantlets grafted on the cashew BRS 226 rootstock presented higher leaf Na^+ and Cl^- concentrations than those grafted on the CCP 09 rootstock, and this higher ion accumulation was associated with greater transpiration when exposed to both mild and high salinity. In citrus plants grafted on the rootstock Cleopatra (excluder) and Carrizo

(includer), it was observed that the higher exclusion of toxic ions Na^+ and Cl^- from the shoot was associated with retention of this ion in roots and the lower transpiration rate (García-Sánchez et al, 2002). Our result showed that the key physiological components involved in photosynthesis such as the transpiration and net assimilation rate of all plant combinations affected strongly by salt treatment and showed differences in term of transpiration intensity and net assimilation rate (Table 3). It is important to note that the differential patterns of salt ion accumulation that induced by the Maxifort F1 rootstock in the HAR/MAX, KAM/MAX, and NER/MAX plants was closely associated with different responses of transpiration and net assimilation rate. The two HAR/MAX and KAM/MAX combinations showed the ability to maintain the essential components of the photosynthetic apparatus and exhibited maximum transpiration (0.81 and 0.74 mM m^{-2} s^{-1}) and net assimilation rate (6.46 and 7.23 μM m^{-2} s^{-1}) at higher salt stress (Table 3). Conversely, NER/MAX plant presented the lowest respective values of both parameters (0.24 mM m^{-2} s^{-1} and 1.41μM m^{-2} s^{-1}). These result clearly demonstrated that the type of scion genotype influence the leaf responses in term of intensity of transpiration rate and assimilation net. As we mentioned above, HAR/MAX and KAM/MAX combinations showed an exclusion mechanism to cope with severe salt and showed high capacity for retaining the Na ion into their roots (Figure 3). Consequently, the lower accumulation of Na^+ in the shoot was accompanied with higher *E* and *A* value (Table 3). Salt ion exclusion by root may result in higher salt resistance of the plant due to lower ionic toxicity, contributing to a metabolic stability and protection of the leaf tissue (Martinez-Rodriguez et al. 2008). On contrary, NER/MAX plant has a halophytic character and showed high ion concentration in its leaf (Figure 3) that associated with the lowest *E* and *A* value (Table 3). High Na^+ concentrations should alter ionic homeostasis in the cytosol and cause toxicity that alters key biochemical K^+-dependent functions such as those involved in photosynthesis, protein synthesis, and stomatal opening (Munns and Tester 2008). Besides, the salt excess in the root medium causes an osmotic stress, rapidly decreasing water uptake and inducing stomata closure. Consequently, transpiration, CO_2 photosynthetic assimilation and leaf growth are strongly restricted (Munns 2002).

Punctual WUE determination revealed that plant production per unit water used was increased in HAR/MAX by (1.6-fold) compared to KAM/MAX and NER/MAX plants under moderated salt conditions (Table 3). It seems that a transpirational water flux, via regulation of *gs* (Thompson et al. 2007) has contributed to partially elucidate the physiological basis of *WUE*. The graft combinations HAR/MAX and NER/MAX reflect a contrast response in

transpiration rate under mild salt stress to that observed under severe, and HAR/MAX plant experienced lower *E* value than NER/MAX (0.49 versus 0.67 mM m^{-2} s^{-1}) at mild salt showed (Table 3). Accordingly, the stomatal adaptation to salinity in HAR/MAX plant was associated with preserving of WUE (16.96) (Table 3) (He et al. 2009). Recent reports suggest that preserved *A* values associated with lower *E* may be considered as reliable indicators of overall salinity tolerance (Orsini et al. 2011, 2012, 2013), and this is substantiated by the greater WUE (16.96 mM CO_2 mM^{-1} H_2O) observed in salt tolerant plants undergoing salinity (Barbieri et al. 2012). From the presented results, it could be hypothesized that the simultaneous regulation of stomatal resulted in differential response in WUE to salinity by the different grafting combinations.

Leaf water potential (Ψ_w) is recognized as an index for whole plant water status (Orsini et al. 2010a) and maintenance of high Ψ_w is considered to be associated with dehydration avoidance mechanisms (Muscolo et al. 2010). The considerable decrease of Ψ_w is probably a result of the structural-functional changes ensuring the plants adaptation to the salt-treatment (Kaymakanova et al. 2008). In such cases, a process of osmotic self-adjustment occurs in the plant cells, directed towards the preservation of the water balance by means of accumulation of osmotically active solutes (Tezara et al. 2003). The higher osmotic potential redaction induced by salinity in both HAR/MAX and NER/MAX plants at 200 mM NaCl (-3.62 and -3.87 MPa) (Figure 2) could point out that these plant would be more tolerant than KAM/MAX plant to the osmotic effect and showed higher accumulation of Na and Cl toxic ions in their stem and leaf (Figure 3) and this strategy is considered a typical of the salt tolerant tomato genotype (Alian et al. 2000; Perez-Alfocea et al 1993). This result was consistent with Santa-Cruze et al. (2001) who mentioned that tolerance induced by a rootstock has been associated with a raised inorganic solute accumulation in leaves of the genotype Pera. However, it seems that the accumulation of salt ions in the leaves of both above plants combined with an efficient compartmentalization mechanism in vacuoles that could help them to maintain higher osmotic adjustment values (1.6 MPa) than KAM/MAX plant (0.9).

The shoot biomass reduction as a consequence to salinity is a well-recognized phenomena (Kaya et al. 2003), either due to osmotic reduction in water availability or to excessive ion (Na^+ and Cl^-) accumulation in plant tissues. In the untaken experiment, the differential growth response was related to the different exclusion ability of saline ions, as the includer

NER/MAX plant showed the highest reduction of shoot FW (-96%) and shoot DW (-84%) regarding the control plant under austere salt (Table 1), and this growth reduction was associated with the highest ion accumulation found in its leaves and root (150 and 113) (Figure 3). Accordingly, the slowest shoot growth of 200 mM NaCl salt treated NER/MAX could be a consequences of the high ion accumulation as reduced growth may be an adaptive strategy (Cuartero and Fernandez-Muňoz 1999), which could permit the plant moreover to maintain higher OA (Figure 2). However, regarding the excluder genotypes Haruki and Kamnium, the rootstock Maxifort F1 showed similar and less reduction of shoot fresh and dry weight of HAR/MAX and KAM/MAX (-85% and -75%, respectively) at 200 mM NaCl in related to their control plant (Table 1), suggesting that the rootstock characteristic able to increase the shoot growth under saline condition depend on the capacity of the shoot genotype to regulate the ion saline concentration (Santa-Cruz et al. 2002). Thus, we might confirm the founding of Oda. (2002) that the grafting-related effect on the development and growth of the scion was probably the result of physiological relationships existing between the scions and rootstocks. Taken together, the efficient reduction in all growth parameters might indicate that the compartmentation of toxic ions at leaf level in NER/MAX plant might have been inefficient mechanism to cope with salt stress. Also, we could indicate that the ability to exclude Na^+ (and Cl^-) from the shoot and to accumulate salt preferentially in the root in KAM/MAX and HAR/MAX plants do not correlate with the maintenance of growth (Tattini et al. 1997).

Rootstock effect on the fruit varied according to the characteristics of the shoot genotype, as the rootstock Maxifort F1 induced higher reduction in fruit fresh weight with kaminum and Nerina F1 scions (-86% and -74%), while it showed less redaction with Haruki scion (-66%) at mild salt stress (Figure 1). The lower fruit FW reduction in HAR/MAX plant could be attributed that the plant maintain high value of WUE (16.96 CO_2 mM^{-1} H_2O) at 100 mM NaCl (Table 3). However, the three scion genotypes exhibited severe similar reduction in fruit fresh weight accounted for 96% at 200 mM NaCl (Figure 1). On other hand, this similar reduction in fruit yield of the three scion plants corroborates the idea that the effectiveness of scions to induce different mechanisms of salt resistant to maintain the fruit yield at long-term salt stress was low.

5.2.1.2. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of graft combinations against Maxifort F1

In this work, there was no effect of both salt and scion genotypes on APX activity as the three scion genotypes: Haruki, Kamonium, and Nerina F1 that have been grafted onto Maxifort F1 rootstock (HAR/MAX, KAM/MAX, and NER/MAX) showed similar APX activity of treated and not treated plants (Figure 9). Similarly, GR activity was not affected either by scion or salt application except Nerina F1 scion that showed great reduction at high salt stress (Figure 9). These results might indicate that the three scions did not exert enough efforts to induce the defence mechanism of these two enzymes. Nonetheless, the effect of scion genotype was clear with Nerina F1 scion that showed higher activity of CAT upon both salt levels that associated with significant increasing in proline accumulation (Figure 10), suggesting that this scion has higher ability to remove ROS comparing with other scions.

Taking everything into account**,** the differential effects of the same rootstock in term of ion accumulation could be attributed to scion genotype characters as the Maxifort F1 rootstock has the ability to operate two different defence mechanisms upon high salt level depending on the character of the scion genotypes used: an inclusion strategy with Nerina F1 scion and an exclusion mechanism presented when Haruki and Kamonium genotypes used as scions. It is important to note that the differential patterns of salt ion accumulation that induced by the Maxifort F1 rootstock was closely associated with different responses of transpiration, net assimilation rate and WUE in shoot genotypes. At high salt stress, the lower accumulation of Na in the shoot of Haruki and Kamonium genotypes was accompanied with higher *E* and *A* value, while Nerina F1scion has a halophytic character and showed high ion concentration in its leaf that associated with the lowest *E* and *A* value. At moderated salt stress, the preserved *A* values in Haruki scion that associated with lower *E* was substantiated by the greater *WUE* which may be considered as reliable indicators of overall salinity tolerance. Moreover, Haruki and Nerina F1 scions operated dehydration avoidance mechanisms by achieving higher osmotic potential redaction upon salinization. The rootstock characteristic was able to regulate the shoot growth under saline condition depend on the capacity of the shoot genotype to regulate the ion saline concentration as the includer Nerina F1 scion that accompanied with high ion accumulation showed the highest reduction of shoot FW and DW which could be an adaptive strategy to cope with saline condition. Moreover, Nerina F1 scion directed towards the preservation of the water balance by means of accumulation of osmotically active solutes and accumulated more proline in the fruit and enhanced the activity of CAT enzyme, suggesting that this scion has higher ability to remove ROS comparing with other scions. However, the three scions did not exert enough efforts to induce

the defence mechanism of APX and GR fruit enzymes which could interpret to some extent the reduction in fruit yield among the three scions used. These results corroborate the idea that the effectiveness of scions to induce different mechanisms of salt resistant to maintain the fruit yield at long-term salt stress was low under the condition of this experiment.

5.2.2. Semi-halophytic shoot genotype and leaf compartmentation mechanism of graft combinations against R1 rootstock

Salt exclusion from the shoot is a key determinant for salt-tolerance in glycophytes (Hasegawa et al. 2000) that have in general a low efficiency of compartmentation at the leaf level (i.e., the ability to sequester Na^+ and Cl^- in the cell vacuole; Glenn et al.1998) and to excrete salts outside of the leaf cells via specialized organs (Shannon et al. 1994). It is well documented that the response of tomato species toward salt varies depending on genotype, salt concentration, and time exposure (Ashraf and Harris 2004; Maggio et al. 2004). In this study when R1 was employed as a rootstock, the three scion genotypes Haruki, Kaminom, and Nerina F1 exert significant effects on salt ion uptake and showed similar ability of withholding the leaf Na ion as root content (105 versus 102 $mg.g^{-1}$ DW, as an average, respectively) under 200 mM NaCl (Figure 3). A like leaf Na ion aggregation, Na stem data showed no significant difference was found among the three combinations and accounted for 58 $mg.g^{-1}$ DW, as an average at 200 mM NaCl (Figure 3). Similarly to the aerial Na accumulation, the root of three grafted plants showed a homogeneousness of Na ion and accumulated about (105 $mg.g^{-1}$ DW), as an average (Figure 3). Moreover, the root and leaf of the three graft combinations partitioned almost the same amount of Na ion (105 versus 102 $mg.g^{-1}$ DW, respectively) which was two times that of the stem Na (58 $mg.g^{-1}$ DW) at 200 mM NaCl. Thus, the three scion genotypes showed a semi-halophytic inclusion mechanism and high efficiency of compartmentation the salt ions at leaf level.

5.2.2.1. Vegetative growth response, water status and gas exchange parameters of graft combinations against R1 rootstock

The data of this experiment give a picture of some integrated mechanisms of acclimation to excess root zone salinity which is responsible to offer the salt tolerant to the plant. However, all growth parameters were inhibited in the three graft combinations that employed against R1 rootstock: HAR/R1, KAM/R1, and NER/R1 (Table 1). High salt treated KAM/R1 plant showed greater inhibition of shoot dry weight (84%) than HAR/R1 and NER/R1 (62% and

74%) in related to control plant, which suggests that the former combination is less tolerant to NaCl stress than the two latter combinations. Furthermore, under 200 mM NaCl, the root dry weight of KAM/R1 plant was affected strongly at high external salt and showed reduction by -67% of total root biomass, respecting its control plant, while root system of HAR/R1 plant showed no significant difference between the non-treated and high salt treated plant (32 versus 33 g $plant^{-1}$) (Table 1). The constant response that observed in high salt treated root dry weight of HAR/R1 (Table 1) are consistent with less significant reduction of evaporation (0.35 mM m^{-2} s^{-1}) than KAM/R1 (0.92 mM m^{-2} s^{-1}) and higher water use efficiency (14.04 versus 8.98 mM CO_2 mM^{-1} H_2O) (Table 3). However, the three graft combinations showed similar reduction in net assimilation rate upon 200 mM NaCl (-66%, as an average) (Table 3), and this reduction is more likely related to limitation of CO_2 diffusion into the leaves (Table 3). These result supported the idea that biochemical limitation of *A* become prevalent in leaves as a cause of massive salt load (Allakherdiev et al. 2000). However, the higher significant reduction of stomatal conductance of HAR/R1 and NER/R1 (-87% and -80%, data not shown) comparing with their control plant, in respect to KAM/R1 (-65%) at 200 mM NaCl, could reduce the water flow into these scions and impeding the flow of unwanted ions (Moya et al. 1999) which explain, at least partly, the lower significant osmotic adjustment that have been achieved in those two plants (0.90 and 0.59 MPa) in respect to KAM/R1 (1.18) (Figure 2) (Moya et al. 1999). Regarding the productivity of the three graft plants, the lower redaction in fruit yield at mild salt stress was in NER/R1 plant (-58%) comparing to control plant, while this redaction accounted for -80% in HAR/R1 and KAM/R1 plants (Figure 1). Consequently, the scion genotypes did not significantly affected the salt induced expression of physiological characters of photosystem apparatus and showed high reduction in fruit yield that ranged from -58% till -80% depending on the scion genotype at 100 mM NaCl, while the fruit fresh weight redaction was higher at 200 mM NaCl (-94%) regardless the scion genotypes.

5.2.2.2. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of graft combinations against R1 rootstock

There was low contribution of scion genotypes in term of enhancing the fruit enzymatic antioxidant system upon salinized condition. Haruki scion in HAR/R1 plant showed a sharp increasing in APX and SOD activities comparing with other scions in KAM/R1 and NER/R1 plants at high salt stress (Figure 9), which might suggest higher ability of this scion to remove ROS. However, there was no effect either of salt or scion genotype on CAT and GR activities

(Figure 9). Regarding the amino acids accumulation, despite that KAM/R1showed significant increasing in fruit protein content regarding the control plant, unexpectedly, the same plant showed less accumulation of proline than HAR/R1 and NER/R1 plants upon both salt stresses (Figure 10). This result confirms that proline not play a determinant role as osmoprotectant and the increased proline concentration may not be associated with salinity tolerance (Colmer et al. 1995). Taking everything into account, fruit free radical scavenging system did not played an important role in salinity tolerance of grafted tomato under the condition of this experiment. The shoot genotypes are relatively salt-sensitive and the antioxidant enzymes activities were more limited, accordingly there was high possibility that the oxidative stressed has been induced by the excess of NaCl which possibly caused high inhibition of fruit yield in all genotypes used.

Briefly, the three scion genotypes showed a semi-halophytic inclusion mechanism upon salinization. However, the scion genotypes did not significantly affect the salt induced expression of physiological characters of photosystem apparatus and showed high reduction in fruit yield either at moderated or high salt stresses regardless the scion genotypes. Additionally, there was low contribution of scion genotypes in term of enhancing the fruit enzymatic antioxidant system upon salinized condition.

5.2.3. Leaf compartmentaion and root extrude mechanisms of graft combinations against Arnold rootstock

It is known that Na^+ ion is the primary cause of ion-specific damage in cucumber, melon, watermelon, tomato, and eggplant, (Tester and Davenport 2003; Varlagas et al. 2010). Plants grafted onto appropriate rootstocks restricted the transport of Na^+ from root to shoot (Romero et al. 1997; Estaň et al. 2005; Goreta et al. 2008; Zhu et al. 2008a). Salt tolerance mechanisms can occur in a wide range of organizational levels from the cellular level (e.g., compartmentation of Na^+ within cells) to the whole plant (e.g., exclusion of Na^+ from the plant and exclusion of Na^+ from the shoot) (Tester and Davenport 2003; Møller et al. 2009). In this study, scion genotypes play a significant factor in Na transporting into shoot, where the leave of HAR/ARN plant accumulate more Na^+ ion by +36% than the root, suggesting that salinity resistance in HAR/ARN plant could be related to leaf compartmentation strategy (Figure 3). On contrary, the other graft plants KAM/ARN and NER/ARN displayed less Na^+ leaf accumulation by 21% than their root and the ion exclusion mechanism might be presented (Figure 3). This result was consistent with Goreta et al.(2008) who reported that the

capacity of 'Strong Tosa' (*C. maxima* Duch.×*C. moschata* Duch.) in watermelons to withstand salt stress better than other tested rootstocks is partially due to efficient Na^+ exclusion from the watermelon shoot.

5.2.3.1. Vegetative growth response, water status and gas exchange parameters of graft combinations against Arnold rootstock

The plant growth has been affected by salt stress depending on the characteristics of shoot genotypes. When Arnold genotype was used as a rootstock, the three scions induced different positive effect in term of root DW and showed increasing by +22%, +11%, and +62%, respectively in HAR/ARN, KAM/ARN, and NER/ARN in related to their control plants at mild salt stress (Table 1). This result was in agreement with Chen et al. (2003) who stated that some resistant scion has a positive effect on root biomass which due to a higher rate of photosynthesis of the more vigorous scions, leading to a greater potential for partitioning of assimilates to the rootstock. However, at 200 mM NaCl HAR/ARN plant show almost the same root biomass as its control plant, while KAM/ARN and NER/ARN showed reduction by (-62% and -32%) comparing with control plants (Table 1). Unexpectedly, despite the plant have received an increasing in root biomass at mild salt which was presume to enhance the translocation of nutrient and water status (Han et al. 2009), there was high reduction in fruit fresh weight as (-82%) in HAR/ARN and NER/ARN and (-62%) in KAM/ARN at 100 mM NaCl, while the medium reduction was higher under high salt stress and achieved 95% comparing the control plant (Figure 1). Additionally, the high exclusion capacity of Na ion from the shoot and extrude it back into the root in high salt stressed KAM/ARN plant was associated with higher reduction in shoot FW (-85%) and shoot DW (-78%) in respect to control plant, while HAR/ARN and NER/ARN plants showed less shoot FW redaction (-79% and -77%) as well as shoot DW reduction (-67% and -56%), respectively (Table 1). One explanation might be the excessive Na ion at root level of KAM/ARN plant restricted the plant growth.

Furthermore, the effect of genotypes variation on photosynthesis parameters was not presented even at high salt stress as all graft plant showed similar reduction in transpiration rate and net assimilation rate (Table 3). This significant reduction in *E* and *A* values respecting the control plants can be largely ascribed to the stomatal limitation (ZgallaÏ et al. 2006; He et al. 2009). Similarly, there was no large variation among these three plants in term of water potential (-2.5 MPa as an average) and osmotic potential (-2.8 MPa) at high salt

stress (Figure 2). However, the lowest osmotic adjustment was achieved in NER/ARN (0.6 MPa) (Figure 2), and this might be due to that this plant contain the lowest Na ion in its leaves (77 $mg.g^{-1}$ DW) (Figure 3) at 200 mM NaCl, in respect to other graft plants HAR/ARN and NER/ARN.

5.2.3.2. Antioxidant enzyme activities, organic solute accumulation and lipid peroxidation of graft combinations against Arnold rootstock

The comparison among the three scion genotypes: Haruki and kamonium and Nerina F1 in term of operating the antioxidant defence mechanism revealed that the Nerina F1 scion has higher ability to elicit increasing in the activities of APX, CAT, and GR in relation to control plant under both salt conditions (Figure 9), which might indicate higher ability to scavenge ROS. However, Haruki scion in HAR/ARN plant showed higher significant increasing in SOD activity than other scions in respect to its control plant (Figure 9). This increasing induced higher tolerance to oxidative stress (Bowler et al. 1991) and accordingly this plant showed lower MDA level upon salinization than KAM/ARN and NER/ARN (Figure 10).

Toward the end, scion genotypes play a significant factor in Na^+ transporting into shoot, where Haruki scion accumulate more leaf Na^+ ion than its root and showed leaf compartmentation strategy to cope with a drastic salt condition, while the other scions Kamonium and Nerina F1 displayed an exclusion mechanism that associated with higher root Na^+ accumulation. However, the root system exhibited significant increasing in root DW upon mild salt level depending on the characteristics of shoot genotypes since Nerina F1 showed the highest root DW increasing followed by Haruki and Kamonium scions. However, there were not too much variation in photosynthesis parameters and water status among these graft combinations. Moreover, the operating system of the antioxidant defence mechanism was depending on shoot genotypes as Nerina F1 scion has higher ability to enhance the activities of APX, CAT, and GR enzymes, while Haruki scion showed higher significant increasing in SOD activity that associated with lower MDA level upon salinization. Nevertheless, the shoot genotypes are relatively salt-sensitive and the antioxidant enzymes activities were more limited, accordingly there was high possibility that the oxidative stressed has been induced by the excess of NaCl which possibly caused high inhibition of fruit yield in all genotypes used.

6. CONCLUSION

The grafting with a salt tolerant rootstock should improve the photosynthesis with higher stomatal conductance and WUE under salt condition, increase the capacity of antioxidant

system by enhancing different enzymes activities particularly the enzymes involved in ascorbate-glutathione cycle, and decrease the level of lipid peroxidation, in turn promoting plant growth should be promoted. The characteristics of rootstocks could be the main factors that result in increased absorption, upward transport of some ions and translocation of these ions into the stem and leaves of the scion. In this study, different rootstocks showed different salt resistance mechanisms associated with different patterns of ion accumulation such as salt inclusion mechanism involving high concentration of sodium ion in the leaves, salt exclusion mechanism consisting in high capacity to extrude the ions from the shoot and retention into the root level, and leaf compartmentation strategy entailing Na accumulation in the leaf. The physiological plant responses such as water relation, net assimilation rate, transpiration rate, and WUE were affected strongly by rootstock genotypes upon salinization. Some rootstocks used the high concentrations of inorganic solutes from the substrate to achieve lower water and osmotic potential, which is considered an osmotically adaptive strategy, while other rootstocks induced reduction in net assimilation rate and stomatal conductance, which could be a mechanism of water conservation in order to maintain leaf turgor and osmotic adjustment under salt stressed. Additionally, other rootstocks enhanced the vigour of root system, which is considered a fundamental issue to sustain the translocation of mineral nutrition into the shoot. However, all used rootstocks did not register high capacity in conferring the resistance to the shoot and yield and did not present a great ability to enhance the enzymatic detoxification mechanism in the fruit; accordingly ROS were not eliminated and oxidative stress took place.

Mechanisms of resistance against salinity in grafted plants display a great complexity; such complexity may be associated with specific interactions between the genotypes of scion as well as rootstock. The plant responses were conditioned significantly by the scion genotypes as well as rootstock characteristics, where both scion and rootstock showed specific effects in ion absorption, upward transport and accumulation of ions, thereby stimulating different physiological and biochemical strategies. The physiological and physical characteristics of rootstock probably affect the absorption of the ions while the characteristics of scion affect their translocation in the plant. Moreover, the characteristics of rootstock were able to regulate the plant growth under saline condition depending on the capacity of the shoot genotypes to regulate the ion uptake. It is important to take into account the salt tolerance mechanisms of both scion and rootstock before proceeding to the selection of grafting combinations. Our results demonstrate that both scion and rootstock exert significant effects

on morphological and physiological parameters linked to the activities of root (root biomass, water and nutrient uptake, and salt ion uptake) or leaves (intensity of transpiration rate and stomatal performance) to cope with salt stress. However, the effectiveness of different mechanisms of acclimation of scions and rootstocks to excess root zone salinity, which is responsible for providing salt tolerance to the plant, were not enough to enhance the plant growth. Moreover, both scions and rootstocks were not able to enhance the fruit enzymatic detoxification mechanism of ROS. Thus, the free radical scavenging system did not play an important role in salinity tolerance of grafted tomato under the condition of this experiment. We could indicate that the salt grafted tomato plants were not tolerant to the stress induced by excess NaCl as the grafted tomato seedling showed lower ability to maintain high net photosynthesis and showed non-effective scavenging system of ROS. Additionally, the three rootstocks used in grafting failed to achieve high productivity where the fruit fresh weight reduction was so high even at mild salt. These results could point out that those rootstocks are not relatively salt tolerant and they are not able to donate resistance to shoots.

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Table 1. Effect of salt stress (0, 100, and 200 mM NaCl) on shoot and root biomass of different tomato graft combinations at 90 DAS. Same letters in each column indicate no significant differences among treatments at $P \leq 0.05$ level. Values are the mean ± SE of three replications.

Scion	Rootstock	Scion/rootstock	NaCl (mM)	Shoot FW (g Plant^{-1})	Shoot DW (g Plant^{-1})	Root DW (g Plant^{-1})
HARUKI	MAXIFORT F1	HAR/MAX	0	765b	96b	101a
			100	242fg	44fg	71b
			200	120gh	27hi	64c
	ARNOLD	HAR/ARN	0	474e	55ef	27fg
			100	165gh	41gh	33ef
			200	99gh	18ij	28fg
	R1	HAR/R1	0	610cd	65de	32ef
			100	241fg	40gh	74b
			200	143gh	25hi	33ef
KAMONIUM	MAXIFORT F1	KAM/MAX	0	1175a	139a	103a
			100	336f	72cd	98a
			200	174gh	35gh	33ef
	ARNOLD	KAM/ARN	0	711bc	102b	55cd
			100	237f	45fg	61c
			200	107gh	22hi	21g
	R1	KAM/R1	0	768b	109b	83ab
			100	232fg	41gh	47cd
			200	85gh	17ij	27fg
NERINA F1	MAXIFORT F1	NER/MAX	0	690bc	81c	83ab
			100	243fg	43fg	49cd
			200	30h	13j	20g
	ARNOLD	NER/ARN	0	622cd	72cd	50cd
			100	327f	57ef	81ab
			200	146gh	25hi	34ef
	R1	NER/R1	0	568de	62de	42de
			100	196fg	29hi	39de
			200	93gh	16j	29fg

Table 2. Analysis of variance of shoots and root biomass: ns, non-significant differences; *, significant differences at $P \leq 0.05$; **, significant differences at $P \leq 0.01$; ***, significant differences at $P \leq 0.001$.

Analysis of variance	Fruit FW	Shoot FW	Shoot DW	Root DW
Main Effect				
Salt	***	***	***	***
Rootstock	ns	***	***	***
Scion	***	***	***	*
Interaction				
Salt * rootstock	ns	***	***	***
Salt * scion	***	***	***	**
Rootstock * scion	***	***	***	***
Salt * rootstock * scion	***	ns	ns	**

Table 3. Effect of salt stress (0, 100, and 200 mM NaCl) on photosynthesis parameters of different tomato graft combinations at 60 DAS. Same letters in each column indicate no significant differences among treatments at $P \leq 0.05$ level. Values are the mean ± SE of three replications.

Scion	Rootstock	Scion/ rootstock	NaCl (mM)	E (mM m^{-2} s^{-1})	A (µM m^{-2} s^{-1})	WUE (mM CO_2 mM^{-1} H_2O)
HARUKI	MAXIFORT F1	HAR/MAX	0	2.69b	17.10bc	6.38cd
			100	0.49fg	7.61ef	16.96a
			200	0.81ef	6.46ef	8.62c
	ARNOLD	HAR/ARN	0	2.88ab	17.66bc	6.23cd
			100	0.38fg	5.68ef	15.01ab
			200	0.73ef	5.81ef	8.34c
	R1	HAR/R1	0	2.73ab	13.79cd	5.22cd
			100	1.47c	9.05e	6.50cd
			200	0.35gh	4.59ef	14.04ab
KAMONIUM	MAXIFORT F1	KAM/MAX	0	1.85c	22.23a	12.19bc
			100	0.81ef	8.37ef	10.37bc
			200	0.74ef	7.23ef	10.28bc
	ARNOLD	KAM/ARN	0	2.96ab	17.35bc	5.87cd
			100	0.53fg	8.19ef	16.51a
			200	0.64fg	6.63ef	11.02bc
	R1	KAM/R1	0	3.19a	16.83bc	5.31cd
			100	0.80ef	8.92e	11.24bc
			200	0.92e	5.08ef	8.98c
NERINA F1	MAXIFORT F1	NER/MAX	0	2.11c	15.54cd	8.04c
			100	0.67fg	6.62ef	10.53bc
			200	0.24gh	1.41g	7.43c
	ARNOLD	NER/ARN	0	2.93ab	18.55b	6.51cd
			100	0.92e	8.16ef	9.02c
			200	0.57fg	5.89ef	10.99bc
	R1	NER/R1	0	2.70b	14.13cd	5.19cd
			100	0.79ef	6.25ef	8.38c
			200	0.54fg	5.26ef	10.73bc

Table 4. Analysis of variance of photosynthesis parameters: ns, non-significant differences; *, significant differences at $P \leq 0.05$; **, significant differences at $P \leq 0.01$; ***, significant differences at $P \leq 0.001$.

Analysis of variance	E	A	WUE
Main Effects			
Salt	***	***	***
Rootstock	***	**	**
Scion	*	***	**
Interaction			
Salt * rootstock	***	***	***
Salt * scion	**	ns	**
Rootstock * scion	***	***	ns
Salt * rootstock * scion	***	***	***

Table 5. Analysis of variance of ions accumulation: ns, non-significant differences; *, significant differences at $P \leq 0.05$; **, significant differences at $P \leq 0.01$; ***, significant differences at $P \leq 0.001$.

Analysis of Variance	Ca^{2+}			K^{+}			Na^{+}			Na^{+}/K^{+}		
	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
Main Effects												
Salt	***	***	***	***	***	***	***	***	***	***	***	***
Rootstock	***	***	***	***	**	***	***	***	***	***	***	***
Scion	***	***	**	ns	***	***	***	***	***	***	***	***
Interaction												
Salt * rootstock	***	*	ns	***	ns	ns	***	***	***	***	***	***
Salt * scion	*	*	ns	*	ns	***	*	***	**	ns	***	***
Rootstock * scion	*	ns	ns	***	*	**	***	***	***	ns	***	***
Salt * rootstock * scion	*	ns	ns	***	ns	***	***	***	***	*	***	**

Table 6. Analysis of variance of Cl^- accumulation: ns, non-significant differences; *, significant differences at $P \leq 0.05$; **, significant differences at $P \leq 0.01$; ***, significant differences at $P \leq 0.001$.

Analysis of variance	Cl^-		
	Root	Stem	Leaf
Main Effects			
Salt	***	***	***
Rootstock	***	***	**
Scion	ns	ns	*
Interaction			
Salt * rootstock	*	ns	*
Salt * scion	ns	***	*
Rootstock * scion	**	***	Ns
Salt * rootstock * scion	*	***	Ns

Table 7. Analysis of variance of antioxidant enzymes and organic solutes: ns, non-significant differences; *, significant differences at $P \leq 0.05$; **, significant differences at $P \leq 0.01$; ***, significant differences at $P \leq 0.001$.

Analysis of Variance	APX	CAT	GR	SOD	MDA	Proline	Protein
Main Effect							
Salt	***	***	***	***	***	***	***
Rootstock	***	***	***	**	ns	*	ns
Scion	ns	***	ns	ns	***	***	***
Interaction							
Salt * rootstock	***	***	**	**	**	***	ns
Salt * scion	***	***	ns	**	***	***	**
Rootstock * scion	**	***	***	**	***	***	***
Salt * rootstock* scion	***	***	**	ns	**	***	***

Figure 1. Effect zero salt (black bar), 100 mM NaCl (gray bar), and 200 mM NaCl (white bar) on fruit fresh weight of different tomato graft combinations at 90 DAS. Values are the mean ± SE of three replications.

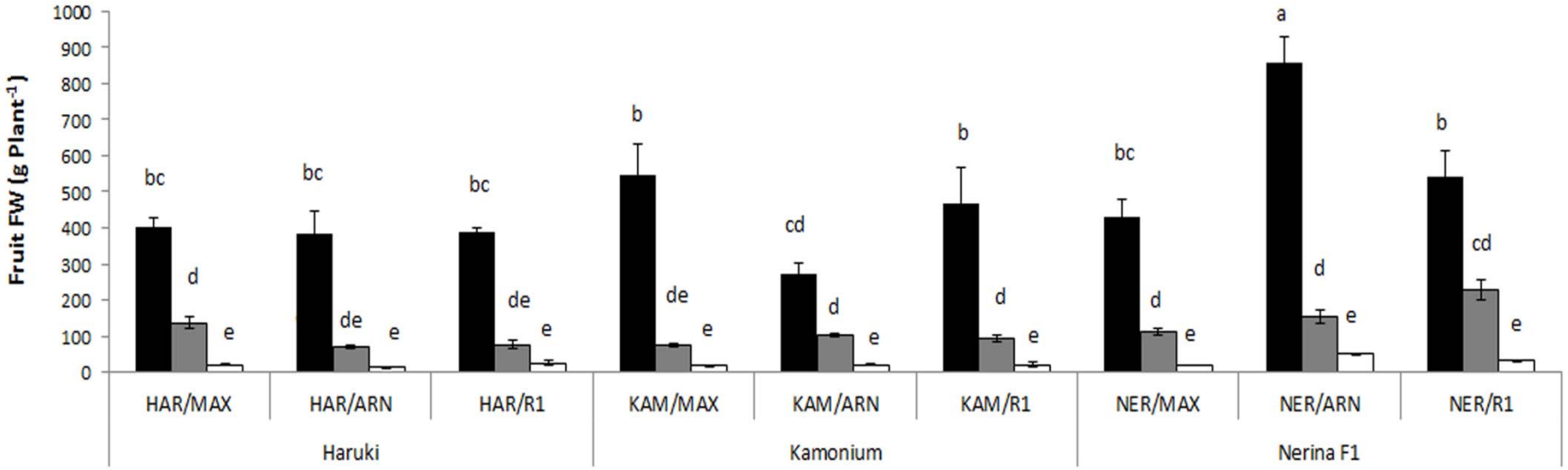


Figure 2. Effect zero salt (black bar), 100 mM NaCl (gray bar), and 200 mM NaCl (white bar) on leaf water relation status of different tomato graft combinations at 60 DAS. Values are the mean ± SE of three replications.

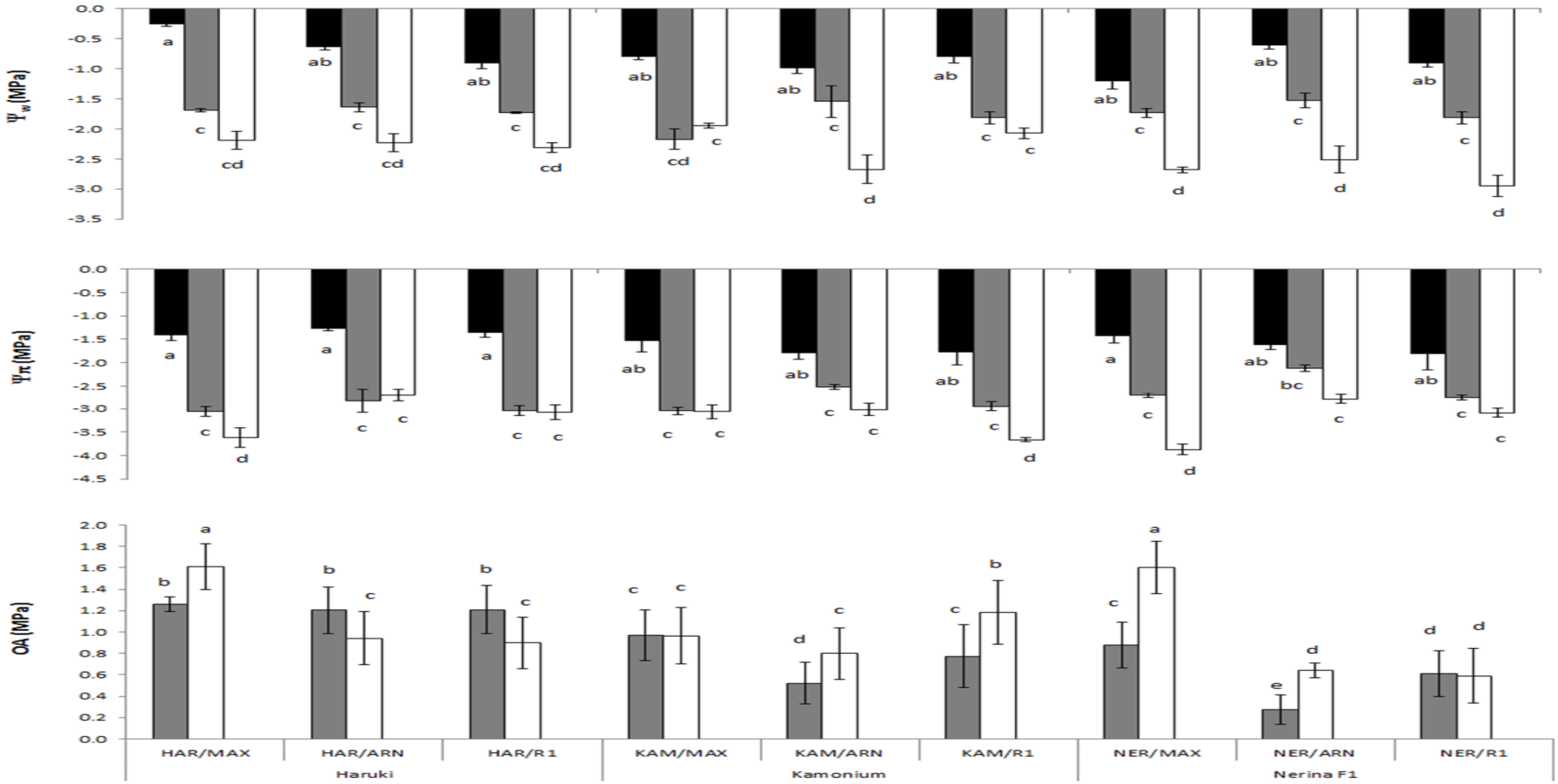


Figure 3. Effect zero salt (black bar), 100 mM NaCl (gray bar), and 200 mM NaCl (white bar) on root, stem, and leaf Na^+ ion accumulation of different tomato graft combinations at 90 DAS. Values are the mean ± SE of three replications.

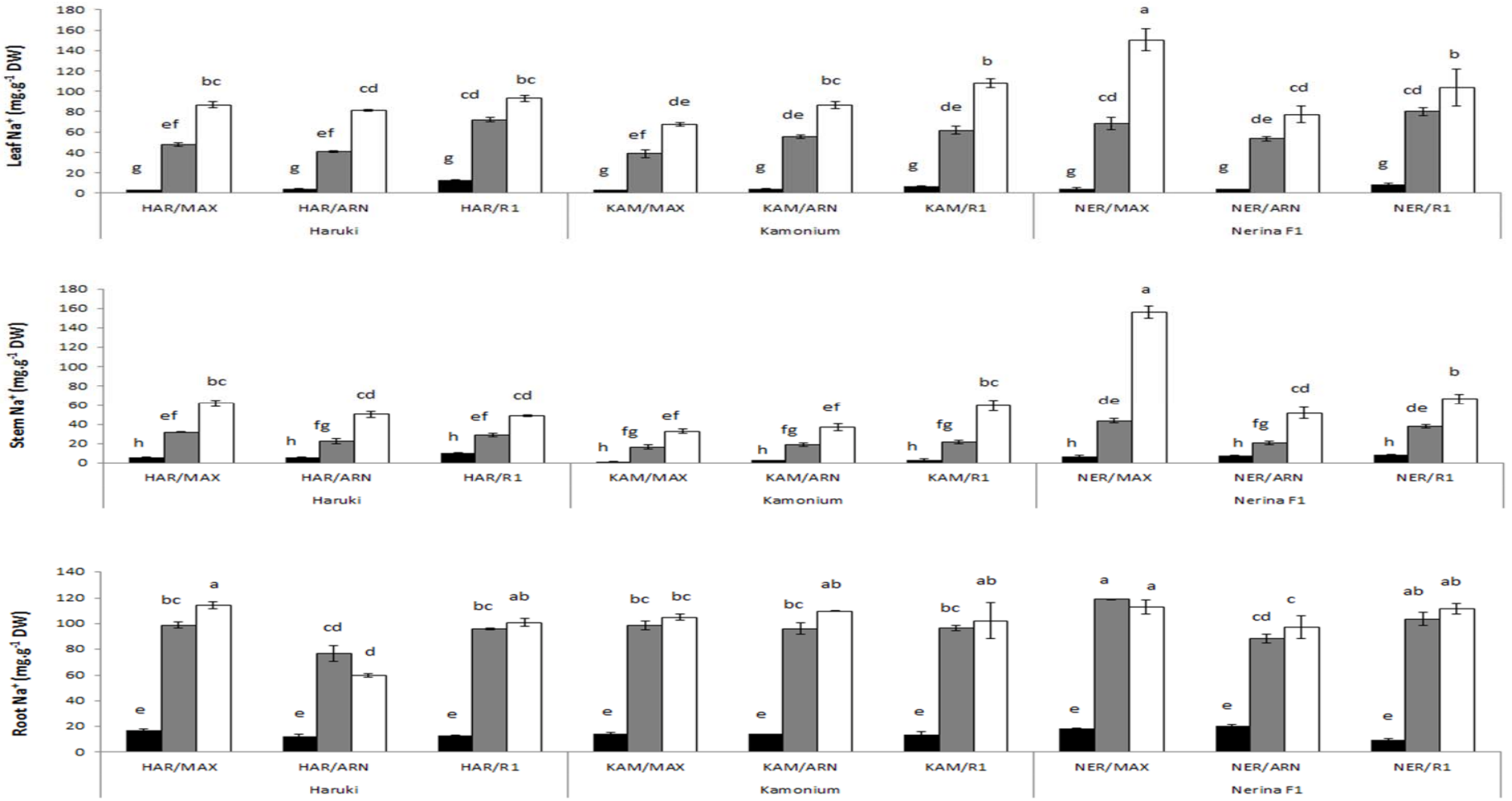


Figure 4. Effect zero salt (black bar), 100 mM NaCl (gray bar), and 200 mM NaCl (white bar) on root, stem, and leaf Na^+/K^+ ratio of different tomato graft combinations at 90 DAS. Values are the mean ± SE of three replications.

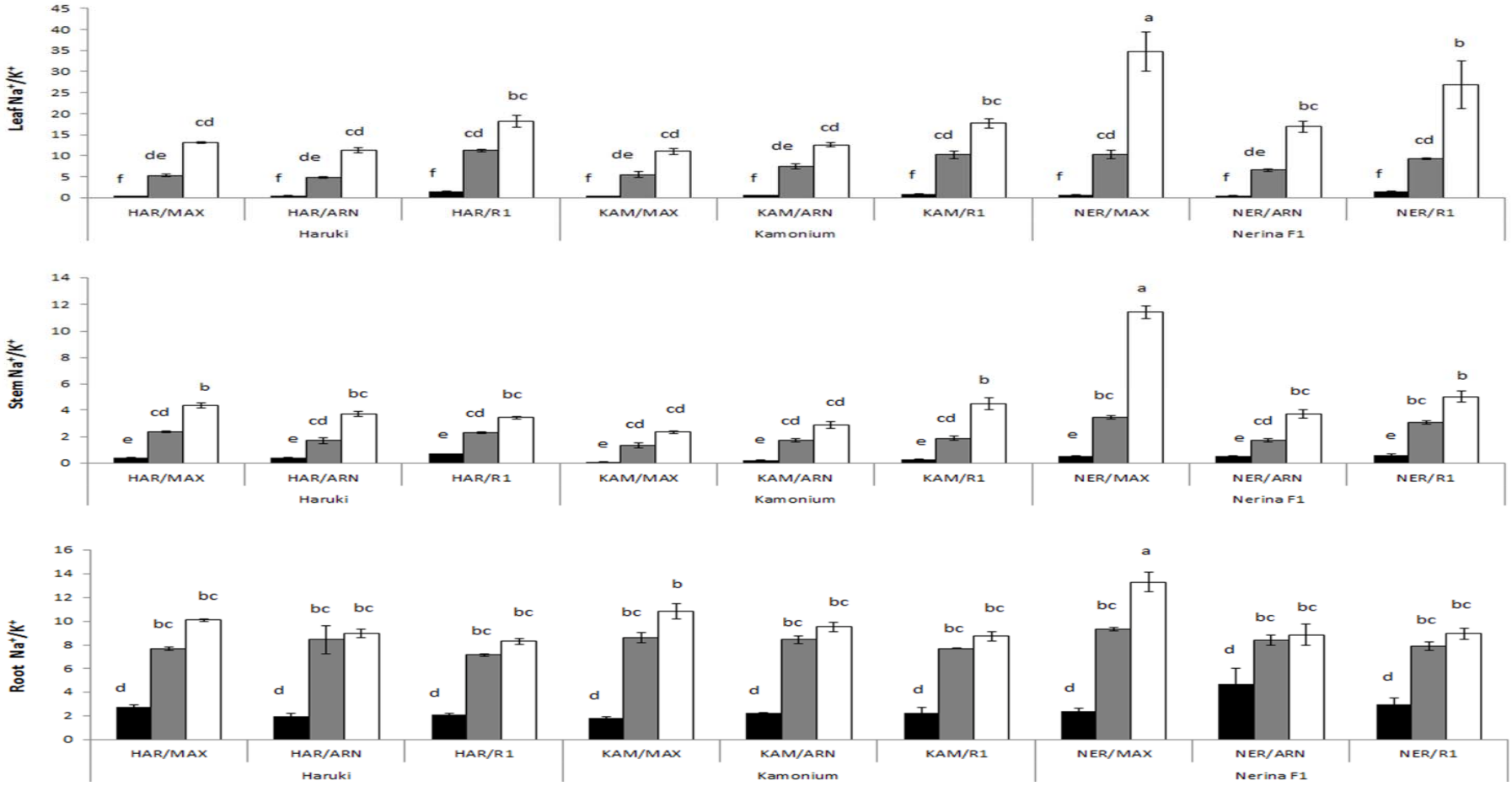


Figure 5. Effect zero salt (black bar), 100 mM NaCl (gray bar), and 200 mM NaCl (white bar) on root, stem, and leaf Ca^{2+} ion accumulation of different tomato graft combinations at 90 DAS. Values are the mean ± SE of three replications.

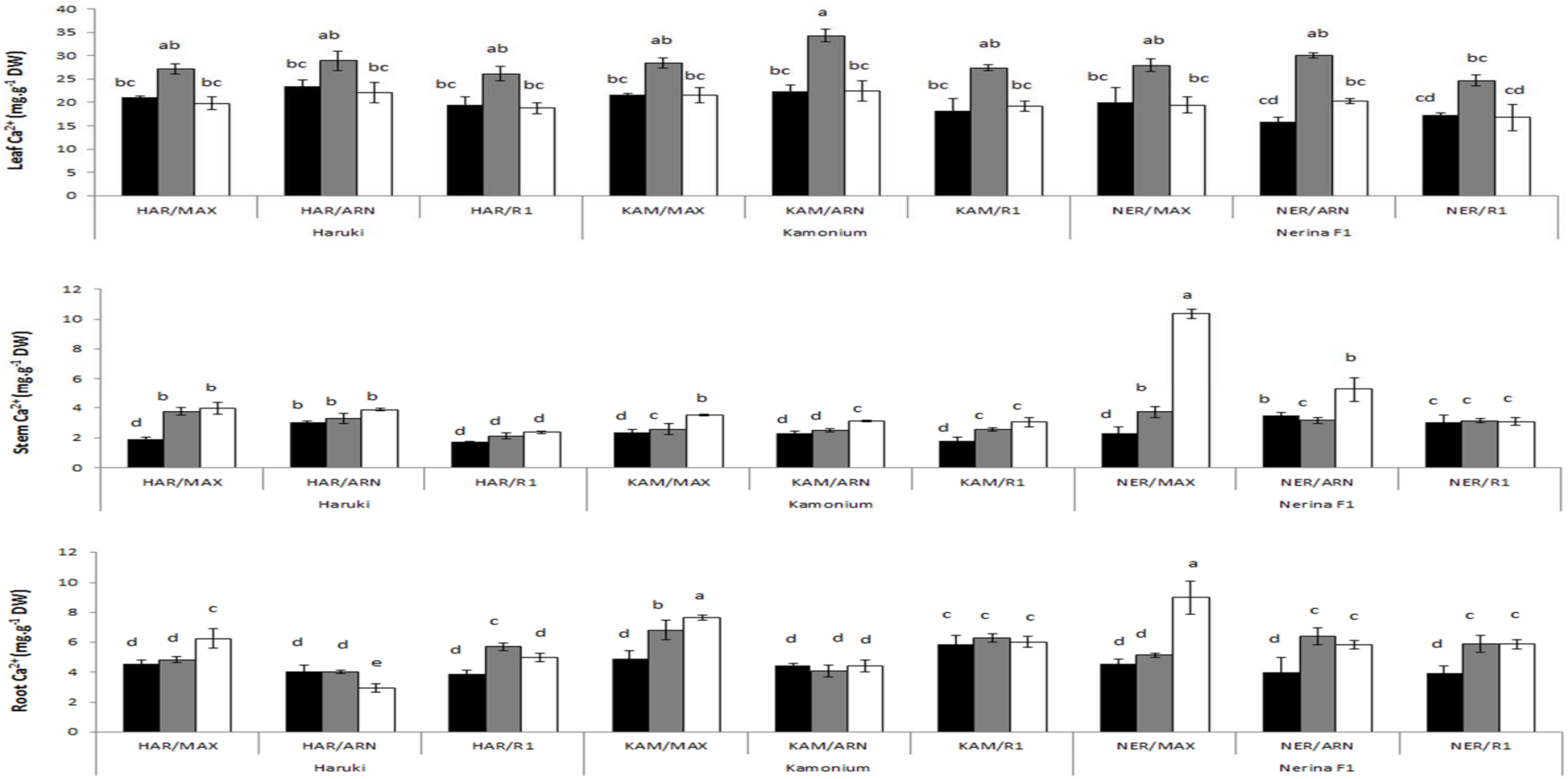


Figure 6. Effect zero salt (black bar), 100 mM NaCl (gray bar), and 200 mM NaCl (white bar) on root, stem, and leaf K^+ ion accumulation of different tomato graft combinations at 90 DAS. Values are the mean ± SE of three replications.

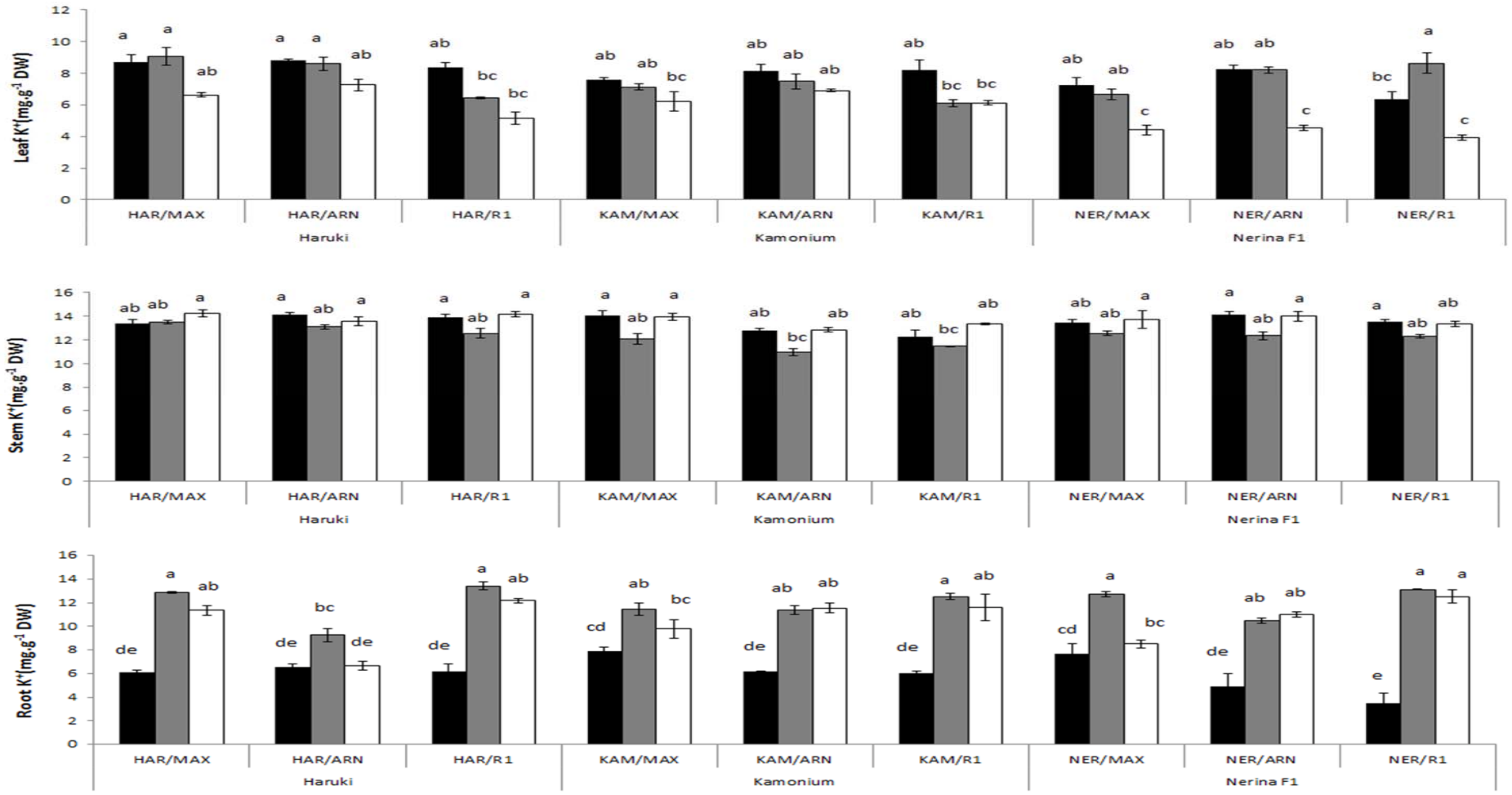


Figure 7. Effect zero salt (black bar), 100 mM NaCl (gray bar), and 200 mM NaCl (white bar) on root, stem, and leaf Cl ion accumulation of different tomato graft combinations at 90 DAS. Values are the mean ± SE of three replications.

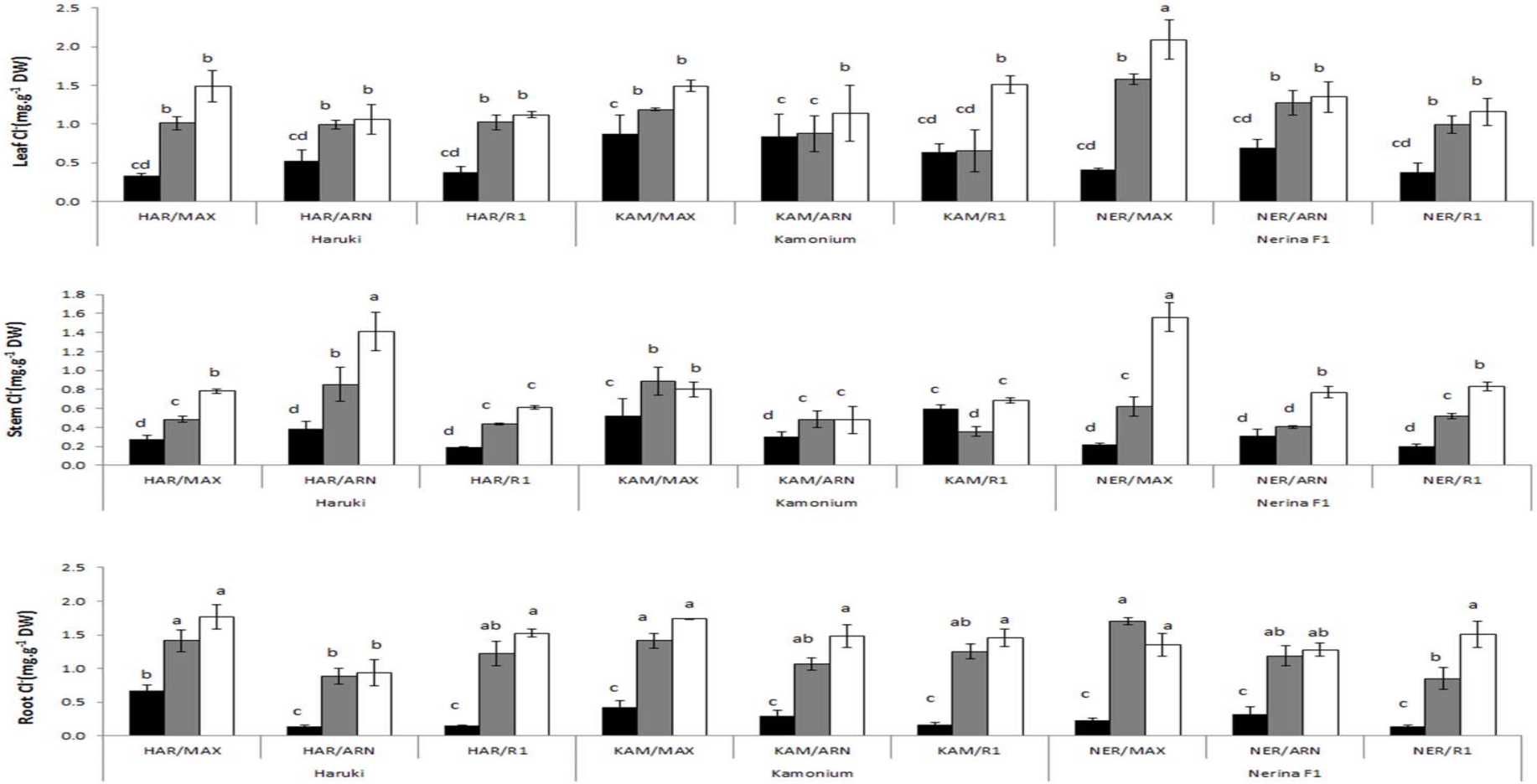


Figure 8. Total plant Na^+ ion partitioning: root Na (black bar), stem Na (grey bar), and leaf Na (white bar) of different tomato graft combinations at 90 DAS. Values are the mean ± SE of three replications.

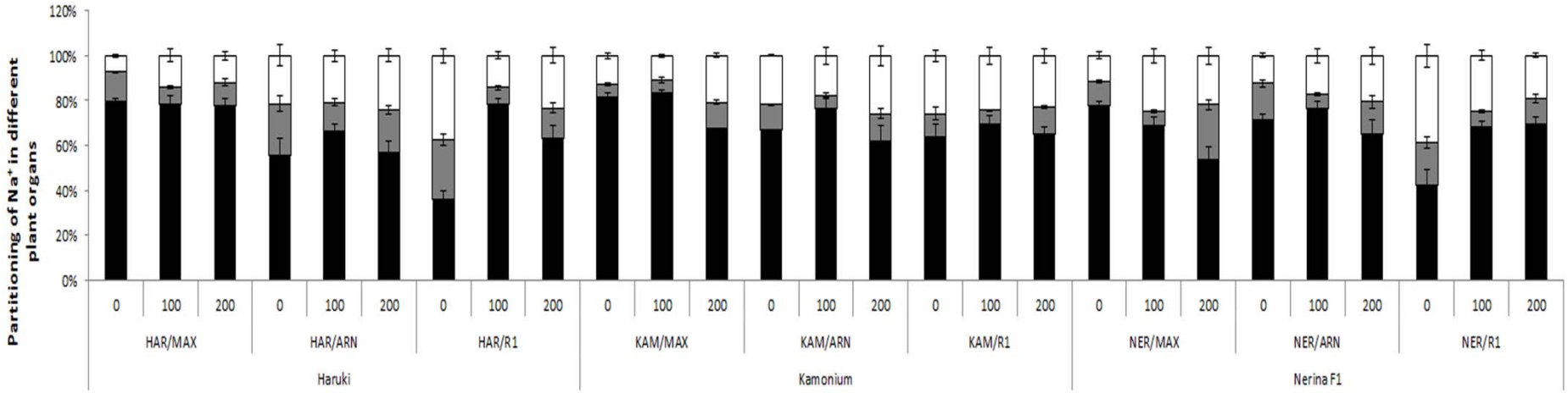


Figure 9. Effect zero salt (black bar), 100 mM NaCl (gray bar), and 200 mM NaCl (white bar) on fruit antioxidant enzymes of different tomato graft combinations at 90 DAS. Values are the mean ± SE of three replications.

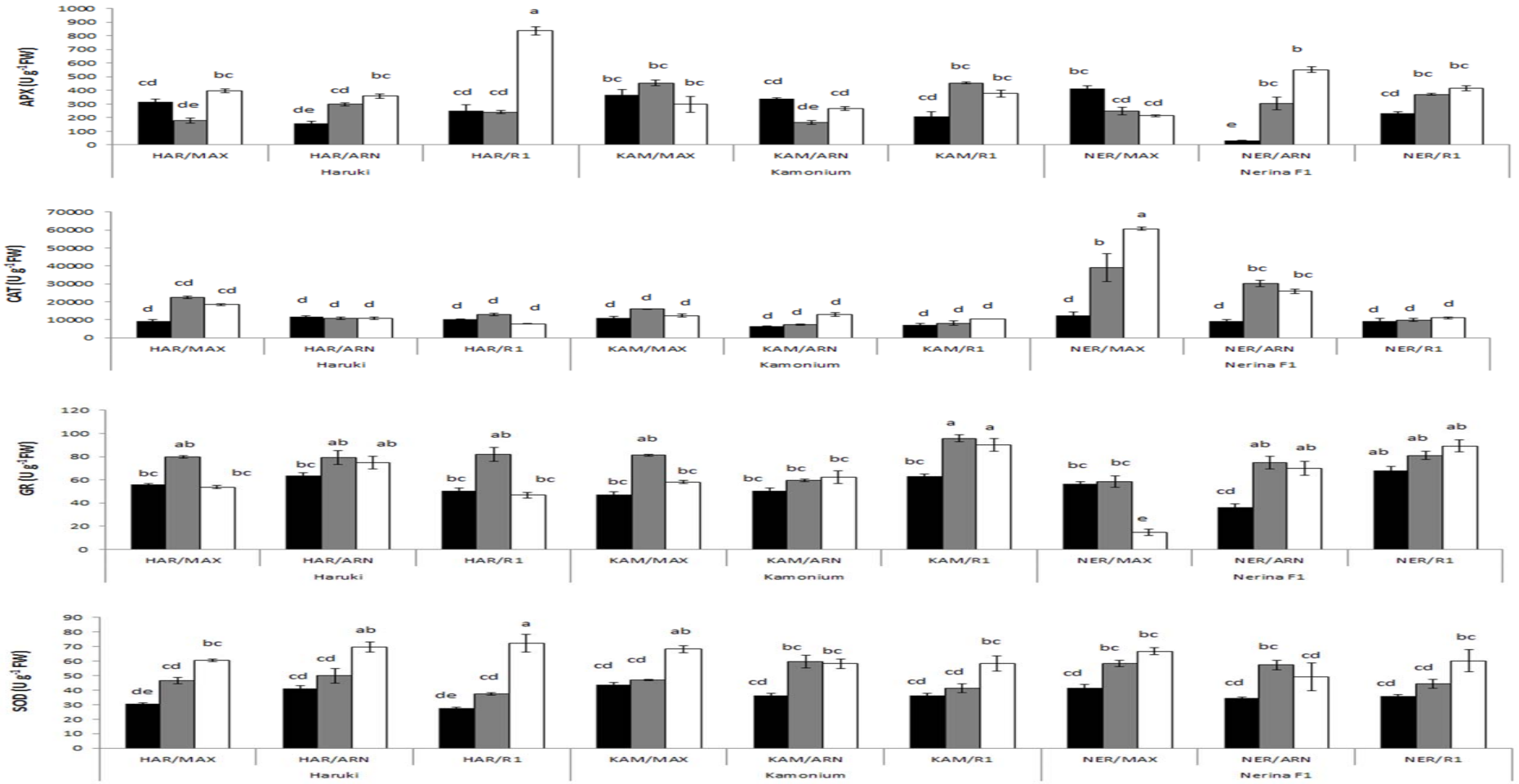
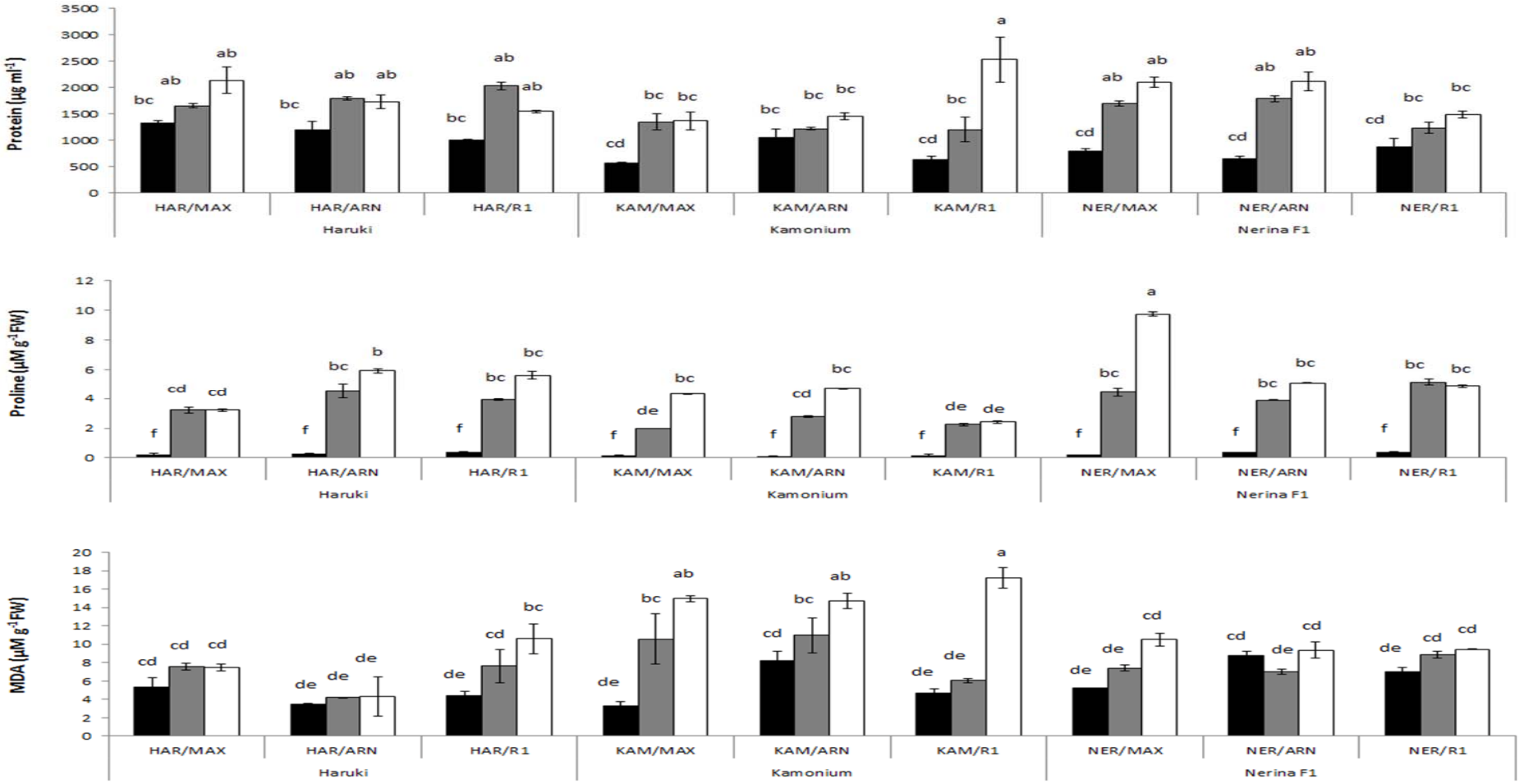


Figure 10. Effect zero salt (black bar), 100 mM NaCl (gray bar), and 200 mM NaCl (white bar) on fruit organic solutes and MDA concentration of different tomato graft combinations at 90 DAS. Values are the mean ± SE of three replications.



CHAPTER 3

TOWARDS UNDERSTANDING OF EVOLUTION OF SALINITY TOLERANCE IN *BRASSICACEAE* SPECIES (CABBAGE AND RADISH)

I. Salinity thresholds and genotypic variability of Cabbage (*Brassica oleracea* L.) grown under saline stress: physiological adaptation and nutritional value

II. Physiological response of white and red radish seedling, metabolites features and antioxidant enzyme activities among seven root radish cultivars (*Raphanus sativus* L.) in salinized medium

CHAPTER 3

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CHAPTER 3

I. Salinity Thresholds And Genotypic Variability Of Cabbage (*Brassica Oleracea* L.) Grown Under Saline Stress: Physiological Adaptation And Nutritional Value

ABSTRACT

Excessive soil salinity, resulting from natural processes or from crop irrigation with saline water, occurs in many semi-arid to arid regions of the world where it inhibits plant growth and yield. The use of saline irrigation water is becoming unavoidable in many horticultural zones thus resulting in increasing salinization of soils and aquifers. In the present study, two botanical varieties of *Brassica*, namely savoy (*Brassica oleracea* var. *sabauda* L.) and white cabbage (*Brassica oleracea* var. *capitata* L.) were grown under saline stress, in order to understand the physiological and biochemical elements of functional salt stress response beyond the salinity threshold. Thirteen salt concentrations (range 0 to 300 mM NaCl) were considered in the first trial, and out of them three (0, 100 and 200 mM NaCl) were selected for use in the second experiment. Measures considered morphological, physiological and biochemical parameters. Exp. 1# enabled to define two salinity thresholds (respectively at 100 and 200 mM NaCl), where the plant response resulted to simultaneously vary in terms of both morphological and physiological elements. In Exp. 2#, moderate salinity (100 mM NaCl) had lower effects on savoy cabbage yield (-16% as compared to control) than in the white cabbage (-62% of control). Concurrently, 100 mM NaCl resulted in a significant increase of antioxidant enzymes (Ascorbate peroxidase, Catalase, and Glutathione reductase) from control conditions, that was greater in savoy (+289, +423 and +88% respectively) as compared to white (+114, +356 and +28% respectively) cabbage. Ions accumulation resulted to be a key determinant in tissue osmotic adjustment (mainly in savoy) whereas the contribution of organic hosmolites was negligible. Overall, the higher antioxidative enzymatic activities in savoy cabbage upon 100 mM NaCl were associated with lower values of water and osmotic potentials as well as higher osmotic adjustment, thus suggesting a possible physiological pathway for alleviating salt stress.

Key words: salt stress, water relations, gas exchange parameters, antioxidative enzymes, *Brassica oleracea.*

1. INTRODUCTION

Salinity stress affects crop growth and yield by reduction of osmotic potential, alterations in plant metabolism, inhibition of enzymatic activities, ionic imbalance, disturbances in solute accumulation, specific ion effects or combination of all these factors (Munns *et al.* 2006). Adverse effects on plant growth and development are experienced at physiological and molecular levels (Vinocur and Altman 2005; Bressan *et al.* 2013). Osmotic adjustment helps plant cells to withstand salt stress and water deficit by maintaining a sufficient turgor for growth (Orsini *et al. 2011*). It involves the transport, accumulation, and compartmentation of inorganic ions and organic solutes (Orsini *et al.* 2013). Under saline conditions, the osmotic withdrawal of water from growing cells may cause their turgor to drop below yield-stress thresholds. Cells must then develop a sufficient low osmotic potential to reverse the flow of water, either through the uptake of ions from the medium or by the synthesis and transport of organic compounds; if none of these actions occur, cell expansion will stop (De Pascale *et al.* 2012*)*. Salt stress reduces gas exchange thereby limiting CO_2 supply to the leaf and causing the over-reduction of the photosynthetic electron transport chain, resulting in production of reactive oxygen species (ROS) (Mateo *et al.* 2004). ROS are highly unstable compounds that can seriously disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids in the absence of any protective mechanism (Mittler 2002). The generation of ROS, including superoxide radical (O_2•-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), and singlet oxygen ($^1O_2$), is generally enhanced in salt stressed plants (Bowler *et al.* 1992; Asada 1999). In order to cope with continuous ROS production, plants have a machinery of enzymatic and non-enzymatic antioxidants, which function as an extremely efficient cooperative system. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione reductase (GR), whereas glutathione (GSH), ascorbate (AsA), carotenoids and tocopherols are non-enzymatic antioxidants (Jogeswar *et al.* 2006). SOD is the front-line enzyme in ROS attack since it rapidly scavenges superoxide, one of the first ROS intermediate to be produced, dismutating it to H_2O_2 (Bowler *et al.* 1992). However, this reaction only converts one ROS to another, and H_2O_2 also needs to be removed since it promptly attacks thiol proteins and reacts with radicals and transition metals to yield the extremely reactive hydroxyl radical (Noctor and Foyer 1998). H_2O_2 is scavenged by peroxidase, especially, ascorbate peroxidase (APX) and catalase (CAT) (Mittler 2002; Karim *et al.* 2012). Glutathione reductase (GR), in the ascorbate/glutathione cycle, has a major role in maintaining the intracellular glutathione pool in the reduced state (GSH) (Jiménez *et al.* 1997). Plants can use the level of steady-state cellular ROS to monitor their intracellular level

of stress (Mittler 2002). However, this steady-state level must be tightly regulated in order to prevent an oxidative burst by over accumulation of ROS, which would ultimately result in extensive cell damage and death (Mittler 2002). Symptoms of oxidative damage (like lipid peroxidation) have been used to assess the increase in ROS production under abiotic stresses (Smirnoff 1993). However, the lack of symptoms is likely to result on the concomitant increase in cellular antioxidant defences. It is generally assumed that salt-sensitive genotypes have low levels of antioxidant enzymes (Logan 2005), although these levels are not necessarily an indicator of salinity tolerance (Munns and Tester 2008). Cabbage (*Brassica oleracea capitata* L.) is a relatively salt-tolerant crop (Maggio *et al.* 2005), although variability among genotypes has been reported (Jamil *et al.* 2007). Consistently, previous reports showed that increased level of salt caused unbalanced nutrient uptake, declined germination, delayed emergence, inhibited seedling growth, root and shoot length, and fresh root and shoot weight in cabbage (*Brassica oleracea capitata* L.) and pak-choi (*Brassica campestris*) (Jamil *et al.* 2005, 2006 and 2007). The objectives of this study were the identification of salinity tolerance thresholds and the assessment of the morphological and productive performances, the phytochemical, secondary metabolite, and enzymatic antioxidative systems in two botanical varieties of cabbage under different salt treatments for the understanding of the salt adaptive mechanisms responsible of the differential response.

2. MATERIALS AND METHODS

The present research consisted of two experiments on *Brassica olereacea*. Exp. 1# was conducted in order to identify and confirm salt tolerance thresholds in white cabbage (*Brassica oleracea* var. *Capitata* L), whereas Exp. 2# used selected salt concentrations for determining differential elements of salt stress enzymatic response in two botanical varieties of cabbage, namely white (same genotype as in Exp. 1#) and savoy (*Brassica Oleracea* var. *Sabauda* L.). Both experiments were conducted in environmentally controlled conditions (T° max 23°C; T°min 13°C; RH: 60%) in the experimental glasshouse at the University of Bologna, Italy. Seeds were sown in polyethylene trays filled with peat moss and transplanted 20 days after germination onto 5 liters-pots filled with a mixture of perlite and vermiculite (1:1, v:v). Plants were grown on a hydroponic system and fed with nutrient solution having the following composition: NO_3^-: 16.5 mM; NH_4^+: 1 mM; $H_2PO_4^-$: 1.50 mM; SO_4^{2-}: 1.50 mM; K^+: 7.0 mM; Ca^{2+}: 5.0 mM; Mg^{2+}: 1.5 mM; Fe^{2+}: 15 μM; Mn^{2+}: 10 μM; B^+: 25 μM; Zn^+: 5.0 μM; Cu^+: 0.5 μM; Mb^{2+}: 0.5 μM. Salt stress treatments started at 20 Days After

Transplanting (DAT). All morphological and physiological measurements were performed at 50 Days After Salt (DAS). Experimental details are reported below.

Exp. 1#

2.1. Experimental design

Thirteen salt concentrations were applied and ranged 0 to 300 mM NaCl, in measure of 0 (2.68 dS m^{-1}), 25 (4.01 dS m^{-1}), 50 (6.33 dS m^{-1}), 75 (7.05 dS m^{-1}), 100 (7.68 dS m^{-1}), 125 (8.04 dS m^{-1}), 150 (8.35 dS m^{-1}), 175 (8.5 dS m^{-1}), 200 (8.72 dS m^{-1}), 225 (8.86 dS m^{-1}), 250 (9.21 dS m^{-1}), 275 (9.28 dS m^{-1}), and 300 (9. 33 dS m^{-1}) mM NaCl dissolved in the nutrient solution. The experiment used a randomized blocks design, with three replications and three plants per replicate.

2.2. Plant growth determinations

Morphological determinations included head, root, shoot and total fresh weights (FW). Total plant leaf area (LA) was measured on digital images by *Image J* processing software (Orsini *et al.* 2011).

2.3. Leaf gas exchanges

Leaf transpiration (E), stomatal conductance (g_s) and net photosynthesis (A) were measured 15 days after salinization on three completely unfolded leaves of nine plants per treatment. Measurements of leaf gas exchange were performed on attached leaf samples using a CIRAS-2 (PPSystem, Hitchin, UK) infrared gas analyser (closed system) with a Parkinson's Automatic Universal Leaf Cuvette (PAR 1000 mmol m^{-2} s^{-1}, 26°C, CO_2 13.63 mmol l^{-1} and 300 cm^3 min^{-1} flow rate) equipped with 18-mm diameter, 2.5-cm^2 area cuvette inserts. Water Use Efficiency (*WUE*) was determined as the ratio between A and E.

Exp. 2#

2.1. Experimental design

Three salt concentrations were considered, 0 (2.68 dS m^{-1}), 100 (7.68 dS m^{-1}) and 200 (8.72 dS m^{-1}) mM NaCl dissolved in the nutrient solution. The experiment used a randomized blocks design, with three replications and six plants per replicate.

2.2. Plant growth determinations

Morphological determinations included head, root, shoot and total fresh (FW) and dry (DW) weights (after drying at 60°C). Total plant leaf area (LA) was measured as in Exp. 1#. At harvest, roots and shoots of all plants were dried and weighed and the root:shoot calculated. Relative Growth Rate (RGR) was determined using the equations:

$$RGR = (\ln DM_2 - \ln DM_1)\,(t_2 - t_1)^{-1}\ (g\ g^{-1}\ d^{-1})$$

Where DM_1 is the initial (15 DAS) total (shoot + root) dry mass, DM_2 the final (50 DAS) total dry mass, and (t_2 - t_1) the difference in time interval between the two samplings. Leaf area index (LAI) was calculated by multiplying specific leaf area by total leaf dry weight then dividing by the ground area occupied by the canopy.

2.3. Plant water relations and leaf gas exchanges

Water potential (Ψ_w) and osmotic potential ($\Psi\pi$) was measured on fresh and frozen/thawed leaf samples using a dewpoint potentiometer (WP4, Decagon Devices, Pullman, WA, USA). Osmotic potential (OA) was calculated using the equation:

$$OA = \Psi_{\pi 0}\, V_0 - \Psi_\pi\, V \text{ (MPa)},$$

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. Leaf gas exchange measurements were conducted as in Exp. 1#.

2.4. Mineral solutes accumulation

For ion analysis, the determination of some cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) and anions (Cl^-, $NO3^-$, and $SO4^{2-}$) was carried out on dry weight basis. 500 mg of leaves dry matter were suspended in 50 ml water and homogenized with a stirrer at 150 rpm for 20 minutes. Samples were then filtered using filter paper (589 Schleicher) and then the extracts were further filtered through cellulose acetate syringe filters (0.20 μm). For cations analysis, the filtrated extract was acidified with 65% nitric acid HNO_3 (1:100 ml, v: v) and quantification of cations was performed using Inductive Coupled Plasma Optical Emission Spectrometry (ICP-OES). Anions determination was done by ion chromatography (IC).

2.5. Enzymes extraction and assays

For protein and antioxidant enzyme extraction, 10 g of fresh leaves were homogenized in 10 ml of 200 mM chilled potassium-phosphate buffer (pH 7.5) containing 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) and 0.1% (v/v) Triton X-100 placed in an ice bath. The homogenate was filtered through a layer of muslin cloth and centrifuged at 10000 × *g* for 20 minutes at 4 °C. The supernatant was collected and eluted through Sephadex G-25 gel column (NAP-25, Amersham Biosciences, Piscataway, NJ, USA) then re-suspended in 10 mM sodium-potassium phosphate buffer (pH 7.0) and used for the determination of the antioxidant enzymes. All enzymatic activities were assayed spectrophotometrically, the analysis was performed in triplicate and the results were normalized by plant fresh weight.

2.6. Total soluble proteins

The soluble proteins concentration of the extract was estimated according to Bradford's method using bovine serum albumin as a standard (Bradford 1976).

2.7. Malondialdehyde (MDA)

The level of lipid peroxidation was determined by measuring malondialdehyde (MDA) formation using the thiobarbituric acid-reactive substance (TBA) method as described by Heat and Packer (1968). For MDA extraction, 100 µl aliquot of enzyme extract was mixed with 900 µl thiobarbituric acid solution containing 0.5 % (w/v) 2- thiobarbituric acid and 0.5M orthophosphoric acid. The mixture was heated in a water bath at 100 °C for 30 minutes then the reaction was quickly stopped by cooling the tubes in an ice water bath. Afterward, the mixture was centrifuged for 1 minute at 13000 × *g* to remove the unspecific turbidity. The absorbance of the supernatant was measured at 532 nm using spectrophotometer Cary-1 (Varian, California, US). Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA-TBA complex (red pigment) was calculated from the difference of the two wavelengths based on standard curve of MDA.

2.8. Assays of enzymes

2.8.1. Ascorbate peroxidase (APX, EC 1.11.1.11)

Ascorbate peroxidase activity was determined using the method of Chen and Asada (1990). Ascorbate reaction solution contained 50 mM sodium-potassium phosphate buffer (pH 7.0), 1 mM ascorbic acid, 0.5 mM hydrogen peroxide and 100 µl enzyme extract in a final assay volume of 1 ml. Ascorbate oxidation was followed at 290 nm. The concentration of oxidized ascorbate was calculated using an extinction coefficient $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. One unit of APX is defined as the enzyme activity catalysing the oxidation of 1µmol of ascorbic acid per minute.

2.8.2. Catalase activity (CAT, EC 1.11.1.6)

Catalase activity was assayed by measuring the initial rate of disappearance of H_2O_2 and determined using the method of Havir and McHale (1987). Catalase reaction solution contained 50 mM sodium-potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 20 µl of enzyme extract in a final assay volume of 1 ml. The reaction was initiated by adding the enzyme extract and the decrease in H_2O_2 was measured following the changes in the absorbance of the reaction solution at 240 nm. The concentration of CAT was calculated using an extinction coefficient $\varepsilon = 0.036\ mM^{-1}\ cm^{-1}$. One unit of CAT is defined as the enzymatic activity that catalyses the degradation of 1 µmol of H_2O_2 per minute.

2.8.3. Glutathione reductase (GR, EC 1.6.4.2)

Glutathione reductase activity was determined using the method of Foyer *et al.* (1991). Glutathione reductase reaction solution contained 50 mM sodium-phosphate buffer (pH 7.5), 5 mM EDTA, 1mM (NADPH), 1mM oxidized glutathione (GSSG) and 300 µl enzyme extract in a final assay volume of 1 ml. NADPH oxidation was determined at 340 nm. Activity was calculated using an extinction coefficient $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$ for NADPH. One unit of GR is defined as the enzyme activity that oxidizes 1 µmol of NADPH per min at room temperature.

2.8.4. Superoxide dismutase (SOD, EC 1.15.1.1)

Superoxide dismutase activity was determined using the method of Masia (1998) by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) to blue formazan by flavins under illumination. Superoxide dismutase reaction solution contained 50 mM sodium-potassium phosphate buffer (pH 8.0), 300 µM methionine, 1.5 mM NBT, 120 µM riboflavin, 100 mM Na_2.EDTA, 300 µM potassium cyanide and 100 µl enzyme extract in a final assay volume of 1 ml. The riboflavin was added last. The reaction was started by illumination the test tubes under 4 fluorescent lamps for 10 minutes. The absorbance of illuminated solution was measured spectrophotometry at 560 nm. One unit of SOD activity is defined as the amount of enzyme that inhibited 50% of NBT photoreduction versus a blank cell containing no enzymatic extract.

2.9. Proline level

Free proline content was determined according to Bates *et al.* (1973). Proline reaction solution contained 3 mM ninhydrin in 60% (v:v) acetic acid. The samples were heated at 100 °C for 1 h in water bath and then cooled in tap water to stop the reaction. The mixture was extracted with toluene and the absorbance of toluene fraction aspired from liquid phase was read at 520 nm. Proline concentration was determined using a calibration curve and expressed as µmol proline g^{-1} FW.

2.10 Chloroplastic pigments

For photosynthetic pigments chlorophyll *a*, *b*, xanthophylls and carotenes, 0.1 g fresh leaves were extracted in 5 ml of chilled 80% (v:v) acetone. The homogenate was centrifuged at 4000 × *g* for 5 min at 4 °C. The absorbance of resulting supernatant was taken at 470, 645, 663 nm. Different pigments were estimated using the formula by Arnon (1949) as follows:

Chlorophyll a = $12.7\ (A_{663}) - 2.69\ (A_{645})$

Chlorophyll b = $22.9\ (A_{645}) - 4.68\ (A_{663})$

C_{x+c} = =1000 (A_{470}) −1.9×chl *a*−63.14× chl *b* /214 (x= xanthophylls and carotenes)

2.11. Statistical analysis

Data were analysed using analysis of variance (ANOVA) by Co-Stat-ANOVA software (CoHort, Monterey, CA, USA). At least three replications per treatment per genotype were used for analysis of all parameters. Treatment means were compared using Student-Newman-Keuls at 5% significance. In exp. 1#, data were plotted and response functions were identified by datasets with significant linear regression, by limiting the number of data considered when additional data would reduce significance.

3. RESULTS

Exp. 1#

3.1. Plant growth and leaf gas exchanges

Upon salinity both plant morphological and physiological performances were decreased (Fig. 1). However, in response to the thirteen salinity levels considered, three different linear functions could be observed for all studied parameters. Greatest reducing slopes were observed between 0 and 100 mM NaCl, whereas above 100 mM NaCl increasing salinity resulted in lower effects on crop performances. Nevertheless, another threshold could be observed around 200 mM NaCl, above which most of the measured parameters would not be anymore affected.

Exp.2#

3.1. Plant growth

Salinity, genotype and their interaction significantly affected all growth parameters (Table 1). Yield of both genotypes decreased significantly upon salinity. However, while 100 mM NaCl caused a 62% reduction in head weight in white cabbage, no significant reduction from control was appreciated in the savoy genotype. On the other hand, a 70% decline in weight was measured in both genotypes when 200 mM NaCl were supplied. Root growth was also impaired by salinity (similarly by 83% at 200 mM NaCl in the two genotypes), although symptoms to moderate stress were less evident in savoy as compared to white, where root weight was reduced in measure of 41% and 81% from control conditions when 100 mM were applied. Salinity (100 mM NaCl) depleted leaf area more dramatically in white as compared to savoy (reduced by 52% and 25% respectively from control conditions), while at 200 mM NaCl no differences among genotypes could be appreciated (average reduction 66% from control). Consistently, both leaf area and LAI were greater (1.6 folds) in savoy as compared to white when 100 mM NaCl were supplied. Salinity-induced reductions were appreciated in terms of relative growth rate (RGR) and root:shoot ratio in both genotypes. Furthermore, savoy plants presented greater leaf area and leaf area index (Table 1).

3.2. Leaf gas exchanges and water relations

Unstressed white cabbage plants exhibited expressively higher values of transpiration rate (*E*) and stomatal conductance (g_s) as compared to savoy plants (1.6 vs 1.2 mM m^{-2} s^{-1} and 115 vs 46 mM m^{-2} s^{-1} respectively) (Table 2). However, the transpiration rate of white cabbage plants was more susceptible to 200 mM NaCl as compared to savoy (reduction from control conditions in measure of -66% and -49% respectively). Similarly, net photosynthesis (*A*) was lower in white cabbage plants as compared to savoy under 100 (-47%) and 200 (-44%) mM NaCl.

Leaf water potential was reduced in both genotypes upon salinization (Fig. 2A). However, such reduction was greater in the savoy genotype at both salt levels (-1.40 and -1.67 MPa, at 100 and 200 mM NaCl) as compared to the white one (-0.75 and -1.42 MPa). Similarly, the highest noteworthy osmotic potential reduction at 100 and 200 mM NaCl was observed in the savoy genotype (-1.74 and -2.13 MPa) relatively to the white one (-1.36 and -1.80 MPa), (Fig. 2B). Higher osmotic adjustment values were achieved in savoy plants (0.7 and 1.0 MPa) compared to the white one (0.2 and 0.4 MPa) at 100 and 200 mM NaCl (Fig. 2C). Likewise, savoy genotype presented higher value of water use effeciency (*WUE)* under 100 mM NaCl, being about 3-fold higher than those measured on white cabbage plants (26 versus 9 mM CO_2 mM^{-1} H_2O) (Fig. 2D).

3.3. Mineral solutes accumulation

The concentration of ions varied between the two *Brassica* genotypes and among salinity treatments. Imposing NaCl stress significantly induced an increase in Na^+ concentration in leaves of the two genotypes compared to unstressed plant (Fig. 3A). Savoy plant accumulated Na^+ in response to 100 (+13fold) and 200 (+23fold) mM NaCl as compared to control plants, whereas lower increases in response to salinity (+7 and +12 fold, respectively upon 100 and 200 mM NaCl) were observed in the white genotype (Fig. 3A). Salinized condition did not affect the Ca^{2+} concentration in savoy genotype as shown by the non-significant difference under different salt treatments (Fig. 3B). In contrary, white plant showed a drastic diminishing in Ca^{2+} ions by (-78%) and (-93%) under 100 and 200 mM NaCl in relation to control plants. The K^+ concentration of the savoy leaves increased apparently by (+268% and +116%) under 100 and 200 mM NaCl respectively, as compared to control plant, while there was no significant effect of salt treatment in white genotype leaves (Fig. 3C). Similarly, the Mg^{2+} content in the leaves of savoy plant increased significantly by +75% and +35% respectively in response to increasing salinity (100 and 200 mM NaCl) (Fig. 3D). However

white plants exhibited a significant reduction in Mg^{2+} content under both salt levels (mean reduction –66% as compared to control conditions). The Na^+/K^+ ratio in savoy genotype leaves was not affected by moderate salinity (100 mM NaCl), whereas the same salt concentration resulted in a 706% increase in white plants (Fig. 3E). Chloride concentration was significantly increased in the white genotype undergoing 100 (138%) and 200 (443%) mM NaCl, whereas a lower response (no significant differences at 100 mM NaCl and increase limited to +202% under 200 mM NaCl was found in the white genotype. (Fig. 3F). SO_4^{2-} concentration was similarly reduced by salinity in both genotypes (Fig. 3G). Nitrate concentration experienced significant increasing as the salinity increased in both genotypes (Fig. 3H). However, white genotype leaves accumulated more NO_3^- content by 2.3 fold as savoy plants at moderate (100 mM NaCl) salt exposure.

3.4. Biochemical response to salinity

APX activity in the leaves of savoy plant was remarkably increased (+123%) as compared to white cabbage plants upon 100 mM NaCl treatment (Fig.4A). However, similar values of APX in the two genotypes were observed under both control and 200 mM NaCl. Similarly, CAT activity was about 2.5 folds higher in savoy compared to white cabbage leaves at 100 mM NaCl (Fig.4B), whereas no genotypic differences appeared either under control or 200 mM NaCl. Glutathione reductase (GR) activity showed a rather constant level in white cabbage plants under control and salinized conditions (69 U g^{-1} FW as an average), while a significant rise in GR activity was observed in savoy, resulting in 1.9 and 2.3 folds increase at 100 and 200 mM, respectively compared to control conditions (Fig.4C). SOD activity increased similarly with salinity, and no genotypic differences could be observed at the studied salinity levels (Fig.4D). However, savoy control plants presented higher SOD (+36%) as compared to white cabbage plants.

Lipid peroxidation level in savoy leaves (measured as MDA content) was increased at 100 and 200 mM NaCl (+201 and +94% from control conditions), whereas no changes would be observed in white cabbage plants (Fig.5A). Salinity enhanced leaf proline accumulation similarly under both salt levels (Fig.5B). No significant responses in protein concentration could be attributed to either genotype or salinity (Fig.5C). Both chlorophyll *a* and *b* were significantly increased by salinity in savoy plants, whereas no changes were observed in white cabbage plants (Fig. 6A and B). Consistently, xanthophylls and carotenes content decreased significantly in savoy plant by (-43%) at 200 mM NaCl as compared to control plant, while no salt-induced changes could be observed in white cabbage plants (Fig. 6C).

4. DISCUSSION

4.1. Identification of salinity tolerance thresholds in white cabbage

Results from Exp. 1# enabled to identify two salinity response thresholds in white cabbage, respectively at 100 mM NaCl (moderate salinity threshold) and 200 mM NaCl (high salinity threshold). Maas (1990) reported that yield declining took place after the tolerance threshold, represented by a single regression line with a species-specific slope. More recently, Maggio *et al.* (2006) proposed that the relationship between yield and salinity was represented by bilinear response that suggested the existence of a *second* physiological threshold that identifies a functional shift between different adaptation mechanisms. This threshold resulted to be at 100 mM NaCl in white cabbage. Furthermore, in the present study, although yield decline is found to occur yet at low salinities, a third threshold (above which the plant still survives although at basal physiological functions) may be placed for cabbage at 200 mM NaCl. Consistently, in the second experiment 0, 100 and 200 mM NaCl concentrations were used.

4.2. Genotypic variability in cabbage physiological and morphological responses to salinity

The greater yield (Table 1) of savoy *vs* white cabbage plants under moderate salinity (100 mM NaCl) is consistent with the concept of a strict genotype-related response to salinity even within the same family (*Brassicaceae*): even close relatives may show great differences in the capability and means to cope with unfavorable growing conditions (Orsini *et al.* 2010). Furthermore, at 100 mM NaCl, not only the cabbage head was preserved, but also root biomass and leaf development were scarcely affected in savoy plants confirming that the stress perceived by these plants was negligible. Recent reports on a wide range of vegetable crops (e.g. Orsini *et al.* 2010a, 2011, 2012 and 2013; Barbieri *et al.* 2012) have pointed out that the capability of the plant to cope with salinity through functional physiological down-regulation may result in preservation of the shoot biomass and, consequently, crop yield. The adoption of qualitative descriptors of the plant response to salinity (e.g. RGR or root:shoot ratio) may provide further indications on the overall architectural response to the stress. The lower RGR observed in the white genotype as compared to savoy plants is consistent with more severe reductions in the net assimilation rate observed in the former (Table 1). Moreover, the general reduction of root:shoot ratio upon salinity may be interpreted as a way for restricting the uptake of toxic ions to the shoot while still maintaining higher turgor and positive growth rate (De Pascale *et al.* 2003a). This may be accomplished by simultaneous reducing root versus shoot development and activating specific metabolic pathways (i.e.,

osmolyte biosynthesis), both of which occur in saline environments (Munns and Tester 2008; Akram *et al.* 2009). Based on these considerations, savoy cabbage appeared to be relatively more tolerant than the white one.

Leaf gas exchanges are generally impaired upon salt stress. This reduction is associated with salt damage of the photosynthetic tissue, changes in stomatal features with the consequent restriction of the CO_2 availability for carboxylation or to the acceleration of senescence (Orsini *et al.* 2010a). The reduced g_s and E observed in savoy plants, as compared to white cabbage yet under control conditions, most likely protected them from tissue dehydration and allowed them to effectivelly adjust to the unfavorable conditions by minimizing transitory cellular turgor loss (Ashraf 2001; Orsini *et al.* 2010; Singh *et al.* 2010). The reduction in transpiration with salinity has been associated with reduced g_s and lower stomatal density of leaves developed under saline conditions (Omami *et al.* 2006). Nonetheless, while similar A values were found in the two genotypes grown under control conditions, a greater reduction in photosynthesis was associated to salinity in white cabbage as compared to savoy. Although salt-induced reductions of A are commonly associated to impaired stomatal opening (Sifola and Postiglione 2002; Bayuelo-Jimenez *et al.* 2003; Omami *et al.* 2006), recent reports suggest that preserved A values associated with lower g_s or E may be considered as reliable indicators of overall salinity tolerance (Orsini *et al.* 2011 and 2012), and this is substantiated by the greater *WUE* observed in salt tolerant plants undergoing salinity (Barbieri *et al.* 2012). The most severe changes in plant water potentials observed in savoy plants may be the result of the structural-functional changes operated by the plant in order to ensure successful adaptation to salinity (Kaymakanova *et al.* 2008). The reduction of the osmotic potential would therein be a consequence of the net increase in solute accumulation which occurs through uptake of solute and/or synthesis of organic compounds in a process called osmotic adjustment (Hasegawa *et al.* 2000; Munns 2002). Osmotic regulation, a phenomena that occurs in both roots and leaves, contributes to maintain water uptake and cell turgor, which are essential to sustain physiological processes such as cell expansion, stomatal opening, photosynthesis (Zhang *et al.* 1999). However, a process of osmotic self-adjustment occurs in the plant cells, directed towards the preservation of the water balance by means of accumulation of osmotically active solutes (Tezara *et al.* 2003). In the hereby presented experiment, savoy plants were able to better preserve the turgor and regulate their osmotic adjustment compared to white genotype. Thus, also from a physiological perspective, the

adaptation of leaf gas exchanges and overall water relations appeared to be more effective in savoy as compared to white cabbage plants.

4.3. Salinity, ion accumulation, and biochemical response in plant tissue: potentials for improved nutritional quality in stressed plants

It is known that deleterious effects of salinity are related to osmotic effects, ion toxicities and ionic imbalance (Munns and Tester 2008; Patel *et al.* 2009). Under salt stress, plants evolved complex mechanisms allowing for adaptation to osmotic and ionic stress. These mechanisms include osmotic adjustment by accumulation of compatible solute and lowering the toxic concentration of ions in the cytoplasm by restriction of Na^+ influx or its sequestration into the vacuole and/or its extrusion (Binzel *et al*. 1988). In this study, sodium accumulation was enhanced in both *Brassica* genotypes when the plants were exposed to salt and showed similar pattern of Na^+ accumulation (Fig. 3A). However, the vast accumulation of Na^+ in relatively salt tolerant savoy plant at moderated salt demonstrated that salinity resistance of this species is not linked to their ability to restrict the uptake and/or transport of sodium accumulation into the aerial parts (Boughalleb *et al.* 2012). K^+ has an important role in osmotic adjustment in the guard cell controlling the stomata movement and thus CO_2 assimilation in photosynthesis (Chartzoulakis *et al*. 2006; Degl'Innocenti *et al*. 2009). Also, K^+ is considered to be an effective agent in salt tolerance mechanisms of the plant through maintenance of Na^+/K^+ homeostasis (Kader and Lindberg 2008; Alemán *et al.* 2009) and osmoregulation (Szczerba *et al.* 2009). A range of studies indicate that an increase in concentration of K^+ and Ca^{2+} in plants under salt stress could ameliorate the deleterious effects of salinity on growth and yield (Grattan and Grieve 1999; Sivritepe *et al.* 2003). In this study, K^+ was the major inorganic ion that accumulated significantly in salt stressed savoy while there was no alteration in its concentration in the relatively salt sensitive white (Fig. 3C). These results suggest that under salt stress, savoy plant may use K^+ for osmotic adjustment (K^+ accumulation plays a key role in salt tolerance mechanism of *Brassica* species by maintaining the ion homeostasis, Alemán *et al.* 2009; Cuin *et al.* 2008). However, under salt stress, plants maintain high concentrations of K^+ in the cytosol by regulating the expression and activity of K^+ and Na^+ transporters and of H^+ pumps that generate the driving force for transport (Zhu *et al*. 1993). Ca^{2+} plays an essential role in processes that preserve the structural and functional integrity of plant membranes, stabilize cell wall structures, regulate ion transport and selectivity, and control ion-exchange behavior as well as cell wall enzymatic activities (Marschner 1995). Salinity dominated by NaCl causes instability of plasma membrane resulting from Ca^{2+} displacement by Na^+ (Santos 2004; Mansour and

Salama 2004), reduces Ca^{2+} availability and Ca^{2+} transporting and mobility to growing regions of the plant, produces extreme ratios of Na^{+}/Ca^{2+} in the plants which increase the plants susceptibly to osmotic and specific ion injury as well as to nutritional disorders that result in reduced yield and quality (Sivritepe *et al*. 2003). In the hereby presented experiment, white genotype exhibited sharp diminishing in Ca^{2+} content associated with increased salt stress (Fig. 3B). The reduction in the concentration of this cation may be related to a lower Ca^{2+} release into the root xylem because of an effect of active loading of these cations into the xylem vessels (Lynch and Läuchli 1985). However, Ca^{2+} concentration in leaves of savoy plant was not affected by increasing salt supply, which was consistent with many previous studies pointing out that an increase in Ca^{2+} concentration in plants challenged with salinity stress could ameliorate the inhibitory effects of salinity on growth (Carvajal *et al*. 2000; Kaya *et al*. 2003). In addition, Mg^{2+} was significantly increased in both levels of stressed savoy genotype (+55%, as an average) compared to unstressed plant, while the concentration of this ion depressed drastically in white genotype by 2.6 fold and 3.5 fold respectively under 100 and 200 mM NaCl (Fig. 3D). This result might suggest the presence of membrane selectivity of savoy plant towards ions uptaking and accumulating which might be utilized in lowering its osmotic potential as a way to cope with stressed condition. Many studies on halophytes and some tolerant glycophytes plants showed that a low foliar Na^{+}/K^{+} ratio is a salt tolerance index and a good indicator of salt tolerance (Tester and Davenport 2003; Ashraf and Orooj 2006). In the present study, leaf Na^{+}/K^{+} ratio was significantly higher in moderated salinized plant of white genotype by 4-fold as in savoy plant (Fig. 3E). It might be possible that the internal accumulation of K^{+} ions in salt stressed savoy plant reduced the Na^{+}/K^{+} ratio which improved the plant salt tolerance. Besides, there are different factors that affect Na^{+}/K^{+} homeostasis such as different gene resources (Huang *et al.* 2008) and over expression of potassium-related genes (Mangano *et al.* 2008). Salt tolerance in plants is associated usually with the ability to restrict the uptake and or transport of saline ions from root to shoot (Hajibagheri *et al*. 1987). At moderated salt stress, Cl^{-} concentration in the leaves of relatively tolerant savoy plant remained similar to those found under control conditions, while its content increased significantly by 2.4 times in white plant comparing to non-treated plant (Fig. 3F). Therefore, the salinity resistance of savoy plant could be related to Cl^{-}exclusion, However, the accumulation of Cl^{-} in the leaves of both genotypes was considerably enhanced with imposition of 200 mM NaCl to the rooting medium and a considerable difference between cultivars was observed (915 mg kg^{-1} DW in savoy versus 1445 mg kg^{-1} DW in white). Excessive accumulation of Cl^{-} results in ion toxicity and growth

inhibition (Ashraf and Harris 2004). Accordingly, the higher inhibition of growth parameters that was observed in white genotype could be related to high concentration of Cl^- (Table 1). Munns (2002) indicated that there are two mechanisms of salt tolerance in plant: those minimizing the entry of salt into the plant and those minimizing the concentration of salt in the cytoplasm. In this regard at 100 mM NaCl, savoy genotype was more able to minimize the entry of nitrate ions into the leaves than white plant (423 mg Kg^{-1} DW versus 971 mg Kg^{-1} DW, Fig. 3H), suggesting that salinity resistance of this species might be related to its ability to restrict NO_3^- accumulation. However, there was considerable reduction in NO_3^- content in white plant at 200 mM NaCl as 2.6 times as moderated salt treated plant. This reduction was associated with vast accumulation of Cl^- ions in leaves (Fig 3 F and H). Theoretically, the reduction of NO_3^- uptake might be related to a decrease in the nitrate reductase activity (NAR) that accompanied with the presence of Cl^- salt in the external medium (Flores *et al*. 2000). According to the hereby presented results, the white cabbage behaved similarly to rocket, a member of *Brassicaeae* family, as a nitrate-accumulation vegetable (up to 4300 mg Kg^{-1} FW, Santamaria *et al*. 1999). The effects of nitrate and its toxic metabolites on human health have been documented (Santamaria 2006). Therefore, decreasing leaf nitrate concentration is critically important for fresh healthy vegetable production (Barbieri *et al*. 2011). Under moderated salinity, the turgor was maintained and osmotic adjustment was achieved in the relatively salt tolerant savoy plant by accumulated higher amounts of Na^+, K^+ and Mg^{2+} values, resulting in lower osmotic potential and higher osmotic adjustment. Consistenly, salt stress tolerance in this species may be associated with ion accumulation (Beltrao *et al*. 2000; Maggio *et al*. 2005).

The alleviation of oxidative damage and increased resistance to salinity and other environmental stresses is often correlated with an efficient antioxidative system (Hasegawa *et al.* 2000; Acar *et al.* 2001; Bor *et al.* 2003). In this study, the activities of the four key antioxidant enzymes (APX, CAT, SOD and GR) appeared to be substantially affected by both salinity and genotypes under assessment (Fig. 2), although each of them showed a specific quantitative and qualitative response (Mittal *et al.* 2012). Similarities, as for other physiological and morphological traits, were generally found among the genotypes either at control conditions or when 200 mM NaCl were supplied. On the other hand, under 100 mM NaCl completely diverse scenarios could be attributed to the two genotypes under study. At this salt concentration, APX, CAT, and GR in savoy resulted to be about 2.2-fold, 2.5-fold, and 1.3-fold respectively greater than in white cabbage (Fig.4A, B and C). CAT and APX are

major enzymes detoxifying hydrogen peroxide (Dionisio-Sese and Tobita 1998). Salinity-induced increase in APX has previously been reported by Hernandez *et al.* (2000). Low GR values have been associated to stress sensitivity (Aono *et al.* 1995), and Shalata *et al.* (2001) found that SOD and CAT activities decreased in roots of a salt-sensitive tomato cultivar while they increased in the roots of a salt-tolerant one under salt stress. Vaidyanathan *et al.* (2003) have found increased activity of CAT, APX, SOD and GR enzymes in leaves of rice under salt, and Mittal *et al.* (2012) reported that salt tolerant *Brassica juncea* cv. Bio902 had a higher activity of SOD, APX, and CAT and showed higher capacity for the scavenging ROS generated by salt in comparison with cv. Urvashi. Furthermore, Zhu *et al.* (2011) showed that SOD activity in leaves of cauliflower (*Brassica oleracea* var. *botrytis* L.) increased first at lower salinity (34, 68 and 102 mM NaCl) and then decreased at higher salinity (136 and 170 mM NaCl), while CAT activities changed reversely with SOD. Bor *et al.* (2003) reported that increased GR activity in leaves of sugar beet plants was closely related with salt tolerance capacity, and Moradi and Ismail (2007) suggested that increased activities of SOD, APX and GR were responsible of the increased tolerance to salinity in two rice cultivars, while those enzymes declined or were not affected by salinity in salt sensitive rice. The observed increased SOD activity (Fig.4D) in both genotypes might be advocated as a common strategy to scavenge O_2^- and counteract membrane damage. As suggested by Munns and Tester (2008), although antioxidative response is generally associated to greater stress tolerance, higher antioxidant capacity is not necessarily an indicator of the overall plant tolerance. Furthermore, according to Noreen and Ashraf (2009) care should be taken when correlating genotypic variability in salt stress response and the relative antioxidant system capability to detoxify ROS. Likewise, salt stress experiments on mutant *Nicotiana tabacum* plants lacking in both APX and CAT showed that plants appeared to be less susceptible to oxidative stress (Rizhsky *et al.* 2002). The observed salt tolerance of savoy genotype may be partially attributed to greater CAT and APX activity resulting in improved detoxification of H_2O_2 to H_2O, coordinated by the additional effect provided by the increased GR activity. Consistently, the present study suggests active involvement of at least catalase and peroxidase among the H_2O_2 scavenging enzymes in determining salinity tolerance of cabbage.

Lipid peroxidation (measured as the amount of MDA produced) is the symptom readily ascribed to oxidative damage and is often used as an indicator of oxidative stress which varies in different plant species (Hernandez *et al.* 2000). Free radicals may induce

peroxidation of lipid membrane, which may also reflect stress induced damages at the cellular level (Jain *et al.* 2001). Greater levels of MDA (as observed in savoy plants under 100 mM NaCl) may be associated to the higher osmotic adjustment observed in leaf tissue of the same plants, suggesting that excess salt accumulation triggered the production of ROS which caused the oxidative damage of plasma membrane. Similarly, Noreen and Ashraf (2009) reported that MDA contents were increased in salt tolerant radish cultivars Red Neck and Mannu Early undergoing salinity, whereas lower levels were found in salt sensitive ones. Also, Zhu *et al.* (2011) found that MDA contents in four cauliflower cultivars were increased gradually with increasing NaCl concentrations.

Proline is generally assumed to serve as a physiologically compatible solute that increases as needed to maintain a favourable osmotic potential between the cell and its surroundings (Orsini *et al.* 2010). In response to drought or salinity stress in plants, proline accumulation normally occurs in the cytosol where it contributes substantially to the cytoplasmic osmotic adjustment (Orsini *et al.* 2012). In addition to its role as an osmolyte for osmotic adjustment, proline contributes to stabilization of sub-cellular structures (e.g. membranes and proteins), scavenging free radicals, and buffering cellular redox potential under stress conditions. In the present experiment, there was a similar significant increase in proline concentration upon salinization in both genotypes (Fig. 5B). Consistently proline seemed not to play a determinant role as osmoprotectant, confirming that increased proline concentration may not be associated with salinity tolerance in *Brassicaceae* (Colmer *et al.* 1995). Although soluble protein content is often claimed to be an important indicator of the plant physiological status (Doganlar and Atmaca 2011), in the hereby presented experiments, the contribution of amino acids accumulation to osmotic adjustment was not significant (Fig. 5C). Consistently, it may be confirmed that amino acids are not the main organic solutes involved in the osmotic adjustment of cabbage plants. Savoy plants presented greater osmotic adjustment in response to salinity as compared to white cabbage plants, indeed soluble protein and proline concentrations appeared to be not dependent on genotype. These results were consistent with previous authors (Ashraf and Sharif 1997; Beltrao et al 2000; Maggio et al 2005) who mentioned that *Brassica* stress tolerance is associated with ions accumulation only, whereas other report stated about a possible accumulation of organic compounds other than ions towards combined drought and salt stresses (Siddiqui et al 2008). Although Mittal *et al.* (2012) and Ashraf and Harris (2004) reported higher levels of soluble protein in salt tolerant cultivars of barley, sunflower, finger millet and rice, it shall be considered that the production

of osmolytes is metabolically expensive and limits plant production by consuming significant quantities of carbon (Greenway and Munns 1980). An alternative pathway is provided by the accumulation of a high concentration of ions from the external medium, solution that results in lower energetic cost for the plant, although it may lead to a toxic effect on the normal biochemical activities within the cell (Volkmar *et al.* 1998). Accordingly, we could correlate the tolerance to salt stress in savoy plant to its ability to maintain self-osmotic adjustment in terms of inorganic ions substances accumulation through uptake of solute, while the synthesis of organic compound was not achieved as protein and proline accumulated in similar pattern in both the salt-tolerant and -sensitive species.

Savoy plant experienced noteworthy increases in photosynthetic pigments (chlorophyll *a* and *b*), while white cabbage plant showed no variation upon salinization (Fig. 6A and B). Higher chlorophyll content may be associated to greater photosynthetic rates, as well to the functional state of leaf tissues, which depends on the content of photosynthetic pigments, the synthesis of the enzymes taking part in the carbon reduction and the formation of the membrane system of chloroplasts (Ivanova and Vassilev 2003). Chlorophyll *a* content has been related to salt tolerance in *Panicum miliaceum* (Sabir *et al.* 2009). Increased total chlorophyll was recorded in *Cucumis sp.,* broad bean and rice plant undergoing salt stress (Kusvuran *et al.* 2008). On the other hand, many reports associated the salt-induced damages occurring at cell and tissue level to the reduction in photosynthetic pigments, chlorophyll *a* and *b* in different crops such as alfalfa (Winicov and Seemann 1990), sunflower (Ashraf 1999; Ashraf and Sultana 2000), and wheat (El-Hendawy *et al.* 2005). In the savoy genotype, the enzymatic adaptation to salinity may have counteracted the ROS induced damages at cell level, thus resulting in greater photosynthetic pigments in the leaves and overall improved crop response to salinity.

5. CONCLUSIONS

The response to salinity of two botanical varieties of cabbage, namely savoy cabbage (*Brassica oleracea* var. *Sabauda* L.) and white cabbage (*Brassica oleracea* var. *Capitata* L.) was hereby addressed. savoy plants were not only more tolerant to the stress than white cabbage ones in term of yield, but also operated functional physiological and biochemical adaptation that resulted in improved plant status and increased nutritional value. Higher activities of APX, CAT, and GR were observed in savoy plants undergoing 100 mM NaCl, resulting in greater detoxification of ROS together with the maintenance of lower water potential and higher osmotic adjustment by accumulation higher amounts of K^+ and $Mg^{2+}$and

lower level of Cl and NO_3^- . These combined factors played a functional role in alleviated salt stress in savoy plant.

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TABLES

Table 1. Effect of different salt content (0, 100, and 200 mM NaCl) on some growth parameters of savoy and white cabbage seedling at 50 DAS. Same letters in each column indicate no significant differences among treatments at $P < 0.05$ level. Values are the mean ± SE of three replications. Data from Exp. 2#.

Cultivar	NaCl (mM)	Head (g plant^{-1})		Root FW (g plant^{-1})		LA (cm^{-2} plant^{-1})		LAI		RGR (mg g^{-1} d^{-1})		Root/Shoot	
Savoy	**0**	109±3.51	A	41±4.93	b	7009±239	a	1.13±0.04	a	69±3.65	a	0.7±0.03	a
	100	92±4.36	Ab	24±1.00	c	5232±369	b	0.83±0.07	b	39±4.53	c	0.2±0.01	c
	200	33±1.15	D	7±0.58	d	2652±174	cd	0.43±0.03	c	58±0.48	b	0.1±0.01	c
White	**0**	167±7.57	A	59±2.33	a	6775±295	a	1.09±0.05	a	38±1.99	c	0.6±0.07	b
	100	63±1.76	c	11±1.15	d	3227±149	c	0.52±0.03	c	40±2.18	c	0.1±0.01	c
	200	51±3.21	c	10±0.88	d	2082±310	d	0.39±0.03	c	36±5.60	c	0.2±0.03	c
Salt (S)		***		***		***		***		**		***	
Var (V)		*		*		**		*		***		*	
S × V		***		***		*		ns		**		ns	

Table 2. Effect of different salt content (0, 100, and 200 mM NaCl) on gas exchange parameters of savoy and white cabbage seedling at 50 DAS. Same letters in each column indicate no significant differences among treatments at $P < 0.05$ level. Values are the mean ± SE of eight replications. Data from Exp. 2#.

Cultivar	NaCl (mM)	E (mM m^{-2} s^{-1})		g_s (mM m^{-2} s^{-1})		A (µM m^{-2} s^{-1})	
Savoy	**0**	1.2±0.13	b	46±5.7	b	17±0.9	a
	100	0.4±0.04	c	15±1.3	c	9±0.7	b
	200	0.6±0.05	c	17±1.5	c	6±0.2	c
	0	1.6±0.08	a	115±7.4	a	17±1.2	a
White	**100**	0.6±0.08	c	21±3.1	c	5±0.5	cd
	200	0.5+0.03	c	17±1.3	c	3±0.4	d
Salt (S)		***		***		***	
Var (V)		*		***		**	
S × V		*		***		**	

FIGURES

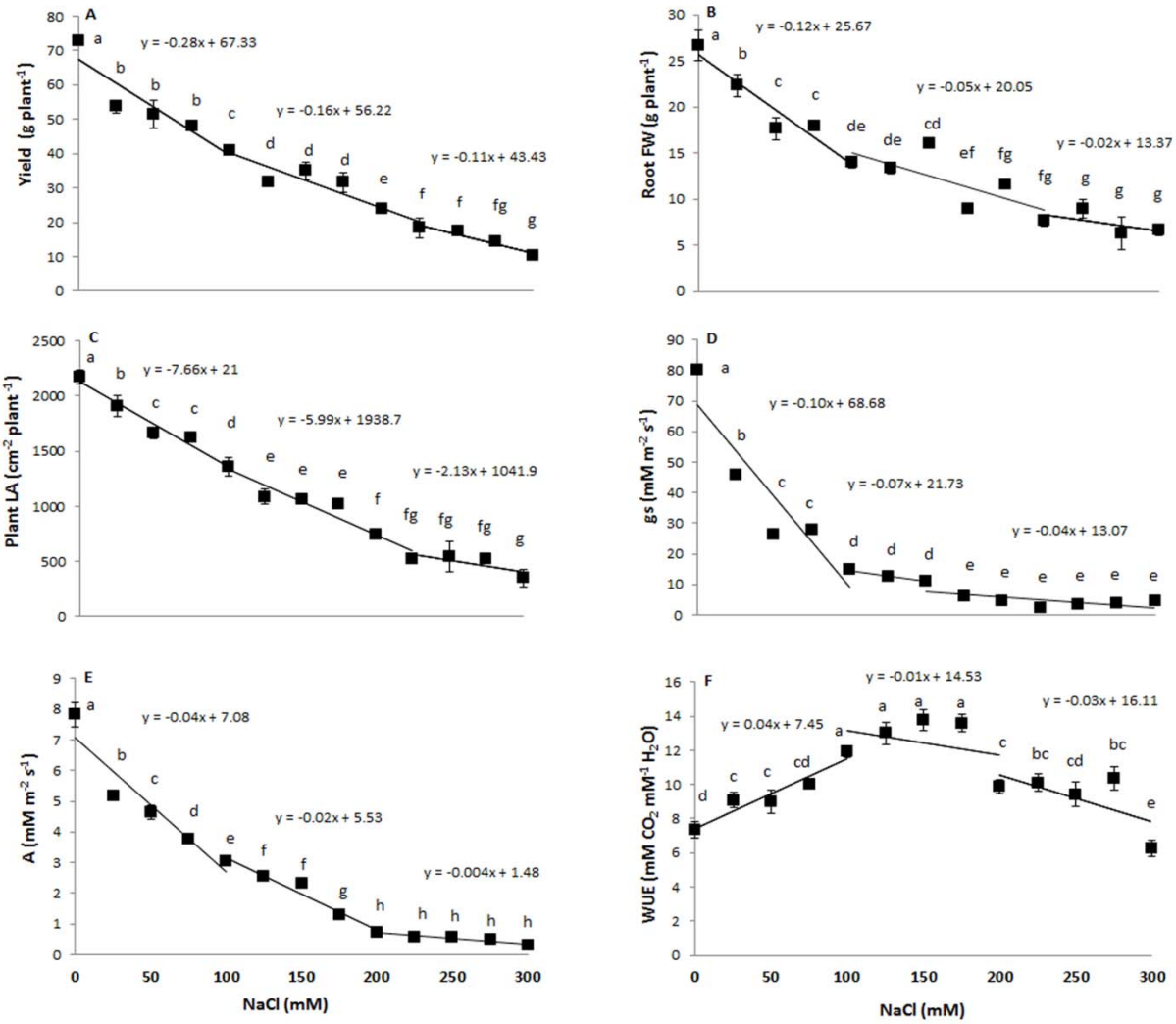

Fig. 1. Identification of salinity tolerance threshold in cabbage seedlings at 50 DAS by means of yield (A), Root FW (B), Plant LA (C), stomatal conductance (*gs*, D), net photosynthesis (*A,* E), and water use efficiency (WUE, F) upon variable salinity (0, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275 and 300 mM NaCl). Values are the mean ± S.E of three replications. Data from Exp. 1#.

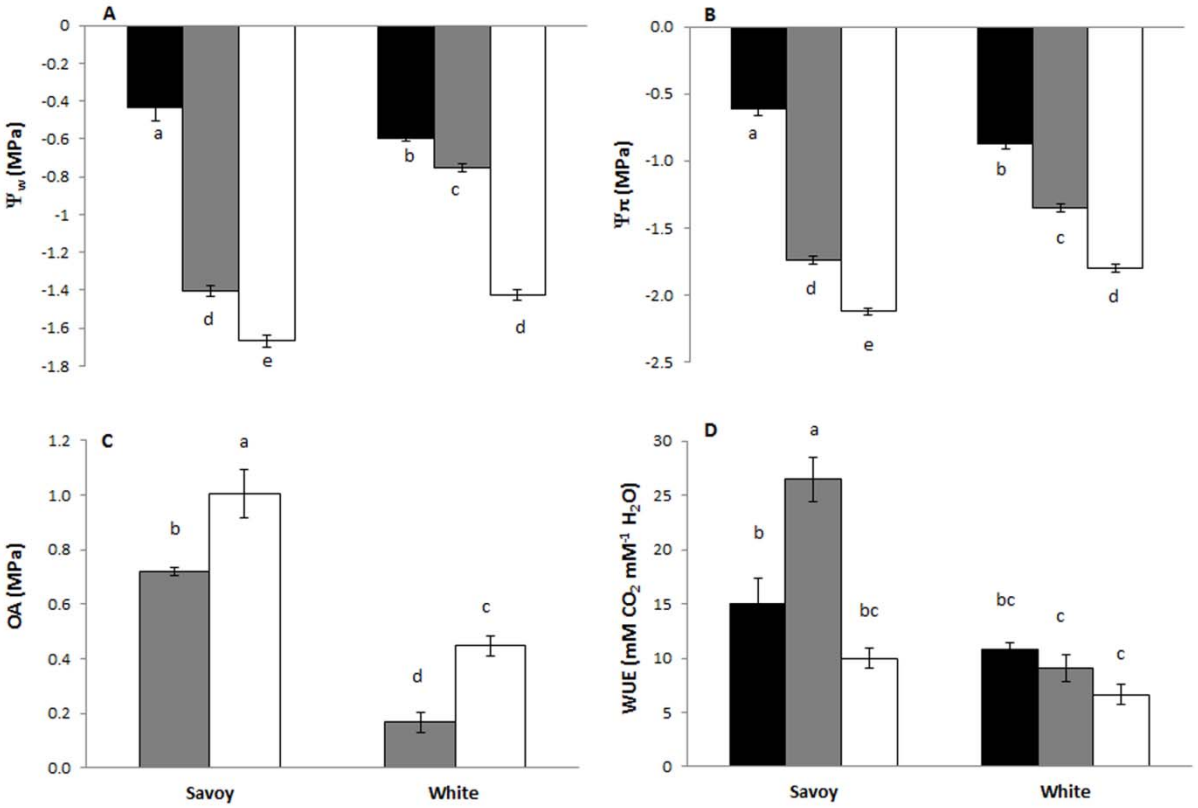

Fig. 2. Effect of zero salt (black), 100 mM NaCl (grey), and 200 mM NaCl (white) on savoy and white cabbage seedling at 50 DAS on water potential (Ψw), osmotic potential (Ψπ), osmotic adjustment (OA), and water use efficiency (WUE). Values are the mean ± SE of nine independent measures. Data from Exp. 2#.

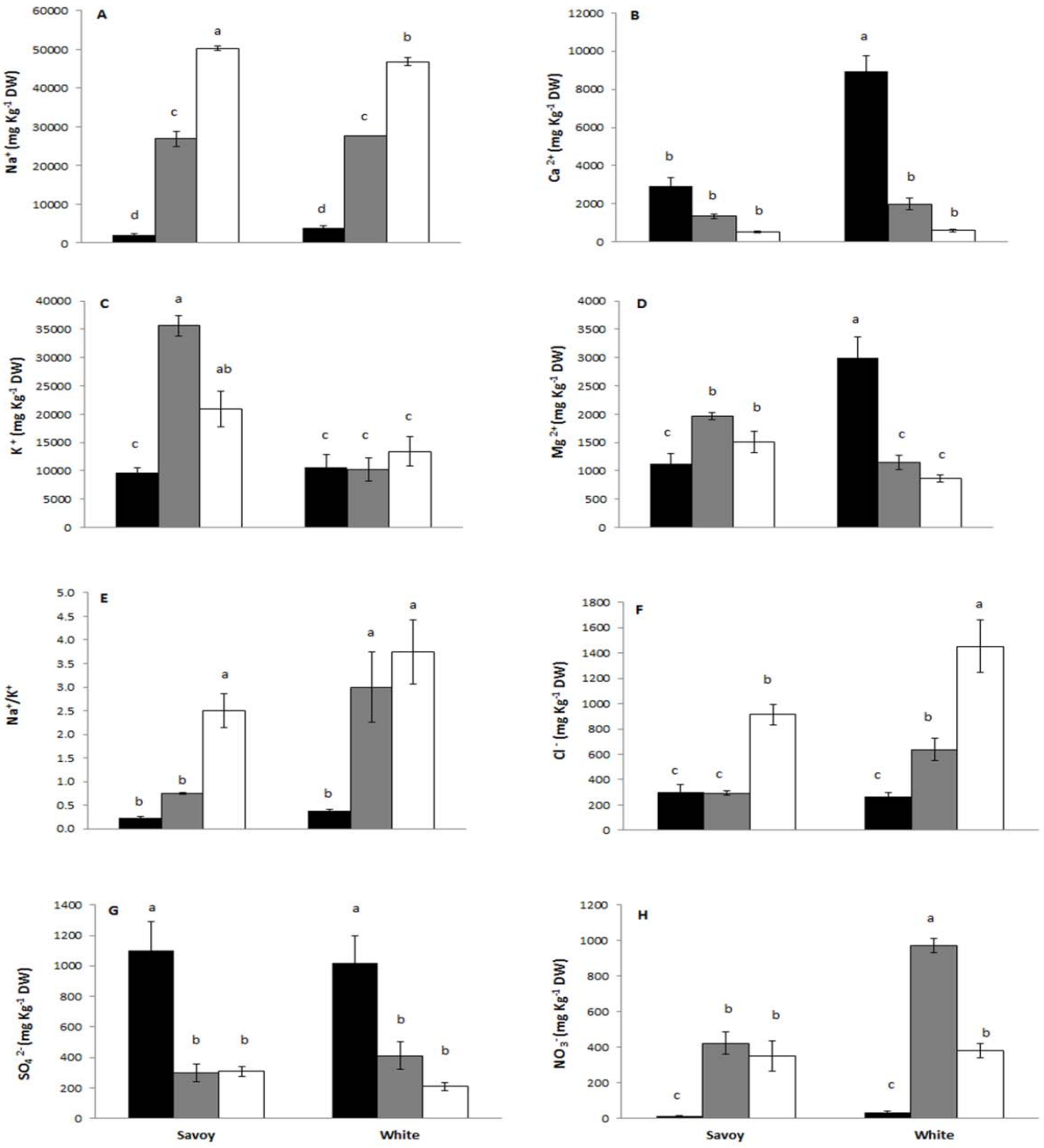

Fig. 3. Effect of zero salt (black), 100 mM NaCl (grey), and 200 mM NaCl (white) on the accumulation of some cations (Na^+, Ca^{2+}, K^+, Mg^{2+}) and anions (Cl^-,SO_4^{2-}, NO_3^-) and the Na^+/K^+ ratio on savoy and white cabbage leaves at 50 DAS. Values are the mean ± SE of nine independent measures. Data from Exp. 2#.

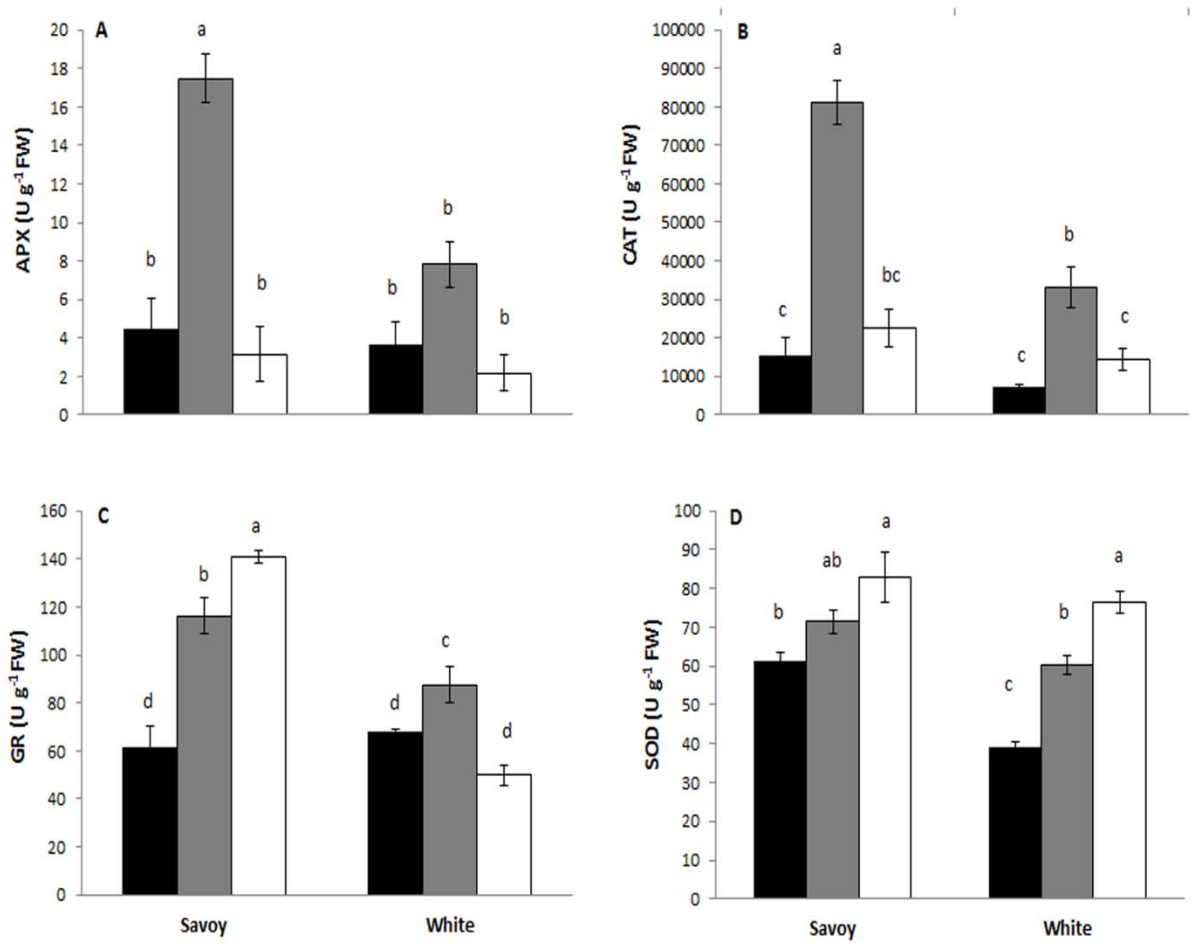

Fig.4. Effect of zero salt (black), 100 mM NaCl (grey), and 200 mM NaCl (white) on ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD) activities in leaves of savoy and white cabbage plants. Values are the mean ± SE of three replications. Data from Exp. 2#.

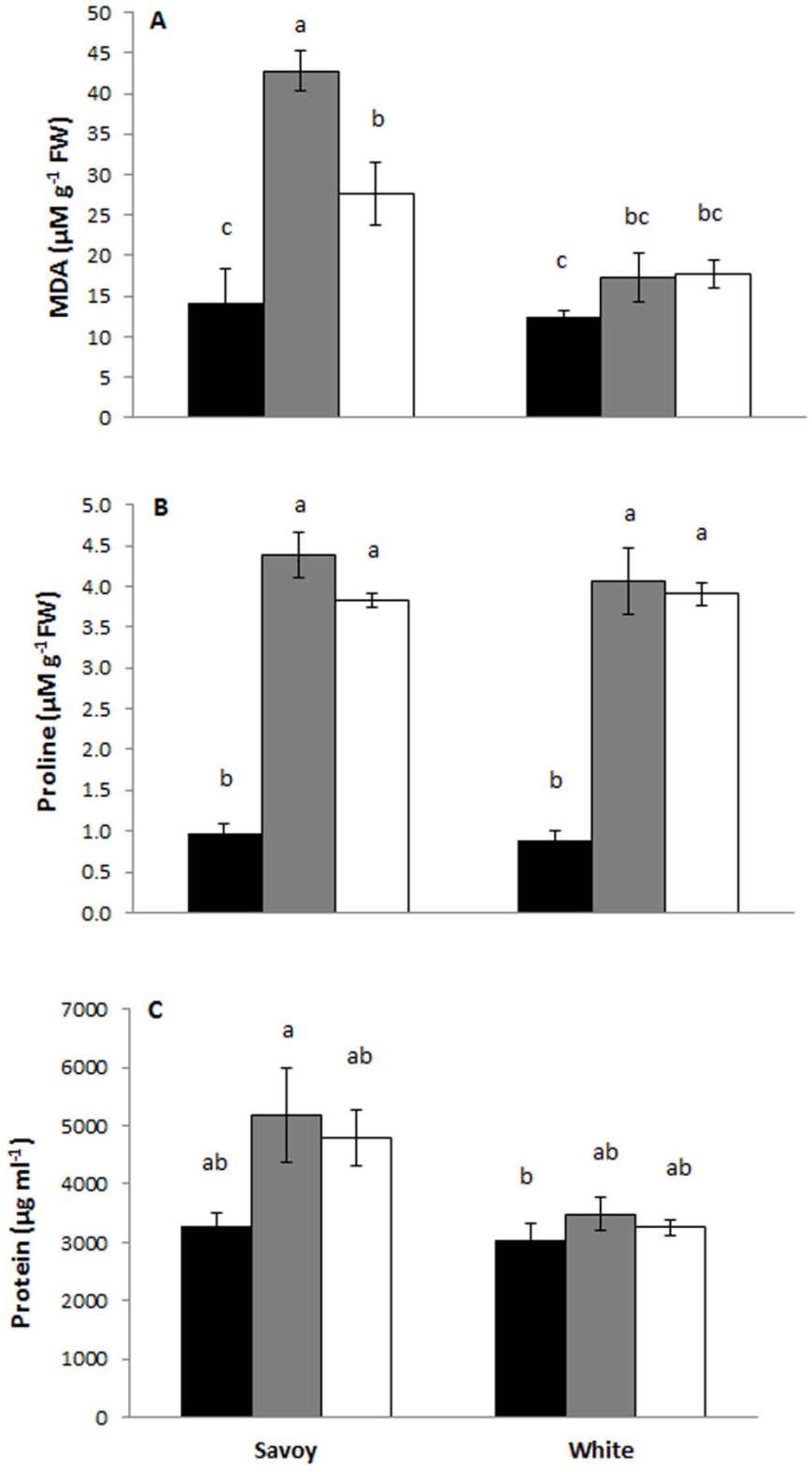

Fig.5. Effect of zero salt (black), 100 mM NaCl (grey), and 200 mM NaCl (white) on malondialdehyde (MDA) and proline level in leaves of savoy and white cabbage plants. Values are the mean ± SE of three replications. Data from Exp. 2#.

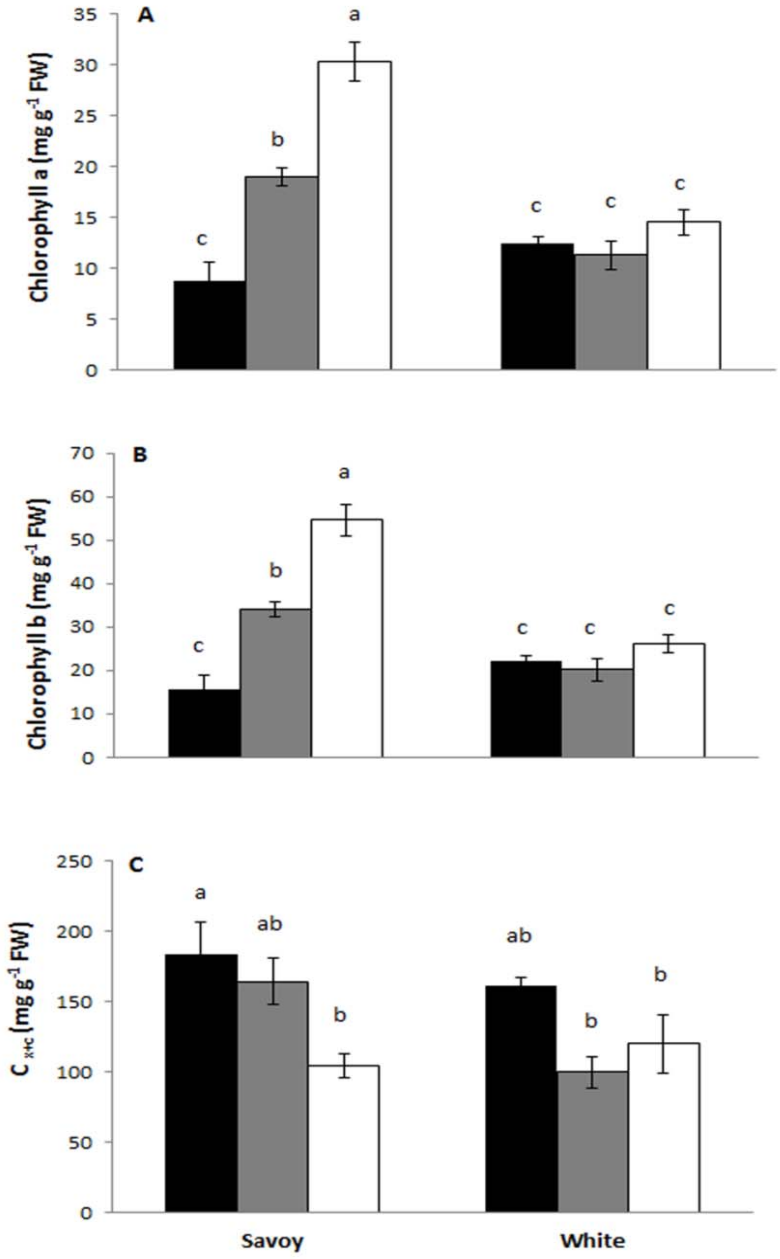

Fig. 6. Effect of zero salt (black), 100 mM NaCl (grey), and 200 mM NaCl (white) on chlorophyll *a* and *b* and *x*anthophylls and carotenoid content in leaves of savoy and white cabbage plants. Values are the mean ± SE of three replications. Data from Exp. 2#.

CHAPTER 3

II. Physiological Response Of White And Red Radish Seedling, Metabolites Features And Antioxidant Enzyme Activities Among Seven Root Radish Cultivars (*Raphanus Sativus* L.) In Salinized Medium

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ABSTRACT

Reducing detrimental effects of salinity on crop production may be achieved through selection of tolerant genotypes within the same species. In the present study a possible method for the identification of salt tolerant radish cultivars is presented. In the first experiment, two morphologically diverse types (long white and round red) are compared morphologically and physiology upon different salinity levels proposed in literature. In a second experiment, the most tolerant genotype from the first experiment was used as control against other seven genotypes therein ranked for their salinity tolerance according to morphological, physiological and biochemical indices such as osmoprotectant biosynthesis (total soluble protein and proline accumulation), oxidative stress indicator (malondialdehyde, MDA and Hydrogen peroxide, H_2O_2), and activities of some key enzymes. This study showed that both genotypes (red and white) experienced unique performance and presented similar significant diminishing in gas exchange parameters after imposing salt, suggesting that photosynthetic parameters is negatively related to assess salt tolerant feature. However, red radish proved better morphological and physiological responds to incremental salinity in term of higher dry matter accumulation, better adaptation of overall water relations, and possessing higher ability to osmotically adjust in saline environments. Thus, round red type has been chosen to be used in the successive steps of the screening. In second trial, among seven radish cultivars, SAXA2 cultivar was relatively considered more salt tolerant in term of having higher yield and shoot fresh weight upon salinization. However, salt stress did not significantly affect MDA accumulation, H_2O_2 content, and APX activity in all seven cultivars. Nonetheless, the relatively salt tolerant cultivar SAXA2 showed higher ability to accumulate more compatible solutes such as proline and protein, and accordingly maintain higher value of osmotic adjustment upon salinization. In addition, this cultivar showed also

considerable increasing in GR activity and higher CAT activity in respect to other cultivars at salt exposure. Thus, our results support the idea that accumulation of proline and protein and having higher activity of GR and CAT are associated with radish salt tolerance, while MDA and H_2O_2 content is negatively related to salt oxidative stress under the condition of this experiment.

Key words: gas exchange, water status, osmotic adjustment, salt stress, oxidative stress, and antioxidative response.

1. INTRODUCTION

Soil salinity is one of the major abiotic stresses that adversely affect plant productivity and quality (*Zhu, 2001*). It was estimated that up to 20% of irrigated lands in the world are affected by different levels of salinity and sodium content (*Mostafazadeh-Fard et al., 2007*).

A high salinity concentration may often occur in Mediterranean areas during the long summer season, as a result of high temperatures and both reduced water availability and quality of irrigation water (*Tattini et al., 2002*). The selection of crop species or cultivars with salinity tolerance traits has been considered an economical and efficient strategy to overcome the problem of salinity stress. Various workers have tried to identify physiological and biochemical differences between salt tolerant and sensitive plants in an effort to develop rapid screening methods for salt tolerance (*Alian et al. 2000*). It is well established that salt tolerance depends on genetic and biochemical characteristics of the species, and crop salinity sensitivity varies with species, genotypes, and growth stages (*Prado et al., 2000; Pujari and Chanda, 2002*), and the genetic diversity may provide useful genes for improving salt tolerance (*Maggio et al., 2005*). Salt stress induces various biochemical and physiological responses in plants and affects almost all plant processes (*Megdiche et al., 2007*) like photosynthesis, nitrogen metabolism, ion homeostasis (*Ashraf, 2004*), proline metabolism and osmolytes accumulation (*Misra and Gupta, 2005*). Salt stress causes stomatal closure, which reduces the CO_2/O_2 ratio inside leaf tissues and inhibits CO_2 fixation (*Hernández et al., 1999*). As a consequence, an over reduction of the photosynthetic electron transport chain occurs, which causes the generation of ROS such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH) and metabolic toxicity (*Jaleel et al., 2007*). In addition, excess of Na^+ and Cl^- ions may lead to changes in the protein structure, while osmotic stress leads to turgor loss and cell volume change (*Errabii et al., 2007*). However, there are different adaptation mechanisms of salt tolerance that mediate the ion homeostasis, osmolytic biosynthesis, toxic radical scavenging, water transport, and long-distance response coordination (*Dalal and Khanna-Chopra, 2001; Jaleel et al., 2007*).

To achieve salt tolerance, plant cells evolve several biochemical and physiological pathways such as the exclusion of Na^+ ions and their compartmentation into vacuoles as well as accumulation of compatible solutes such as proline, glycinebetaine, and polyols (*Errabii et al., 2007*). Plants are equipped with an array of enzymatic and non-enzymatic antioxidant molecules to alleviate cellular damage caused by ROS (*Foyer and Noctor, 2000; Apel and*). Commonly known antioxidant enzymes that involved in the enzymatic scavenging of ROS include Superoxide dismutases (SOD, EC 1.15.1.1) that react with the superoxide radical to

produce H_2O_2 (*Scandalios, 1993*). Catalase (CAT, EC 1.11.1.6) has been found predominantly in leaf peroxisomes where it functions chiefly to remove H_2O_2 formed in photorespiration or in β-oxidation of fatty acids in the glyoxysomes (Dat *et al.*, 2000). Ascorbate peroxidase (APX, EC 1.11.1.11) ,which uses ascorbic acid as a reductant in the first step of the ascorbate glutathione cycle, and glutathione peroxidase (GPX, EC 1.11.1.9) that uses glutathione as electron donors are the most important plant peroxidase involved in H_2O_2 detoxification (*Noctor and Foyer, 1998*). Glutathione reductase (GR, EC 1.6.4.2) is responsible for the reduction of oxidized glutathione for the chain reactions of scavenging H_2O_2 by APX and GPX to be completed and continued (*Mittler, 2002; Apel and Hirt, 2004*). *Mittler et al*. (2004) reported that all these interactive processes are regulated by complex biochemical pathways controlled by more than 150 genes in *Arabidopsis*. However, how these pathways are regulated, it is still not clearly full understood (*Mittler et al., 2004*).

It is generally assumed that salt-tolerant genotypes of most plant species have higher activities/levels of antioxidant enzymes than those of salt sensitive ones (*Logan 2005*), but in some cases the reverse is true (*Munns and Tester 2008*). Many authors proved that the antioxidant enzyme systems are altered under abiotic stresses, including salinity, and the quantitative and qualitative aspects of changes are often related to the levels of resistance to salinity. In rice, the salt-tolerant varieties have higher SOD activity and lower lipid peroxidation than the salt-sensitive varieties (*Dionisio-Sese and Tobita, 1998*). In tomato and citrus, salt-tolerance is attributed to the increased activities of SOD, APX, and CAT (*Mittova et al., 2004*). Also, in the model plant *Arabidopsis thaliana*, mutants lacking one or both cytosolic and chloroplastic APX, involved mainly in H_2O_2 removal, were found to be more tolerant to salt stress (*Mittler et al. 2004*).

Recently, most radish reports have been restricted to determine phytochemical content that have been associated with beneficial health effects such as phenolic (*Sgherri et al 2003*), anthocyanins (*Liu et al 2008; Wang et al 2010*), amylase activity (*Hara et al., 2009*), antioxidant activity (*Lugasi et al 2005; Wang et al., 2010*), antimutagenic (*Nakamura et al., 2001*), and antiproliferative effects (*Yamasaki et al., 2009; Beevi et al., 2010*). In addition, many studies have been conducted for comprising phytochemical composition between radish sprouts (*Kim et al., 2006; Papi et al., 2008*) and mature radishes (*Salah-Abbes et al., 2009; Shukla et al., 2010; Wang et al., 2010*). Furthermore, *Noreen and Ashraf*, (2009) have been studied the effects of salt in the leaves of six radish cultivars. They mentioned that NaCl adversely affected shoot fresh weight and soluble protein, while increased the level of

proline, and SOD and CAT activities. In view of all mentioned reports, there is lack of studies about the effect of salt stress on physiological and metabolites features and enzymes activities of root radish. Thus, in this work, our objectives were evaluating the performance of two botanical varieties of radish (round red or long white) for understanding the salt adaptive mechanisms in radish and defining the most salt tolerant genotype between them; assessing the differential response of seven genetically different radish cultivars upon salinization in term of some phytochemical, secondary metabolite, and enzymatic antioxidative systems to choose the most promoting cultivar(s) under stressed conditions.

2. MATERIAL AND METHODS

2.1. Plant material and culture

Two experiments were carried out at the research station of Bologna University, Italy (44°30‟54‟ N, 11°24‟24‟ E, 39 m a.s.l.), in an experimental glasshouse under controlled conditions (T° max 23°C; T°min 13°C; RH: 60%). In Exp. 1#, two genotypes of round red (cv. SAXA2, Blumen Seed, Milano, Italy) and long white (cv. Candela di ghiaccio, Blumen Seed, Milano, Italy) radish were considered. In Exp. 2#, seven round red radish cultivars was used as follow: cv. Tondo PRECOCI SSIMO 1:TP; cv. SAXA DA FORZARE3: SAXA; cv. Lungo Apunta Bianca, LPB; cv. Lungo Rosso Apunta Bianca3, LRPB; cv. Tondo Apunta Bianca, TPB; cv. SAXA2, and cv. Tondo Apiccola Punta Bianca, TPPB (Blumen seed, Milano, Italy). Seeds were sown in polyethylene trays filled with peat moss. Seven days after germination, seedlings were transplanted onto small plastic pots (250 × 250) filled with perlite: vermiculite (1:1 v: v). Hydroponic irrigation system was used with nutrient solution containing the following composition: NO_3^-: 16.5 mM; NH_4^+: 1 mM; $H_2PO_4^-$: 1.50 mM; SO_4^{2-}: 1.50 mM; K^+: 7.0 mM; Ca^{2+}: 5.0 mM; Mg^{2+}: 1.5 mM; Fe^{2+}: 15 μM; Mn^{2+}: 10 μM; B^+: 25 μM; Zn^+: 5.0 μM; Cu^+: 0.5 μM; Mb^{2+}: 0.5 μM. Salt treatment was begun at 4 days after transplanting (DAT). Three salt concentrations were applied in both trails: 0 (2.68 dS m^{-1}), 100 (7.68 dS m^{-1}) and 200 (8.72 dS m^{-1}) mM NaCl and dissolved in the nutrient solution. At 40 and 30 days after salt treatment, the plants of Exp. 1# and 2# were harvested, respectively.

2.2. Growth measurement

Nine plants from each treatment (three per replicate) were sampled; shoots and roots were separated and their fresh weight (FW) were directly determined. Leaf area index (LAI) and leaf area (LA) was measured on digital images by *Image J* processing software (*Orsini et al., 2011*). Growth indication was calculated, as the Relative Growth Rate (RGR), and the Net Assimilation Rate (NAR) (*Benincasa, 1988*) using the equations:

$$RGR = (\ln DM_2 - \ln DM_1)\,(t_2 - t_1)^{-1} \quad (g.g^{-1}.d^{-1})$$

$$NAR = [(DM_2 - DM_1)\,(LA_2 - LA_1)^{-1}]\,[(\ln LA_2 - \ln LA_1)\,(t_2 - t_1)^{-1}] \quad (g.m^{-2}.d^{-1})$$

Where DM_1 is the initial (15 DAS) total (shoot + root) dry mass, DM_2 the final (30 DAS) total dry mass, LA_1 the initial leaf area, LA_2 the final leaf area, and $(t_2 - t_1)$ the difference in time interval between the two samplings (15 d). Total leaf water potential (Ψw) was determined by using a dew-point psychrometer (WP4, Decagon Devices, Washington, WA). The osmotic potential ($\Psi\pi$) was estimated on frozen/thawed leaf samples. OA =$\Psi_{\pi 0}$ V_0 - Ψ_π V, where $\Psi_{\pi 0}$ V_0 is the product of (osmotic potential) × (osmotic volume) of unstressed plants and Ψ_π V is the product of (osmotic potential) × (osmotic volume) of leaves from salinized plants. Leaf gas exchange measurements as photosynthetic rate (A), stomatal conductance (gs), and transpiration (E) were measured by CIRAS-2 infrared gas analyser (PP-system Hitchin, UK). Punctual water use efficiency (WUE) was calculated as the ratio of A to *E*. Water loss (WL) determinations were performed at 15 DAS. Three plant pots for each treatment were sealed with a plastic film to prevent water loss from the soil surface, leaving the shoot protruding from the film. Before sealing, plants were re-watered to pot capacity with water (control), or water plus 100 or 200 mM NaCl. Each plant was then placed on an electronic balance under glasshouse condition, and the weight loss was measured after 24 h. WL values were normalized respect to whole plant dry weights taken at the end of the measurements.

2.3. Enzymes extraction and assays

The investigation of antioxidant enzymes regulation that provides the protection against NaCl induced oxidative damage in plant took place to assess just the performance of different seven root red radish cultivars in experiment 2.

For protein and antioxidant enzyme extraction, 10 g of fresh leaves were homogenized in 10 ml of 200 mM chilled potassium-phosphate buffer (pH 7.5) containing 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) and 0.1% (v/v) Triton X-100 placed in an ice bath. The homogenate was filtered through a layer of muslin cloth and centrifuged at 10000 × *g* for 20 minutes at 4 °C. The supernatant was collected and eluted through Sephadex G-25 gel column (NAP-25, Amersham Biosciences, Piscataway, NJ, USA) then re-suspended in 10 mM sodium-potassium phosphate buffer (pH 7.0) and used for the determination of the antioxidant enzymes. All enzymatic activities were assayed spectrophotometrically, the analysis was performed in triplicate and the results were normalized by plant fresh weight.

The soluble proteins concentration of the extract was estimated according to Bradford's method using bovine serum albumin as a standard *(Bradford 1976).*

2.4. Malondialdehyde (MDA)

The level of lipid peroxidation was determined by measuring malondialdehyde (MDA) formation using the thiobarbituric acid-reactive substance (TBA) method as described by Heat and Packer (1968). For MDA extraction, 100 µl aliquot of enzyme extract was mixed with 900 µl thiobarbituric acid solution containing 0.5 % (w/v) 2- thiobarbituric acid and 0.5M orthophosphoric acid. The mixture was heated in a water bath at 100 °C for 30 minutes then the reaction was quickly stopped by cooling the tubes in an ice water bath. Afterward, the mixture was centrifuged for 1 minute at 13000 × *g* to remove the unspecific turbidity. The absorbance of the supernatant was measured at 532 nm using spectrophotometer Cary-1 (Varian, California, US). Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA-TBA complex (red pigment) was calculated from the difference of the two wavelengths based on standard curve of MDA.

2.5. Hydrogen Peroxide H_2O_2

The H_2O_2 content was determined as described by *Wolff* (*1994*) measuring the colorimetric reaction of xylenol orange with Fe(III) that generated after the oxidation of Fe(II) by H_2O_2.

Fresh leaf tissue was homogenised with 4ml of 36 mM H_2SO_4 + 1% PVPP and the homogenate was centrifuged at 10000 ×*g* for 20 minutes at 4 °C. The Fe-xylenol orange reagent (FOX) contained 100 µM xylenol orange, 250 µM500 µM $(NH_4)_2Fe\ (SO_4)_2$, 100 mM sorbitol that solved in 25 mM H_2SO_4. For H_2O_2 extraction, 50 µl aliquot of plant sample extract was mixed with 950 µl of FOX reagent and the mixture was incubated for at least 30 min. The absorbance of the supernatant was measured at 560 nm. The amount of H_2O_2 was calculated based on standard curve of H_2O_2.

2.6. Assays of enzymes

2.6.1. Ascorbate peroxidase (APX, EC 1.11.1.11)

Ascorbate peroxidase activity was determined using the method of Chen and Asada (1990). Ascorbate reaction solution contained 50 mM sodium-potassium phosphate buffer (pH 7.0), 1 mM ascorbic acid, 0.5 mM hydrogen peroxide and 100 µl enzyme extract in a final assay volume of 1 ml. Ascorbate oxidation was followed at 290 nm. The concentration of oxidized ascorbate was calculated using an extinction coefficient $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. One unit of APX is defined as the enzyme activity catalysing the oxidation of 1µmol of ascorbic acid per minute.

2.6.2. Catalase activity (CAT, EC 1.11.1.6)

Catalase activity was assayed by measuring the initial rate of disappearance of H_2O_2 and determined using the method of Havir and McHale (1987). Catalase reaction solution contained 50 mM sodium-potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 20 µl of enzyme extract in a final assay volume of 1 ml. The reaction was initiated by adding the enzyme extract and the decrease in H_2O_2 was measured following the changes in the absorbance of the reaction solution at 240 nm. The concentration of CAT was calculated using an extinction coefficient $\varepsilon = 0.036\ mM^{-1}\ cm^{-1}$. One unit of CAT is defined as the enzymatic activity that catalyses the degradation of 1 µmol of H_2O_2 per minute.

2.6.3. Glutathione reductase (GR, EC 1.6.4.2)

Glutathione reductase activity was determined using the method of Foyer *et al.* (1991). Glutathione reductase reaction solution contained 50 mM sodium-phosphate buffer (pH 7.5), 5 mM EDTA, 1mM (NADPH), 1mM oxidized glutathione (GSSG) and 300 µl enzyme extract in a final assay volume of 1 ml. NADPH oxidation was determined at 340 nm. Activity was calculated using an extinction coefficient $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$ for NADPH. One unit of GR is defined as the enzyme activity that oxidizes 1 µmol of NADPH per min at room temperature.

2.6.4. Superoxide dismutase (SOD, EC 1.15.1.1)

Superoxide dismutase activity was determined using the method of Masia (1998) by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) to blue formazan by flavins under illumination. Superoxide dismutase reaction solution contained 50 mM sodium-potassium phosphate buffer (pH 8.0), 300 µM methionine, 1.5 mM NBT, 120 µM riboflavin, 100 mM Na_2.EDTA, 300 µM potassium cyanide and 100 µl enzyme extract in a final assay volume of 1 ml. The riboflavin was added last. The reaction was started by illumination the test tubes under 4 fluorescent lamps for 10 minutes. The absorbance of illuminated solution was measured spectrophotometry at 560 nm. One unit of SOD activity is defined as the amount of enzyme that inhibited 50% of NBT photoreduction versus a blank cell containing no enzymatic extract.

2.7. Proline level

Free proline content was determined according to Bates *et al.* (1973). Proline reaction solution contained 3 mM ninhydrin in 60% (v:v) acetic acid. The samples were heated at 100 °C for 1 h in water bath and then cooled in tap water to stop the reaction. The mixture was extracted with toluene and the absorbance of toluene fraction aspired from liquid phase was

read at 520 nm. Proline concentration was determined using a calibration curve and expressed as μmol proline g^{-1} FW.

2.8. Statistical analysis

The experiment design was randomized block with three replications and 9 plants per replicate. A two-way analysis of variance (ANOVA) was performed using Co-Stat-ANOVA software program. The means and calculated standard errors are reported. The significant was tested using Student-Newman-keuls at 5% significant.

3. RESULTS

Exp. 1#

3.1. Plant growth and physiological response

Reduction in plant growth was appreciated in two radish cultivars upon salinity (Table 1). However, while a greater reduction of yield and leaf area (-94%) was observed in white radish exposed to 200 mM NaCl, the red cultivar seemed to better stand the stress and showed reduction by (-74% and -72%) compared to control plant. There was a sharp diminution in RGR in white radish (-141%) versus (-49%) in red one at 200 mM NaCl referring to control plant (Table 1). Similarly, the highest reduction in NAR was recorded in white cultivar (-186%) versus (-39%) in red one at 200 mM NaCl related to non-treated plant (Table 1). LAI had been affected by salinity and was most evident for white radish that showed decreasing by (-94%) compared with (-72%) in red one at 200 mM NaCl. Though all gas exchange parameters showed significant reduction upon salinity in both cultivars, there wasn't any appreciated difference between their performances even at higher salt level (Figure 1A, B and C). Total leaf water potential (Ψw) and osmotic potential (Ψπ) in both cultivars showed similar pattern of redaction at 100 mM NaCl (Figure 1E and F). However, at exaggerated salt (200 mM), white radish showed higher significant reduction in both parameters by (75% and 21%, respectively) in related to red plant. Conversely red cultivar showed noteworthy higher turgor potential by (+245%) compared to white one at 200 mM NaCl. Also, water use efficiency (WUE) increased substantially by (+242%) in red cultivar while it showed sharp reduction by (-72%) in white one at 200 mM NaCl compared to zero salt plant (Figure 1D). Indeed, total transpiration in white radish was higher by two times as red plant under control condition (Figure1H). Upon salinization, both cultivars showed a significant reduction in water loss. However, the rate of water loss in white radish was increased to a greater extent by (+381%) compared to red plant at 100 mM NaCl.

Exp. 2#

3.1. Plant growth and physiological response

Salinity suppressed significantly the shoot fresh weight and yield of all radish cultivars (Table 2). However, the response of the radish cultivars was expressively different at varying NaCl level. For example, SAXA2 and TP cultivars had relatively higher yield in comparing with other cultivars by (+29% and +143%, as an average) under control and 100mM NaCl, respectively. Similar results confirmed by shoot fresh weight (Table 2). However, there was no appreciated difference in yield and shoot fresh weight among cultivar's performance at 200 mM NaCl. Upon salinity, all cultivars showed unique reduction in net photosynthesis (Figure 2A), evaporation rate and stomatal conductance (data not shown) and yet without any pronounced genotypic variation among them. On other hand, TP and SAXA2 cultivars showed higher values of water use effciency by (+80% and +141%, as an average) at 100 and 200 mM NaCl, respectively in related to all other cultivars (Figure 2B). Leaf water potential (Ψw) and osmotic potential ($\Psi \pi$) reduced significantly with increased salt content in all cultivars (Figure 3A and B). However, the differences among the cultivars was not noteworthy observed except for cvs TP and SAXA2 that showed higher reduction in both parameters upon 200 mM NaCl. Likewise, all cultivars showed similar ability to maintain their osmotic adjustment except cv SAXA2 that showed the highest OA value at 200 mM NaCl (Figure 3C).

3.2. Lipid peroxidation, H_2O_2, protein, and proline content

Increasing supply of NaCl did not change clearly the level of MDA, and all salinized root radish cultivars showed similar MDA content compared to control condition (14 $\mu M\ g^{-1}$ FW as an average) (Figure 4A). Likewise, salt stress did not significantly affect root H_2O_2 content and all cultivars responded without variation in this biochemical attribute (Figure 4B). In the same way, there was no significant effect of salt treatment on total soluble protein of all radish cultivar roots except in relatively salt tolerant cv. SAXA2 that showed a pronounced increasing by (+190% ,as an average) compared to all other cultivars at 200 mM NaCl (Figure 4C). Adding salt to growth medium caused a significant increasing of proline level in all root radish cultivars (Figure 4D). However, both cultivars SAXA2 and TP showed the highest proline content (11 and 14 $\mu M\ g^{-1}$ FW, as an average) under 200 mM NaCl, respectively in respect to other cultivars.

3.3. Enzymes activities

The performance of plant under control condition showed similar values of APX activity compared to both salinized growth mediums (119 versus 122 $Ug^{-1}FW$, as an average) (Figure 5A). In spite of salt treatment did not enhance APX activity in all radish cultivar roots, still cv. SAXA2 showed higher APX activity by (+131%, as an average) at 200 mM NaCl compared to other cultivars. Under control condition, the cultivar SAXA2 followed by TP showed the highest CAT activity by (+255%, as an average) as compared to other cultivars (Figure 5B). Despite that both saline levels significantly reduced CAT activity of all radish cultivar roots, cv. SAXA2 showed higher value of CAT activity by (+480%, as an average) in related to all other cultivars at 200 mM NaCl. On contrary, saline growth medium experienced an appreciated increasing in GR activity in all radish cultivar roots (Figure 5C). However, SAXA2 cultivar followed by TP showed higher GR value by (+151% and +395% as an average) compared to all other cultivars under 100 and 200 mM NaCl, respectively. Likewise, salt treatment enhanced SOD activity and all roots showed a similar significant increasing in SOD activity under 100 mM NaCl by (+210%) compared to control plant (Figure 5D). However, at higher salinized level (200 mM NaCl), SOD activity reduced in all roots less than control but was still insignificant reduction (35 versus 46 $Ug^{-1}FW$ as an average).

4. DISCUSSION

Recent study indicated that radish could possibly be grown with salty solutions at EC (12.7 dS m^{-1}) (*Yildrim et al., 2008*). It is well documented that high salinity causes inhibition of plant growth, impaired metabolism, imbalance of nitration in plant (*Cavagnaro et al., 2006; Jie et al 2006*), instability of plasma membrane resulting from calcium displacement by sodium (*Santos 2004, Mansour and Salama, 2004*). Furthermore, many physiological phenomena such as stomatal regulation, photosynthesis, protein synthesis and turgor-pressure-driven solute transport in xylem depend upon the availability of potassium to plants (*Ashraf, 2004; Marschner, 1995*). Under salt stressful environments, the availability of nutrients to plants including K is hampered (*Ashraf, 2004; Munns, 2005*), and that leads to diminished plant growth and development (*Chen et al., 2007*). Typical agronomic selection parameters for salinity are yield, leaf area, and relative growth rate (*Ashraf and Harris, 2004; Okhovation-Ardakani et al 2010*). In this investigation in Exp. 1#, the white cultivar recorded greater reduction in yield by (-78%) compared to red one at imposition of 200 mM NaCl (Table 1). Likewise, 200 mM salinized white cultivar showed drastic reduction in both leaf area and leaf area index by (-76%) as compared to red one (Table 1). However, *Marcelis and*

VanHooijdonk, (*1999*) reported that 80% of growth reduction in *Raphanus sativus* (radish) at high salt content attributed to a reduction in leaf area and consequently reduced light interception, while 20% of growth reduction referred to reduction in stomatal conductance. It is known that net assimilation rate (NAR) is considered as a good physiological marker for salt tolerance (*Azevedo Neto and Tabosa, 2000a*). The photosynthetic capacity in crop plants is vital for dry matter production (*Akram et al 2009*). Thus, the final biological yield or economic yield can be increased either by increasing the rate of photosynthesis or by optimizing assimilate partitioning (*Natr and Lawlor, 2005*). In this work, we assessed the biomass production of both cultivars under salt in term of relative growth rate (RGR) and net assimilation rate (NAR). The application of 200 mM NaCl reduced significantly those both parameters in white plants by (-141% and -186%) versus (-49% and -39%, respectively) in red cultivar related to control plant (Table 1). Based on these considerations, red radish appeared to be relatively more tolerant than the white one. Furthermore, it is well known that crop salinity sensitivity varies with species, genotypes, and growth stages (*Prado et al 2000, Pujari and Chanda 2002*). Accordingly, in Exp. 2# seven different red radish cultivars were investigated to identify some candidate susceptible/ resistant genome(s) toward salt stress. Salinity markedly suppressed the shoot and yield of seven radish cultivars (Table 2). However, similar growth reduction was recorded for all cultivars except cvs. SAXA2 and TP were found to be consistently higher in growth and yield particularly at 100 mM NaCl. Accordingly, we could suggest that SAXA2 followed by TP are the most tolerant cultivars. These results showed an intraspecific variability in the response to salt stress of *Raphanus sativus* which is considered as a promising tool for future screening. Similar results of growth variation among cultivars have been reported in canola (*Ulfat et al., 2007*), radish (*Noreen and Ashraf, 2009*) and tomato (*Foolad, 1996*).

Stomatal regulation is an important factor in controlling photosynthetic rate as well as water balance of plant growing under stress condition (*Athar and Ashraf 2005*). Salt stress reduces the rate of water uptake by the root, accordingly, an imbalance between water uptake by root and water loss by transpiration causes plant to wilt (*Lafitte 2002*). Therefore, plant defends itself from water loss by closing the stomata to avoid fast dehydration and allowed them to adjust to unfavorable conditions (*Boughalleb et al., 2009; Orsini et al 2010*). Furthermore, a reduced photosynthesis rate is considered as normal reaction of plant under salt which due to stomatal closure with the consequent restriction of CO_2 availability for carboxylation or toxic effect of salinity on the photosynthesis apparatus (*Praxedes et al., 2010; Orsini et al, 2010a*).

In our work in Exp. 1#, both cultivars showed unique performance and presented similar significant diminishing in gas exchange parameters after imposing salt (Figure 1A, B and C), suggesting that photosynthetic parameters are negatively related to assess salt tolerant feature in radish. Similarly in Exp. 2#, net photosynthesis of seven radish cultivars showed uniform significant reduction upon salt application compared to control plants (Figure 2A). This result was consistent with many reports of suppression net photosynthesis under salt in tomato (*Romeroaranda et al., 2001; Maggio et al., 2007*), soybean (*Kao et al 2003*) and barley genotypes (*Jiang et al., 2006*).

The ability of plant to control transpiration water flux versus growth (i.e water use efficiency) under salinized condition is a critical tolerance determinant (*Maggio et al., 2006; Orsini et al 2010; Barbieri et al 2012*). In Exp. 1#, both cultivars showed significant reduction in water uptake, which expressed in term of water loss by (-65%) in white plant versus (-55%) in red one at 200 mM NaCl (Figure 1H). Study by *Bayuelo-Jimenez et al*. (*2003*) have shown that water uptake and transpiration declined as the salt concentration in the irrigation water increased, and this reduction could be related to reductions in leaf area and stomatal conductance. However, transpiration water flux is associated with ion loading in which reduced transpiration flux will restrict the accumulation of ions to the shoot (*Zhu et al 2002*). Consequently, this remark could interpret that white genotype might uptake and load more ions to its shoot than red one which affected its growth development (Table 1). Furthermore, red cultivar was more efficient in water use and showed significant increasing in its WUE as 13 folds as white plant under 200 mM NaCl (Figure 1D). This might be explained, at least in part, by the fact that red cultivar had higher WUE that associated with lower reduction in total transpiration rates (Figure 1H). In Exp. 2#, both cultivars SAXA2 and TP showed higher WUE upon salt stress (Figure 2B). In previous studies, *Pagter et al.* (*2009*) reported that saline conditions increased the WUE (*Gorai et al 2010*) and did not alter photosynthetic parameters derived from light response curves, supporting the assumption of a well-functioning CO_2 utilization even if plants were salt stressed. *Ashraf* (*2001*) linked the increasing of WUS in salt-tolerant *Brassica* species to having higher assimilation rates and lower stomatal conductance, while *Masle et al.,* (*2005*) and *Barbieri et al.,* (*2012*) reported that lower stomatal density is a critical determinant for having higher WUE in *Arabidopsis* and *Ocimum basilicum* L.

It is known that the ability of plant to maintain its osmotic potential at levels below of the osmotic potential of the soil surrounding the plant considers as a successful tolerated mechanism to face the harmful effect of salt (*Tester and Davenport, 2003; Zhu, 2001*) in a process called osmotic adjustment (*Munns 2002, Serraj and Sinclair 2002*). It is accepted that during osmotic adjustment the cells try to compartmentalize most of absorbed ions in vacuoles that associated with synthesising and accumulating of compatible organic solute in the cytoplasm in order to maintain the osmotic equilibrium between these two compartments (*Hasegawa et al., 2000*). However, in some cases accumulation of solutes is so high that it goes beyond the limits of regulation of cytoplasmic content with associated impairment of growth (*Pitman 1984*). In this study in Exp. 1#, higher decreases in water and osmotic potential was recorded in white radish (Figure 1E and F) and this might be attributed to fact that white plant uptake more water (Figure 1H) and accordingly had higher Na^+ and Cl^- accumulation which possibly exceeded the amount needed for the osmotic adjustment. Consequently, this process accelerated tissue dehydration and causing injury to metabolic systems that affected negatively plant development of white radish (*Munns, 2002*). Similar result was recorded by *De Lacerda et al* (*2003*) who mentioned that higher reduction in osmotic potential was recorded in salt sensitive sorghum. Conversely, leaf turgor potential significantly increased in red cultivar as three times compared to white plant at 200 mM NaCl (Figure 1G). However, the maintenance of high leaf turgor was possibly functional to support the plant growth of red cultivar and yield upon salinization, whereas white plant was subject to salt induce disturbance of water balance and loss of leaf turgor (*Wang and Nil 2000*). Several authors have associated tissue turgor maintenance to the plant's ability to osmotically adjust in saline environments (*Munns 2002; Maggio et al 2005*). Thus, these physiological perspectives in term of better adaptation of overall water relations appeared to be more effective in red radish as compared to white plants, suggesting that salt adaptive mechanism in relatively salt tolerant red radish was associated with plant's ability to osmotically adjust under salt stress. In Exp. 2#, there was a substantial reduction in water and osmotic potential in all seven cultivars upon exposure to both salt levels (Figure 3A and B). However, radish cultivars showed significant genotypic variation in which TP and SAXA2 showed higher reduction in both parameters by (27% and 16%, as an average) respectively in related to other cultivars at 200 mM NaCl. Our results were in the same trend with other researchers who mentioned about significant reduction in osmotic potential under salt stress in barley leaves (*Yagmur et al. 2006*) and bean seedlings (*Gama et al., 2009; Stoeva and kaymakanova, 2008*). Many previous reports showed variation in OA response between cultivars upon salt

application such as soybean (*Ping et al, 2002*), wheat (*Abdel- Aziz & Reda 2000*) and tomato (*Santa-Cruz et al., 1999*). In our work, the relatively salt tolerant cultivar SAXA2 showed higher ability to adjust larger value of OA (1.94 MPa) at 200 mM NaCl in related to other cultivars (Figure 3 C). This finding was in line with (*Flowers, 2004; Munns and tested, 2008; Ashraf and Harris, 2004*) who documented that osmotic adjustment is a fundamental response of plant under salt condition and it is considered one of the important mechanisms of salt tolerance. Thus, we could suggest that salt tolerant feature was achieved possibly by contribution of inorganic ions and or/ organic accumulation in this cultivar, while the performance of this plant in term of photosynthetic parameters and gas exchange was similar to other cultivars. Consistently, the selection of the radish types to be used in the successive steps went to the round red type in order to assess its enzymatic adaptation to salinity and its role in counteraction the ROS induced damages at cell level.

MDA, a product of lipid peroxidation in plants exposed to adverse environmental conditions, is a reliable indicator of free radical formation and peroxidative damage to cell membranes (*Logan 2005*). *Shafi et al*. (*2010*) stated that MDA level was increased under salt condition in wheat plant. *Aksoy and Seçkin Dinler* (*2012*) mentioned that increased MDA level in salt treated soybean was related to increasing reactive oxygen species. Many previous reports mentioned that lipid peroxidation was strongly variable among cultivars upon salinization such as pea (*Noreen and Ashraf*, 2009), *Crithmum maritimum* L. (*Karim et al 2012*), and Iranian wild almond species (*Sorkheh et al, 2011*). However, our results showed that the level of lipid peroxidation was similar in stressed and non-stressed root plants and there was no noteworthy effect of salt on MDA level (Figure 4A). Consequently, the extent of lipid peroxidation was similar among different radish cultivars, suggesting that membrane peroxidation is not the main detrimental effect caused by salt stress and it was not associated with salt tolerance of red radish. This result was agreed with *Jouve et al.* (*2003*) who found that the endogenous level of MDA did not vary in the control and salt stressed Aspen (*populous tremula* L.). Also, *Noreen and Ashraf* (*2009*) reported that MDA content decreased in all radish cultivars upon salinization except in salt tolerant one and they suggested that lipid peroxidation was negatively associated with salt tolerance of radish cultivars. Likewise, *Sorkheh et al*, (*2011*) reported that MDA is not affected harmfully by salt stress and its level was increased in salt sensitive almond species *p. arabica* while it reduced in other salt sensitive almond species upon salt treatment.

It is known that salt stress induces the production of singlet oxygen, superoxide onion, hydrogen peroxide, and hydroxyl radical in plant (*Mateo et al., 2004*). H_2O_2 is a strong oxidant that can initiate localized oxidative damage in cells leading to disruption of metabolic and loss cellular integrity (*Sairam and Srivastava, 2000*) and it must be eliminated by conversion to H_2O in reaction involving APX and CAT. In the present study, root H_2O_2 remained unchanged upon salt stress in all studied cultivars (Figure 4B). Similar results were reported by *Sorkheh et al.,* (*2011*) who mentioned that there was no significant change in leaf H_2O_2 of almond species upon salinization. On contrary, *Mittova et al.,* (*2004*) found that the mitochondria and peroxisomes of salt stressed tomato root presented increased levels of lipid peroxidation and H_2O_2.

Protein synthesis is considered as one of the mechanisms that have been affected by salt stress in plant and a possible primary target of salt toxicity (*Gulen et al., 2006*). In addition, soluble protein content is an important indicator of physiological status of plant (*Doganlar and Atmaca, 2011*). In our results, there was significant increasing in protein content just in cultivar SAXA2 by (+366%) at 200 mM NaCl (Figure 4C). This increasing is likely attributed to increase protein synthesis of enzymes upon stress activation and might suggest that there was a stress-induced protein upon stress activation in this cultivar. *Mittal et al.,* (*2012*) and *Ashraf and Harris*. (*2004*) reported higher levels of soluble protein in salt tolerant cultivars of barley, sunflower, finger millet and rice. Also, *Kapoor and Srivastava* (*2010*) observed an increasing in protein content associated with salt stress in *Vigna mungo* L. Consequently, in consistent with all previous reports, we might suggest that protein could be one of the main organic solute involved in the osmotic adjustment in this cultivar.

One of the most important mechanisms of higher plants under salt stress is the accumulation of compatible solutes like proline (*Hasegawa et al., 2000*). Proline is known to be an osmoregulatory solute in plant under hyperosmotic stress, and plays a functional role as primary defense response to maintain osmotic adjustment and protecting cell structure (*Misra and Gupta, 2006; Al-Saady et al, 2012; Koca et al., 2007*). It acts as a free radical scavenger (*Chen and Dickman, 2005*) to alleviate salt stress, and it is considered as enzyme protectants against abiotic stresses (*Sharma and Dubey, 2005*) by stabilizing many functional units such as complex II electron transport (*Hamilton and Heckathorn, 2001*), membranes and proteins (*Holmström et al., 2000*). In this investigation, although all radish cultivars showed a significant increasing in proline content upon salinization, SAXA2 cultivar followed by TP

possessed comparatively higher amount of proline accumulation (Figure 4D). This result was in agreement with (*Hoseini et al., 2010; Najafi et al., 2006*) who reported that increased proline level is a common response in root and shoot of *Carthamus tinctorius* and *Pisum sativum*. Indeed, the variation in proline accumulation among radish cultivars was analogous to what have been reported by *Ashraf and Foolad*. (2007) that the contribution of proline varies among species and cultivars of a same species. However, higher level of proline might due to expression of genes encoding enzymes of proline synthesis such as pyrroline-5-carboxylate or decrease in enzymes of proline oxidative such as proline dehydrogenes which affected by osmotic and salinity stress (*Amini and Ehsanpour, 2005; Misra and Gupta, 2006*). Accordingly, we could conclude that the observed salt tolerant in SAXA2 was attributed, at least in part, to the osmoprotectant effect of compatible osmolytes such as protein and proline accumulation that helped the plant to achieve its osmotic adjustment.

Induction of antioxidant enzyme activities is a general adaptation mechanism that the plant used to overcome oxidative stress (*Foyer and Noctor, 2003*). It is well reported that to endure salt-induced oxidative damages, constitutive and/or induced activity of antioxidants such as SOD, POX, CAT and GR is crucial (*Foyer and Noctor, 2005*). Generally, salt tolerant cultivars showed higher activities of these antioxidant enzymes as compared to salt-sensitive ones (*Sairam et al., 2002*). *Sofo et al*. (*2010*) suggested that higher antioxidant enzyme activity are present in tolerant than in sensitive woody species under environmental stress. It is known that H_2O_2, which produced by the activity of SOD, is a strong inhibitor of the Calvin Cycle and it must be eliminated by conversion to H_2O in reactions involve APX and CAT (*Sorkheh et al., 2011*). APX plays a vital role in plant defence against oxidative stress by scavenging H_2O_2 in chloroplast, cytosol, mitochondria, and peroxisome of plant cells (Asada, 2006). Many reports have shown increased activity of APX upon salt treatment (*Hernandez et al., 1995; Oidaire et al., 2000*). In the present work, APX activity did not vary between salt stressed and control plants (Figure 5A). According to *Mittova et al.,* (*2004*), high APX activity may show high level of H_2O_2 production in cell walls and/or cytosol it is localized in. Accordingly, referring to have a steady activity of APX upon salinization in regard to control plant, H_2O_2 production may be low where this enzyme is located (*Doğan, 2001*). In addition, the decreasing of APX activity under salt stress may be related to its detoxification (reduction) capacity being below the oxidation capacity of H_2O_2 (*Doğan, 2001*).

CAT enzyme, the main H_2O_2 scavenging enzyme, also plays role in detoxifying H_2O_2. There are many studies stated that stress has a preventing effect on CAT activity, and the destructive cells, as a cause of salt, has a distinctive role in decreasing CAT activity (*Doğan, 2001; Sairam et al., 2005; Lee 2001*). However, *Shalata et al*. (*2001*) reported that CAT has a fundamental role on protecting against salt stress. In this work, CAT activity was down-regulated by salt stress in all cultivars (Figure 5B). Accordingly, the decrease in CAT activity that associated with stable regulation of APX activity upon salinization could indicate that a little H_2O_2 is formed (0.9 nM g^{-1} FW , as an average Figure 4B) in the root of salt treated radish, so that CAT and APX activation are not required to detoxify H_2O_2 (Figure 5A and B). Our result was consistent with *Ben Hamed et al.* (*2012*) who found that salt stress reduced the CAT activity while APX activity did not change in *Crithmum maritimum* L. plant. On contrary to our result, *Bor et al.* (*2003*) and *Sekmen et al.* (*2007*) observed that salt induced activities of CAT and APX in sugar beet and plantain, respectively. However, *Sandalio et al.* (*2001*) attributed the reduction of CAT activity in pea plant upon salinization to a decrease in protein content. Also, CAT is associated with peroxisomes that contain proteases and CAT being a target of the peroxisomal protease activity (*Distefano et al., 1999*).

GR, the last enzyme of ascorbate-glutathione cycle (ASC-GSH), plays an essential role in plant defence against ROS by catalyzing NADPH-dependent reduction of oxidized glutathione and maintaining the GSH level (*Madhava Rao et al., 2000; Ben Hamed et al, 2012*). It is known that decreased GR activity enhances plant sensitivity to environmental stress (*Aono et al., 1995*). In our experiment, NaCl treatment induced remarkably GR activity in all radish cultivars and particularly in SAXA2 and TP (Figure 5C). The oxidative stress in SAXA2 followed by TP appeared to be prevented by the ascorbate-glutathione cycle as shown by the increased GR activity under both salt levels, suggesting that both cultivars might have employed non-enzymatic routs for conversion of $O_2^{\bullet-}$ to H_2O_2 using antioxidant like GSH and ascorbate. Similar result was found by *Vaidyanathan et al.* (*2003*) on NaCl-stressed rice (*Oryza sativa* L.). The induction of GR by salinity could increase the NADP/NADPH ratio, promoting the availability of NADP to accept electrons from the chain of electron transfer and limiting ROS formation in chloroplast (*Ben Amore et al., 2006*).

SOD plays a significant role in protecting living cells against the toxicity of active O_2 species due to their capacity to scavenge superoxide (*Scandalios, 1993*). *Ashraf* (*2009*) found the variation in the activity of SOD in response to salinity appeared at the inter-specific or intra-

specific level. In this study, salt stress enhanced steady and similar increasing in SOD activity among all radish cultivars at 100 mM NaCl and there was no significant change in SOD activity detected in the most tolerant cultivar, SAXA2 (Figure 5D). It is known that catalyzing the dismutation of superoxide radical by SOD to H_2O_2 and O_2 caused superoxide concentration low and stable and thus minimizing the creation of hydroxyl radicals by Haber-Weiss reaction (*Elstner, 1982; Bowler et al., 1992*). Similar result was reported by *Oidaire et al*.(*2000*) who found SOD activity increased steadily after salt stress on rice seeds. The increased SOD activity might increase the plant ability to scavenge $O_2^{\bullet-}$, which might lessen membrane damage, and this partly could interpret that there was no variation in MDA and H_2O_2 contents between stressed and relieved plants (Figure 4A and B). Our results was inconsistent with many reports that indicated differential response of this enzyme between cultivars such as potato (*Rahnama and Ebrahimzadeh, 2005*), wheat (*Sairm et al., 2002*), Brassica (*Kumar et al., 2008*), and strawberry (*Turhan and Gulen Erics, 2008*).

5. CONCLUSION

in the first experiment, applying different salt on round red and long white radish showed interestingly that round red radish had less reduction in yield, dry matter accumulation and leaf area. However, both cultivars showed unique significant diminishing in gas exchange parameters after imposing salt, suggesting that photosynthetic parameters is negatively related to assess salt tolerant feature. However, higher decreases in water and osmotic potential was recorded in white radish and this might indicated that the accumulation of solutes was so high that it went beyond the limits of regulation of cytoplasmic content that caused impairment of its growth. Also, white genotype showed less efficient in water use and higher transpiration and water loss which could be attributed to fact that white plant uptake more water and accordingly load more ions that accelerated tissue dehydration and affected negatively its development. Based on these considerations, red radish appeared to be relatively more tolerant than the white one. In second expiremnt, the investigation of intraspecific variability of seven different red radish cultivars upon salt stress was assessed to identify some candidate susceptible/ resistant genome(s) toward salt stress. Upon salinity, similar growth reduction was recorded for all cultivars except cvs. SAXA2 and TP were found to be consistently higher in growth and yield. Also, radish cultivars showed significant genotypic variation in which SAXA2 cultivar showed the sharpest reduction in water and osmotic potential and the highest ability to adjust greater value of OA. Consequently, this reflects the ability of this cultivar to osmotically adjust. Furthermore, the biochemical data suggested that the induction of antioxidative enzymes activity is an important component of

the tolerance adaptation mechanism of radish to salinity (*Mittal et al, 2012*).We have found steady and similar response of stressed and control plants in term of MDA and H_2O_2, suggesting that these two components are negatively associated to salt oxidative stress. Regarding the enzymes that converted H_2O_2 to water, APX showed steady activity in stressed and non-stressed plant, while CAT decreased upon salinization which could suggest that a little H_2O_2 is formed in the root of salt treated radish, so that CAT and APX activation are not required to detoxify H_2O_2. Nonetheless, there was no variation in SOD activity among different cultivars upon salt application, suggested that the variation could appear at inter-specific rather than intra-specific. However, SAXA2 followed by TP showed up-regulation of GR activity among different enzymes and metabolites involved in oxidative stress. This could indicate that both cultivars might have employed non-enzymatic routs for conversion of $O_2^{\bullet-}$ to H_2O_2 using antioxidant like GSH and ascorbate. In addition, SAXA2 showed higher activity of CAT enzyme upon salinization in comparison with other cultivars. Accordingly, the higher activity of GR and CAT in SAXA2 could be related with prevention of the creation of ROS more effectively. Thus our finding suggests that the cultivar SAXA2 that induce anti-oxidative enzymes in response to salt may greatly contribute to its ability to sustain growth and give higher productivity in presence of stressed condition.

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Table 1. Effect of different salt concentration (0, 100 and 200 mM NaCl) on vegetative growth of red and white radish seedling at 40 DAS. Same letters in each column indicate no significant differences among treatments at $P < 0.05$ level.

Cultivar	Salt (NaCl)	Yield (g plant^{-1})		Leaf area (cm^{-2} plant^{-1})		RGR (g g^{-1} d^{-1})		NAR (mg cm^{-2} d^{-1})		LAI	
White	0	28±3.49	a	749±25.23	a	14±3.18	a	0.24±0.06	a	1.87±0.06	a
	100	8±1.08	c	247±16.43	cd	8±2.32	a	0.16±0.04	a	0.62±0.04	cd
	200	2±0.14	c	43±5.10	e	-6±2.98	b	-0.21±0.11	b	0.11±0.01	e
Red	0	28±2.99	a	657±54.93	b	11±2.43	a	0.22±0.03	a	1.64±0.14	b
	100	15±1.10	b	330±7.57	c	17±2.25	a	0.37±0.02	a	0.82±0.02	c
	200	8±0.15	b	181±24.88	d	5±3.15	a	0.13±0.08	a	0.45±0.06	d
Salt (S)		***		***		***		**		***	
Var (V)		*		ns		*		**		ns	
S×V		ns		**		ns		*		**	

Table 2. Effect of different salt concentration (0, 100 and 200 mM NaCl) on shoot fresh weight (FW) and yield of seven red radish seedlings at 30 DAS. Same letters in each column indicate no significant differences among treatments at $P < 0.05$ level.

	Cultivar	**0**		**100**		**200**	
Shoot FW (g plant^{-1})	**TP**	25±1.51	a	11±0.54	de	6±0.62	hi
	SAXA	15±1.10	bc	6±0.28	hi	3±0.30	hi
	LPB	11±0.85	def	4±0.78	hi	2±0.23	i
	LRPB	15±0.77	bc	5±0.83	hi	3±0.32	i
	TPB	13±1.34	cd	5±0.02	hi	2±0.41	i
	SAXA2	17±0.50	b	7±0.67	e	5±0.62	hi
	TPPB	9±1.01	efg	3±0.29	hi	2±0.33	i
Yield (g plant^{-1})	**TP**	22±1.51	a	7±0.77	e	4±0.84	gh
	SAXA	19±1.16	b	3±0.71	gh	4±0.66	gh
	LPB	16±0.86	c	4±0.51	gh	3±0.24	gh
	LRPB	20±0.33	b	2±0.41	gh	4±0.39	gh
	TPB	14±0.67	d	2±0.40	gh	1±0.05	h
	SAXA2	23±0.58	a	6±0.33	e	4±0.33	gh
	TPPB	18±0.88	b	3±0.31	gh	2±0.64	gh

	Shoot FW (g plant^{-1})	**Yield (g plant^{-1})**
Salt (S)	***	***
Var (V)	***	***
S×V	***	***

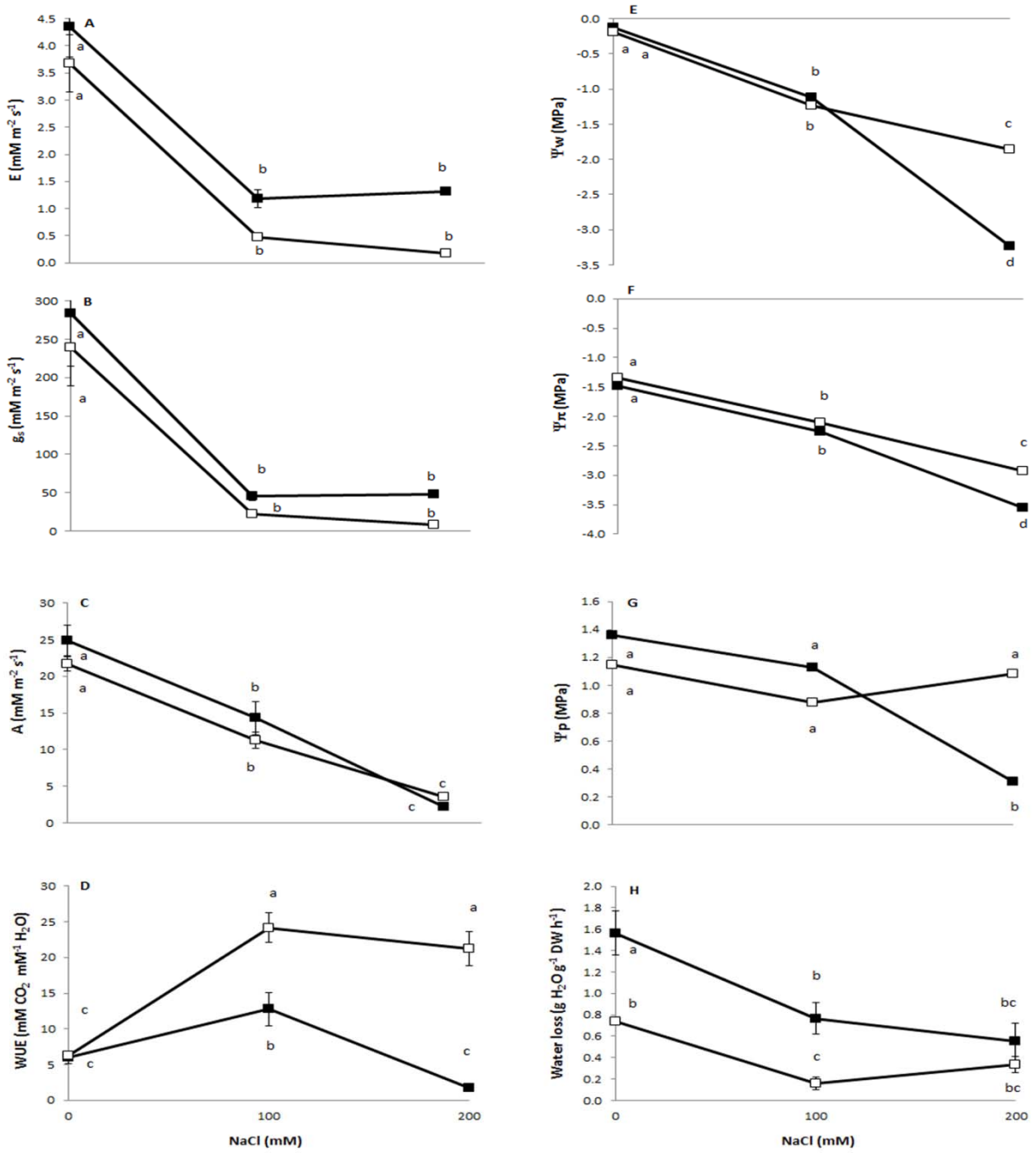

Figure 1. Effect of different salt concentration (0, 100 and 200 mM NaCl) on transpiration rate (E), stomatal conductance (gs), net photosynthesis (A), water use efficiency (WUE), water potential (Ψw), osmotic potential (Ψπ), turgor potential (Ψp) and water loss (WL) of red (open squares) and white radish seedling (closed squares) at 40 DAS. Values are the mean ± SE of three replications.

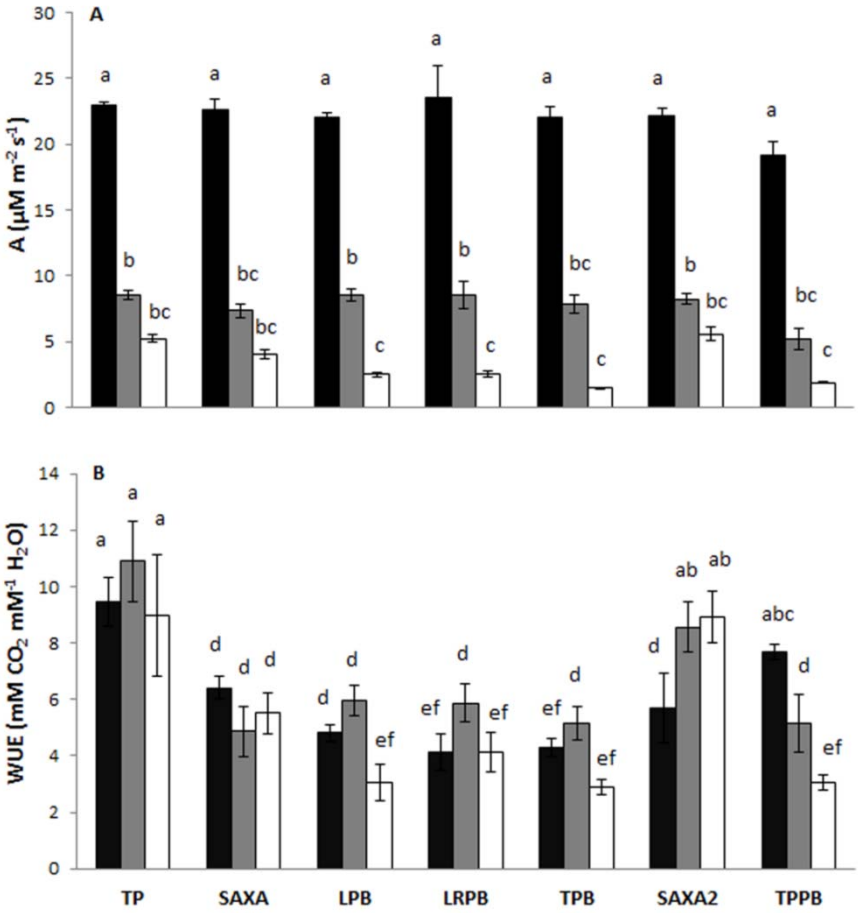

Figure 2. Effect of different salt concentration (0, black bar; 100, gray bar; and 200 mM, white bar NaCl) on net photosynthesis (A) and water use efficiency (WUE) of seven red radish seedlings at 30 DAS. Values are the mean ± SE of three replications.

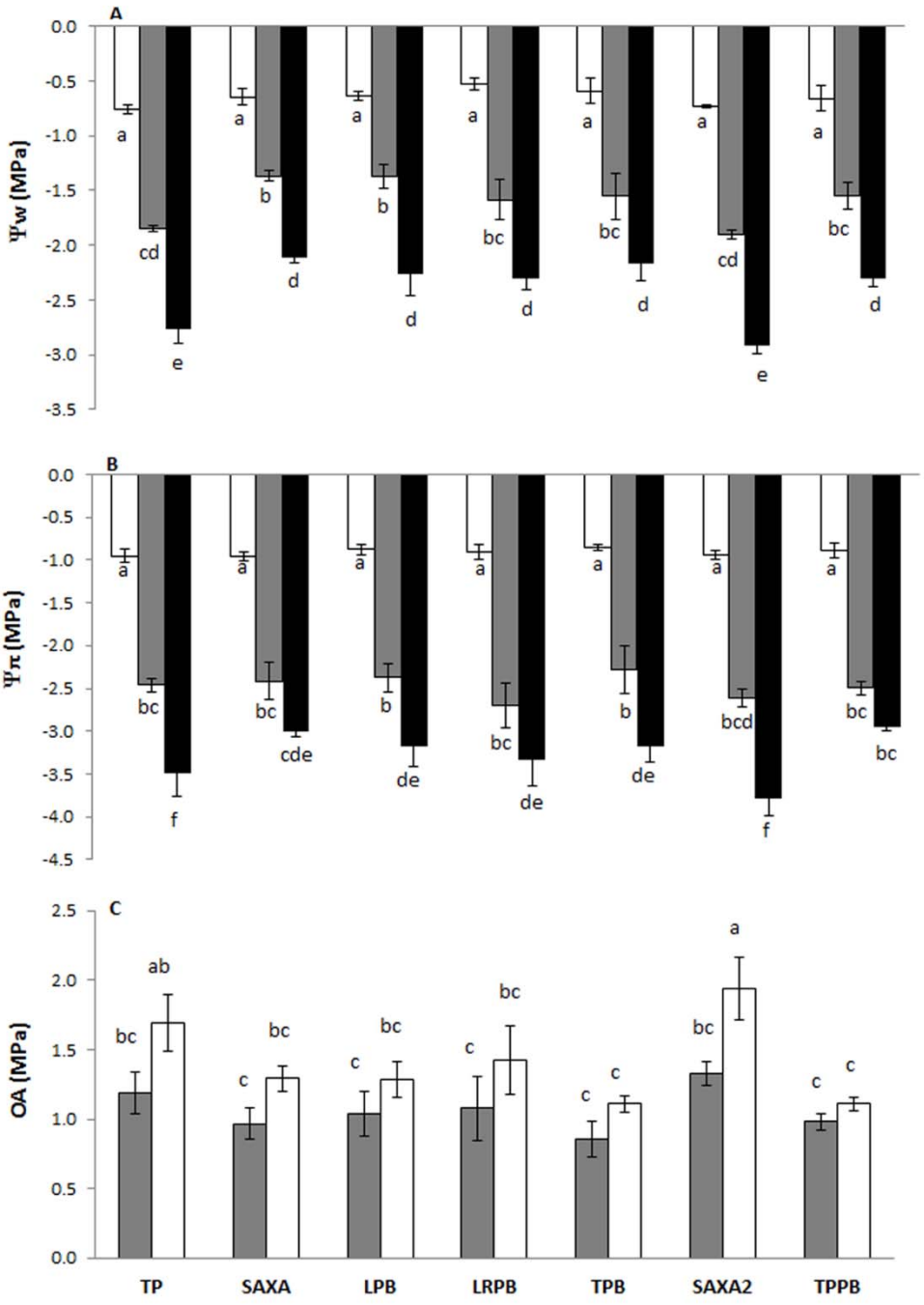

Figure 3. Effect of different salt concentration (0, black bar; 100, gray bar; and 200 mM, white bar NaCl) on water potential (Ψw), osmotic potential (Ψπ), and osmotic adjustment (OA) of seven red radish seedlings at 30 DAS. Values are the mean ± SE of three replications.

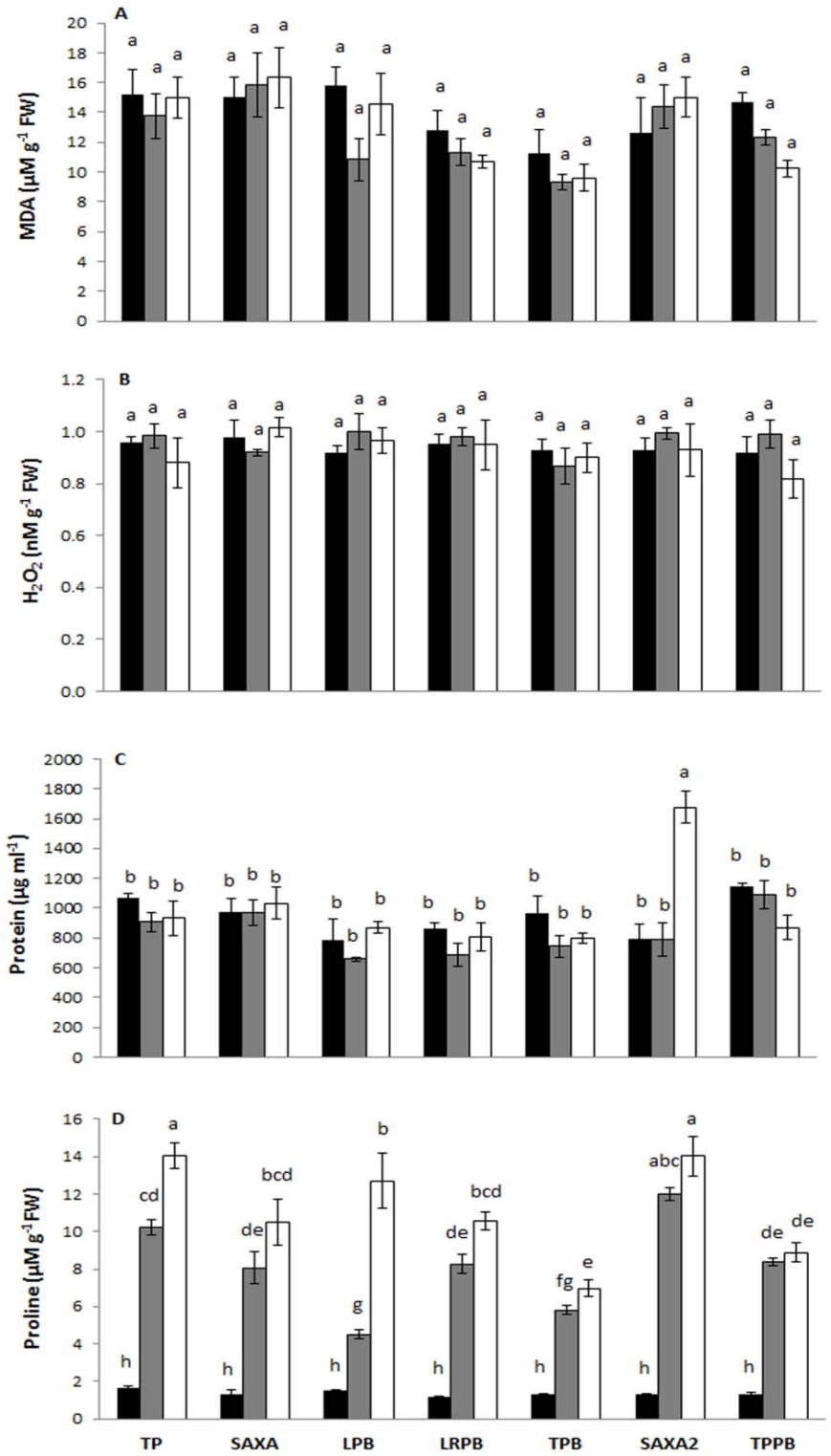

Figure 4. Effect of different salt concentration (0, black bar; 100, gray bar; and 200 mM, white bar NaCl) on MDA, H_2O_2, protein, and proline content of seven red radish root at 30 DAS. Values are the mean ± SE of three replications.

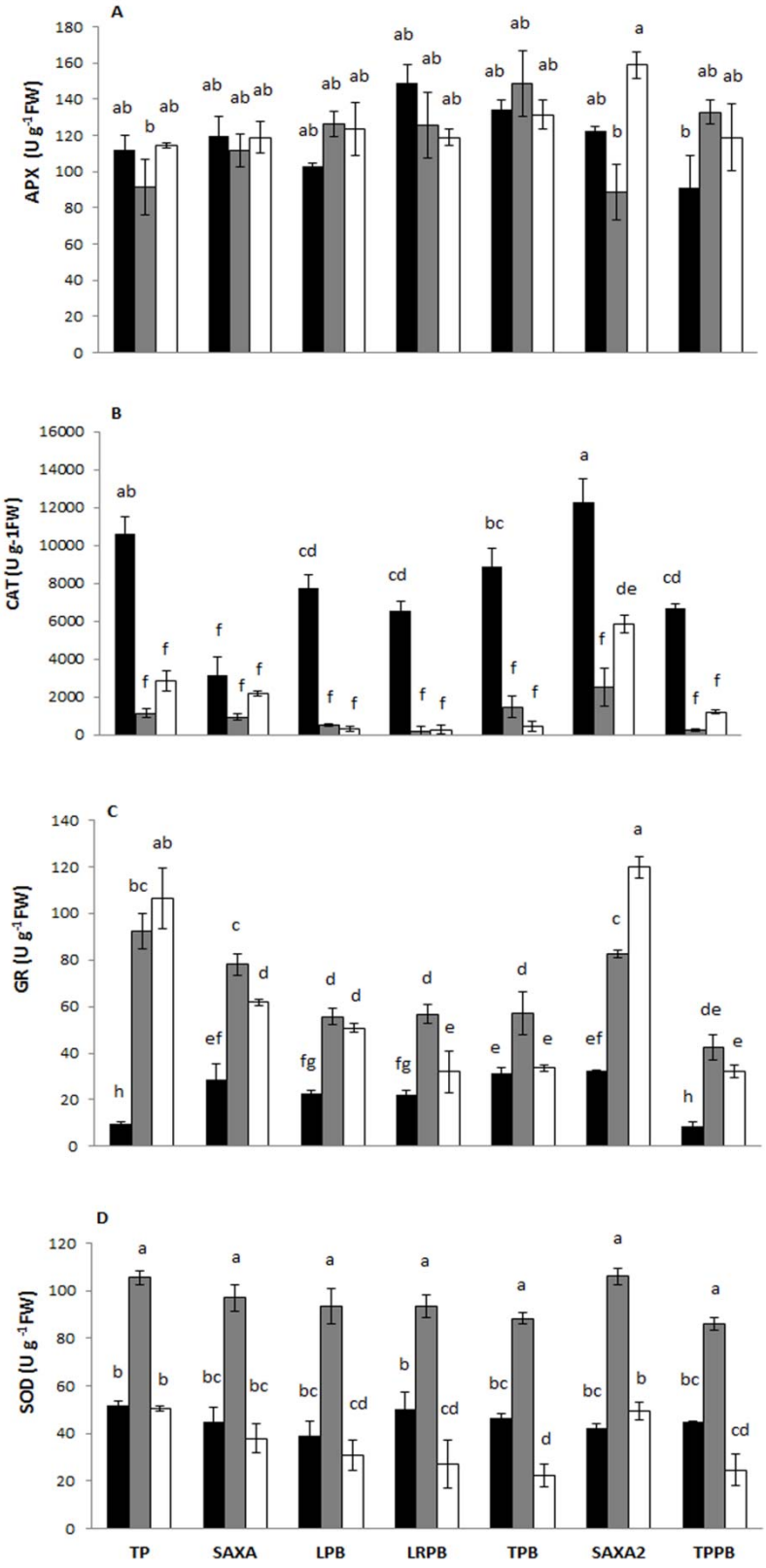

Figure 5. Effect of different salt concentration (0, black bar; 100, gray bar; and 200 mM, white bar NaCl) on APX, CAT, GR, and SOD activities of seven red radish root at 30 DAS. Values are the mean ± SE of three replications.

CHAPTER 4

TRANSFERRING THE CURRENT KNOWLEDGE OF MOLECULAR PROCESSING INVOLVED IN SALT TOLERANCE FROM MODEL PLANT OF *ARABIDOPSIS* TO THE CROPPED SPECIES OF *BRASSICA RAPA*

I. Physiological responses between two *Brassica rapa* cultivars under moderated salt stress and the transcript abundance for SOS pathway and some transcription factor genes

I. Physiological Responses Between Two *Brassica Rapa* Cultivars Under Moderated Salt Stress And The Transcript Abundance For SOS Pathway And Some Transcription Factor Genes

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ABSTRACT

The response to a moderate salt stress was analysed in two cultivars of *Brassica rapa: B. cimosa* and *B. rapa var di Milano guetto* under growth chamber condition, taking into consideration both physiological parameters and gene expression. The seeds were sown in multiwell polyethylene trays filled with peat moss. Twenty days old seedling plants were subjected to 0 and 100 mM NaCl and sampled at an early (6h) and a late phase (72 h). The physiological measurements included gas exchange parameters and ion accumulation analysis, while the expression analysis was focused on genes of the SOS pathway and a group of abiotic-stress responsive transcription factors. The results showed in *B. rapa cimosa* a down-regulation of the gene SOS1 at 72h, while SOS2 expression was increased in the early stage of the response (6h), decreasing then to the constitutive levels after 72h; SOS3 showed a >5-fold up-regulation after both 6h and 72h. However, the transcript levels of both non-induced and induced plants of *B. di milano* resulted extremely unstable. Similarly, the expression pattern of transcription factor genes in most cases showed a very unstable expression resembling, at least for some families, an on/off mechanism in both cultivars. However, *B. rapa cimosa* showed a relatively stable expression level for two transcription factors belonging to the bHLH and Homeobox families; both of them resulted up-regulated in response to salt stress, suggesting a role in triggering the cellular mechanisms involved in salt tolerance. The physiological and molecular data obtained are important for planning future experiments aimed to the analysis of gene expression in response to abiotic stresses.

1. INTRODUCTION

Salinity is a major abiotic stress affecting crop productivity and limits expansion of agriculture land. Salt stress involves cellular osmotic stress, ion toxicity and their consequences secondary stress (nutritional deficiency and oxidative stress (Zhu 2001). Toxic effects of Na^+ include inhibition of enzyme activity (Hasegawa et al., 2000) and disruption of K^+ nutrient acquisition (Zhu 2003). Thus, it is essential for plant survival to transport or compartmentalize Na^+ to maintain non-toxic levels of cytosolic Na^+ (Manabe et al., 2008). Three mechanisms function cooperatively to prevent the accumulation of Na in the cytoplasm: restriction of Na influx, active Na efflux, and compartmentalization of Na in the vacuole (Tester and Davenport, 2003). The salt-overly-sensitive (SOS) signal-transduction pathway is important for ion homeostasis and salt tolerance in plants (Hasegawa et al., 2000; Zhu, 2003). The SOS pathway in *Arabidopsis* is defined by three main protein components, *SOS1, SOS2*, and *SOS3*. Na^+/H^+ antiporters located in both the plasma and vacuolar membranes are ubiquitous membrane proteins that catalyze the exchange of Na^+ for H^+ across membranes; they play major roles in removing Na^+ from the cytosol or compartmentalizing it in vacuoles for maintenance of a low Na^+ concentration (Shi et al., 2002), energized by electrochemical H^+ gradients generated by H^+-pumps in the plasma membrane, i.e., H^+-ATPase, and the tonoplast, i.e., H^+-ATPase and H^+-PP*i*ase (Wang et al., 2007). Manipulating genes responsible for Na^+/H^+ antiporters to maintain ionic homeostasis in plants is an important strategy to deal with salt stress. SOS1encodes for a plasma membrane Na^+/H^+ antiporter, responsible for the exclusion of sodium in the apoplast. SOS2 gene encodes a serine/threonine type protein kinase, which activates SOS1(Liu et al., 2000). Salt stress elicits a transient increase of Ca^{2+} that is sensed by SOS3, a myristoylated calcium-binding protein, which physically interacts with and activates SOS2 (Halfter 2000). SOS2 and SOS3 define a regulatory pathway for Na^+ and K^+ homeostasis and salt tolerance in plants, and the SOS2/SOS3 kinase complex phosphorylates and activates the SOS1 protein (Qiu et al., 2002; Zhu 2003). It is thought that SOS1 is in *Arabidopsis* a plasma membrane Na^+/H^+ antiporter (Shi et al., 2000) and it is mediated Na^+ efflux at the root epidermis and long-distance transport from roots to shoots while protecting individual cells from Na^+ toxicity. Furthermore, a large percentage of genes that show altered expression following exposure to abiotic stress have been reported to be involved in stress tolerance, in the regulation of other gene expression, and in stress signal transduction (Shinozaki et al., 2003; Xiong et al., 2002). Therefore, the study of these genes is important for understanding

mechanisms involved in regulating the stress-response as well as to fully elucidate the mechanisms of tolerance against abiotic stress. It is though that various transcription factors are involving and functioning in the responsive mechanisms under abiotic stress in *Brassica raba* (Lee et al., 2008). They identify 56 genes encoding putative transcription factors responsive to abiotic stresses (salt, cold and drought) in *B. raba* and divided them into 15 transcription factor families on the basis of the classification of their *Arabidopsis* homologues. Some of these genes are most likely to be involved in stress-signaling pathway.

The genus *Brassica,* which is closely related to *Arabidopsis thaliana* (belong to the same taxonomy family) occupy third place among the various oilseed species due to its considerable economic and nutritional value. The cultivated *Brassica* species include both diploid and polyploid species. The high chromosome number of species *B. napus* (2n = 38, AACC), *B. juncea* (2n = 36, AABB), and *B. carinata* (2n =34, BBCC) are amphidiploids, while the low chromosome number species *B. nigra* (2n = 16, BB), *B. oleracea* (2n = 18, CC), and *B. rapa* (2n = 20, AA) are diploid (Morinaga 1934). Most of the *Brassica* species have been classified as moderated salt tolerant (Purty et al., 2008). However, the polyploid species can generally withstand adverse environmental factors better than their respective diploid ancestors (Stebbins 1966). It has been further suggested that the salt tolerance of amphidiploids has been acquired from the (*B. campestris)* and (*B. oleracea* L.) genomes (Ashraf et al., 2001). Indeed, the species *B. rapa* accounts for most of the oilseed production in Europe and North America. However, their growth, yield, and oil production are markedly reduced due to salinity. In particular, seed germination and early seedling growth have been reported to be relatively more sensitive towards salinity. There is significant inter and intraspecific variation for salt tolerance within *Brassica* which needs to be exploited through selection and breeding for enhancing salt tolerance (Ashraf and McNeilly 2004). The main objective of this study is to characterize the response to salt stress in *B. rapa* using both physiological parameters and an expression analysis for a pool of selected genes; this approach may lead to identify genes involved in salt tolerance. Given the importance of the SOS pathway in the response to abiotic stresses, the expression pattern of genes *SOS1*, *SOS2*, and *SOS3* will be analysed. Moreover, it is known that transcription factors, by activating or suppressing the expression of stress responsive genes, are direct mediators of the plant response to exogenous and endogenous stimuli; many transcription factors, belonging to several different families, have been reported to be up- or down-regulated in *Brassica* in response to cold, drought or salt stress (Lee et al., 2008), and are likely to be responsible for

triggering the cellular response to abiotic stresses. A selection of transcription factors from different families, whose expression was reported to be modulated in response to salt stress, were included in the pool of genes to be analysed to investigate their possible role.

2. MATERIAL AND METHOD

2.1. Plant material and salt stress treatment

This experiment was carried out in growth chamber at the University of Bologna, Italy. Seeds of two cultivars of *Brassica raba* ssp. *cimosa* and *var di milano gouetto viola* were germinated in small well polystyrene trays filled with peat moss substrate and grown for approximately three weeks in growth chamber at 24 °C (16 h day/8 h night, RH 65%, light intensity 290 µmol m^{-2} sec^{-1}). Two NaCl salt treatments (0 mM, 2.68 dS m^{-1} and 100mM, 7.68 dS m^{-1}) were initiated at 20- day old seedling plants under small cyclic hydroponic irrigation system. The timecourse of salt stress was lasted for 6h and 72h. At harvesting mentioned time, full expanded young uniform size leaves of three individual plants were collected and considered as one biological replicate.

2.2. Measurements of plant growth and photosynthesis rate

Shoot fresh weight (FW) and dry (DW) weights were determined for each salt treatment. Leaf transpiration (*E*), stomatal conductance (g_s) and net photosynthesis (*A*) were measured at 6h and 72h on three completely unfolded leaves of nine plants per treatment. The measurements of leaf gas exchange was performed using a CIRAS-2 (PPSystem, Hitchin, UK) infrared gas analyser (closed system) with a Parkinson's Automatic Universal Leaf Cuvette (PAR 1000 mmol m^{-2} s^{-1}, 26°C, CO_2 13.63 mmol l^{-1} and 300 cm^3 min^{-1} flow rate) equipped with 18-mm diameter, 2.5-cm^2 area cuvette inserts.

2.3. Measurements of mineral solutes concentration

Sodium, potassium, and calcium accumulation were determined based on dry weight. 500 g of leaves dry matter were suspended in 50 ml water and homogenized with a stirrer at 150 rpm for 20 minutes. Samples were then filtered using filter paper (589 Schleicher) and then the extracts were further filtered through cellulose acetate syringe filters (0.20 µm). Later, the filtrated extract was acidified with 65% nitric acid HNO_3 (1:100 ml, v: v) and quantification of cations was performed using Inductive Coupled Plasma Optical Emission Spectrometry (ICP-OES).

2.4. Gene expression studies

To identify salt responsive genes that could be involved in the plant tolerance mechanism, two main approaches have been used. Firstly, we investigated the expression levels of SOS pathway genes (*SOS1, SOS2,* and *SOS3*) in response to salinity stress in the two *Brassica raba* cultivars. The second approach was aimed to examine the expression level of some salt stress responsive transcription factor genes that have been already identified in *Brassica rapa*

ssp. *Pekinensis* and presumably involved in the regulation of salt resistance genes expression (Lee et al., 2008). Seven genes encoding putative transcription factors responsive to salt stress in B. *rapa* ssp. *Pekinensis* that belong to six different transcription factor families were used (Table 5). Two housekeeping genes for the expression analysis, Elongation Factor 1- α (EF1alpha) and Gliceraldehyde-3-Phosphate Dehydrogenase (GAPDH) were selected for their reported stability under abiotic stresses (Qi et al., 2010). Nucleotide sequences for all the genes were obtained from GenBank (Table 4 and 5) (http://www.ncbi.nlm.nih.gov/genbank/). The Basic Local Alignment Search Tool (Altschul 1997) was used to identify their homologues in the *Brassica rapa* Chiifu-401 v1.2 genome sequence (Wang et al., 2011) as implemented in Phytozome (http://www.phytozome.org). Primer pairs were designed using Primer3 (Untergasser et al., 2012) close to the 3' end of the coding sequence of each gene, for the amplification of fragments sized approximately 90 to 120 bp.

2.4. 1. RNA extraction and reverse transcription

For gene expression studies, 20 days old plants were subjected to two salt treatments (0 and 100 mM NaCl) and sampled after 6h and 72h from the treatment. Each sample was formed from the leaves of three different plants, and three biological replicates were harvested and analysed for each sample. Total RNA was extracted as described by Zamboni et al. (2008); RNA integrity was checked by loading 1µl of the extracted sample in a 0,8% agarose gel in SB buffer (Brody and Kern 2004). RNA was then treated with DNase I (Sigma) following manufacturer's instructions. One µg of total RNA was reverse transcribed using an oligo-dT primer and SuperScript II Reverse Transcriptase (Invitrogen); cDNA was diluted 1:10 in sterile distilled water and tested through PCR using gene-specific primers for two housekeeping genes (GAPDH and EF1alpha). The PCR tubes contained 1µl diluted cDNA, 1× PCR reaction buffer, 2 mM $MgCl_2$, 0.5 U Amplitaq Gold DNA Polymerase (Applied Biosystem), 0.2 mM dNTPs, and 0.5 µM each primer. PCR was carried out as follows: 1 cycle of 10 min at 94 °C; 30 cycles of 20 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C; and the final extension of 7 min at 72 °C. PCR products were visualized in a 1.2% agarose gel in SB buffer.

2.4.2. Real-time PCR

Gene expression levels were analyzed through Real-Time PCR using a StepOne Plus Real-Time PCR instrument (Applied Biosystems) using SYBR Select Master Mix (Applied Biosystems). Each reaction was performed in a total volume of 10µl, containing 5µl of SYBR Select Master Mix, 100 nM of each primer, 3µl of the 1:10 dilution of the cDNA and PCR-

grade water. The reactions were incubated at 50°C for 2 min and at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, with data collection at each annealing step. Two technical replicates were loaded for each sample; gene expression data were normalised to the EF1-alpha housekeeping gene and Relative Quantity (RQ) of each transcript was calculated using the delta-delta C_T model (Livak and Schnittgen 2001):

$$RQ = 2^{-\Delta\Delta C_T}$$

where ΔC_T is the difference between the threshold cycle of the tested gene and that of the housekeeping in the same sample, and $\Delta\Delta C_T$ is the difference between the ΔC_T of the tested sample and that of an arbitrarily chosen reference sample, for which RQ = 1 ($\Delta\Delta C_T = 0$) is assumed. The mean RQ was first calculated between the two technical replicates of each sample then the average value of three biological replicates was obtained.

2.5. Statistical analysis

For plant growth, photosynthesis measurements and ion analysis, three biological replicates of three individual plants per each replicate were considered for each salt treatment and timecourse of salt application. The data subject to analysis of variance (ANOVA) by Co-Stat-ANOVA software program. Treatment means were compared using Student-Newman-keuls at 5% significant.

3. RESULTS

3.1. Plant growth and photosynthesis rate

Two *Brassica* species showed that imposing 100 mM NaCl to the growth medium has no significant effect on shoot fresh and dry weight after 72h of salt application comparing with 0h (Figure 1); however the difference between two genotypes were significant as were the interaction between genotype and salt level (Figure 1 and Table 1). *Brassica*. *rapa cimosa* showed higher shoot FW either for non-treated or salt treated plants (Figure 1).

The transpiration rate (E) and stomatal conductance (gs) of salt treated plants of two *Brassica* species were not affected by applicable salt either at short time (6h) or elongation time (72h) of salt exposure and showed similar responses as the control plants (Figure 2). However, it is worthy to mention that both cultivars showed similar range of E and gs values in both control and salt stress conditions and the difference between two genotypes were not significant as well as all interaction between them: genotype ×salt, genotype ×time of salt exposure, and salt level×time exposure were not significant (Table 2). On other hand, two Brassica species exhibited significant difference in A value under control condition, where *B.cimosa* showed higher net assimilation rate as approximately 2-fold as *B. di milano* (Figure 2). However,

upon salinization, only *B.cimosa* trend to reduce A value by approximately two times as the control plants even at early time of salt establishing (6h), while *B. di milano* did not exert any efforts under salt and showed similar manner as non-treated plants (Figure 2).

3.2. Ion analysis accumulation

Two *Brassica* cultivars showed genotype and time-dependent differential Na accoumulation effects and the significant difference were recorded between two genotypes (Figure 3 and Table 3). At first 6h of salt exposure, *B.cimosa* showed slighter ability to accumulate more Na^+ ion in the leaf tissues either under control or saline conditions. However, under elongation salt period (72h), *B.cimosa* showed higher essential Na ion accumulation than *B. di Milano* in both treated and non-treated plants (Figure 3). Intrestingly, these results showed that the salt stress did not affect Na accumulation as almost unique pattern of Na accumulation were recorded in two Brassica cultivars under control and salt stressed conditions. Moreover, potassim analysis showed neither Brassica cultivars, salt treatment, nor lasted period of salt application have any effect on K accumulation and the K concentration avaraged 57 mg g-1 DW in leaf tissues of two cultivars under control or saline condation (Figure 3 and Table 3). Similar to Na accumulation, Ca concentration showed differences in term of genotipic variation, where *B. di milano* accumulated more Ca ion particularly at 6h of salt exposure (Figure 3 and Table 3).

3.3. Gene expression studies

3.3. 1. Integrity of total RNA extraction and amplification of housekeeping gene

Isolation of intact RNA is essential for many techniques used in gene expression analysis, particularly when cDNA has to be synthesized. RT-PCR assays involve the analysis of small regions of RNA (generally less than 200bp), preferably placed close to the 3' extremity of the gene, which is reverse-transcribed more efficiently when using oligo (dT) primers annealing on the polyadenilated 3' tail of mRNA. Therefore, it is convenient to check RNA integrity before starting a gene expression analysis. Figure 4 showed an aliquot of the RNA sample that has been run on a denaturing agarose gel stained with ethidium bromide. In general, the most samples showed clearly sharp intensity two bands of the 28S and 18S rRNA band; when the correct profile was not visible, suggesting a loss or degradation of RNA in the sample, RNA extraction was repeated.

Moreover, housekeeping genes are typically constitutive genes that are required for the maintenance of basic cellular function, and are expressed in all cells of an organism under

both normal and patho-physiological conditions (Butte et al., 2001). The relative quantification of mRNA levels by RT-PCR is currently an extensively used technique, in which reliable quantification depends on the use of one or more stably expressed endogenous genes, usually housekeeping genes, as internal controls. However, because even the expression of housekeeping genes can be modulated in some tissues depending on the physiological, developmental or health status, it is necessary to first study the stability of several endogenous gene expressions in order to select suitable internal references (Huggett 2005). In this study, we examined the stability of two potential reference genes: Elongation Factor 1- α (EF1alpha) and Gliceraldehyde-3-Phosphate Dehydrogenase (GAPDH), whose expression was reported to be the most stable under abiotic stresses (Qi et al. 2010). The PCR showed a better efficiency in the amplification of EF1alpha compared to GAPDH (Figure 5); furthermore, a Real-time PCR comparison of the two housekeepers confirmed a higher stability for the EF1alpha transcript among the different samples. Thus, all our gene expression analysis was normalized based on EF1alpha housekeeping as a reference gene.

3.3.2. Real-Time PCR analysis of SOS genes expression

In this experiment, the expression level of SOS pathway genes from *Brassica rapa* were under investigation .Constitutive (un induced) and salinity-induced transcript levels were determined by Real-time PCR in two *Brassica* cultivars: *Brassica rapa cimosa* and *Brassica rapa var di milano gouetto viola*. The expression levels of *SOS1, SOS2*, and *SOS3* were assayed in 3-week-old seedling plants after 6 and 72 hours of (0 and 100) mM NaCl salt exposure (Figure 6). In the case of SOS1, a down-regulation (approx. one fourth compared to not treated plants) was detected in *B. rapa cimosa* at 72h from salt exposure, while no significant deviation from constitutive expression levels was found at 6h (Figure 6). On the contrary, *B. di milano* showed an up-regulation of *SOS1* transcripts at both 6h and 72 h in relation to control plants (Figure 6), but a difference in transcript levels was also found between the negative controls (untreated plants at 6h and 72h). The expression patterns of *SOS2* of *B. rapa cimosa* showed an early up-regulation by 3- fold in respect to the control plants at 6h of salt exposure, while the transcript level returned at constitutive levels after 72h (Figure 6). In *B. di Milano* exhibited a similar expression in treated and control plants at both 6h and 72h, but the transcript levels resulted significantly higher at 72h in both treated and non-treated plants (Figure 6). Finally, the expression patterns for *SOS3* in *B. rapa cimosa* cultivar exhibited a strong up-regulation in response to salt stress (5 and 9 folds comparing to control plants at 6h and 72h, respectively) (Figure 6). On the other hand, *B. di Milano*

revealed a down regulation by 5 times comparing to the control plants only at short salt stress time (6h).

3.3.3. Real-Time PCR analysis of transcription factors expression

The expression levels of the 7 transcription factors analysed are reported in (Figure 7). TF01 (belonging to the bHLH family of transcription factors) in *B. rapa cimosa* resulted to be up regulated in response to salt stress and showed a significant increasing in transcript abundance by 6 and 7 times as control plants respectively at 6h and 72h of salt exposure (Figure 7). On the contrary, *B. di Milano* showed lower expression levels of TF01 in treated samples compared to non-treated, but a high variation was also observed between the control plants at 6h and 72h (Figure 7). In the case of TF02 (Cupine superfamily), TF03, TF04 (C2C2-CO-like superfamily) and TF06 (MYB family), a dramatic variability in expression levels was observed between the different samples, including in the comparison of control (non-treated) plants of the same cultivar (Figure 7). TF05 (belonging to the Homeobox family) showed a strong up-regulation in the early response to salt stress (6 times as the control plant at 6h), that later decreased to almost 2 times at 72h (Figure 7). On the contrary, *Brassica di Milan* plant showed similar transcript levels in treated and control plants at both salt periods. Finally, for the transcript factor TF07 (NAC family) the only significant difference between treated and non-treated plants was found at 6h in *B. rapa cimosa*, which showed an early up-regulation in the salt-exposed sample (5-folds as the control plants) However, despite an overall more stable expression level of TF07 compared to the other transcription factors analysed, even for this gene very strong differences were observed between untreated control plants of the same cultivar..

4. DISCUSSION

4.1. Plant growth and physiological responses

It is well documented that crop salinity sensitivity varies among species and genotypes (Prado et al., 2000; Pujari and Chanda 2002), and the genetic diversity is considered a useful tool for screening of genes that involved in salt tolerance (Maggio et al., 2005). Munns. (2002) pointed out that the adaptive responses toward salt stress varied depending on plant development, salt concentration and time of exposure. Houle et al. (2001) mentioned that seedling stage as well as the reproductive stage represents the two most sensitive stages in the life cycle of plants. In our investigation, the result of shoot fresh weight of two *Brassica* species showed 100 mM NaCl did not affect the seedling growth at 72h of salt initiation (Figure 1). This result might indicate that the salt dose was not enough to elicit the reduction in plant growth. However, *B. cimosa* was able to maintain higher significant shoot FW under

control and even after 72h of salt exposure, which might suggest that the *B. cimosa* is more resistant to salt stress than *Brassica. di Milan* and present an interesting research genetic material.

It is well known that leaf gas exchange parameters were significantly impaired upon salt stress. This reduction is generally associated with salt damage of the photosynthetic tissue, changes in stomatal features with the consequent restriction of the CO_2 availability for carboxylation or to the acceleration of senescence (Orsini et al., 2010a). However, our result showed the two Brassica cultivars displayed no significant change in E and gs values upon salinization regardless the time of salt exposure (Figure 2). Our results were inconsistent with Ashraf. (2001) who stated that salt stress causes decreases in transpiration rate with increasing salinity with *Brassica* species. Also, other author reported that the reduction in transpiration with salinity was related to the reduced *gs* and the lower stomatal density of leaves developed under saline conditions (Omami et al., 2006). One explanation of our results could be 100 mM NaCl or 72h of salt exposure were not enough to drive those plants to initiate closure of stomatal. Controversially scenario has been recorded in term of net assimilation rate, where *B. cimosa* showed about 2 times higher A value under control condition than *B. di Milan*; in addition, *B. cimosa* showed significant reduction in A values by (-45% and -102%) at 6h and 72h of salt exposure (Figure 2). It is worthy to mentioned that upon salinization *B. cimosa* had similar value of stomatal condactance and evaporation rate as *B. di Milan,* but the former showed lower significant value of A regarding the control plant while the latter plant did not show any change in A value even at extended period of salt stress. Ashraf. (2001) linked the increasing of salt-tolerant *Brassica* species to having higher assimilation rates and lower stomatal conductance. Thus, we might suggest that *B. cimosa* is more salt resistant than *B. di Milan* as it has the ability to up-down regulate this feature depending on surrounding condition.

Mineral nutrients are essential for plant growth and they are virtually involved in all metabolic and cellular function like energy metabolism, primary and secondary metabolism, cell protection, gene regulation, signal transduction and plant reproduction (Hansch and Mendel 2009). Osmotic adjustment helps the plant cells to withstand salt stress and water deficit by maintaining sufficient turgor for growth. It involves the transport, accumulation, and compartmentation of inorganic ions and organic solutes (Mustard and Renault 2004). This processing allows increasing the water potential gradient between the soil and plant and

improving the water absorption under soil water deficit (De Herralde 2000). In this study, *B.cimosa* showed higher capacity than *B. di Milano* to accumulate more Na ion at 72h of salt exposure (Figure 3). However, this increasing was recorded under both control and salt conditions. Thus, this result most likely pointed out that the differences in Na ion accumulation was belong to genetic variability rather than salt effect or it could indicate that the salt dose of 100 mM NaCl was not sufficient to induce any specific mineral accumulation in both Brassica cultivars. However, we might link the higher Na accumulation in *B.cimosa* with higher shoot fresh weight growth (Figure 1) as the plants use high concentrations of inorganic solutes from the substrate which is considered as an osmotically adaptive strategy to cope with salt stress since the energetic cost of inorganic solute accumulation is less expensive process than the use of organic solutes (Yeo 1983). Potassium accumulation showed no differences were presented between the two Brassica cultivars regardless the salt concentration of time exposure of salt stress (Figure 3). Similar to Na concentration, Ca level showed the difference in this ion accumulation most likely was owing to genetic variability and not because of salt side effect (Figure 3 and Table 3).

4.2. Differential expression of SOS genes after salt exposure

Salt tolerant plant can be defined as the ability of the plants to complete their growth cycle with an acceptable growth and yield (Colmer and Flowers 2008). However, salt tolerance is a complex phenomenon involving a numerous mechanisms that operate at cellular, tissue, organ or whole plant levels (Yeo 1998). Some traits may only be functional at one time in a particular species and the effect of one mechanism may mutually exclude the effect of the others at a of certain stage development (Yeo 1998; Carvajal et al., 1999). The importance of the SOS pathway in plant salt tolerance has been fairly established (Zhu 2002, 2003; Shi et al., 2003). *SOS1, SOS2, and SOS3* genes are the candidate genes in studies related to scoring the genetic variability that occurs naturally in *Arabidopsis* genotype (Quesada et al., 2002). SOS1 encodes for a plasma membrane Na^+/H^+ antiporter, responsible for the exclusion of sodium in the apoplast. SOS2 gene encodes a serine/threonine type protein kinase, which activates SOS1 (Liu et al., 2000). The SOS3 gene encodes an EF-hand type calcium binding protein (Liu 1998; Mahajan et al., 2008). In our study, the expression levels of the three SOS genes were determined in the two cultivars *B. rapa cimosa* and *B. rapa var di Milano guetto* (Figure 6). In the case of *B. rapa cimosa* all the three SOS genes showed a relatively stable constitutive expression, resulting in similar transcript levels after 6h and 72h in non-treated samples. In response to salt explosion, SOS1 expression did not vary significantly after 6h but strongly decreased after 72h, suggesting a down-regulation in the late response to salt

stress. SOS2 expression on the other hand seemed to be enhanced (~3 fold) in the early response (6h), decreasing then to constitutive levels after 72h. Finally, SOS3 showed the greatest variation, being strongly (>5 fold) up-regulated after both 6h and 72h. SOS2 is known as a major salt-tolerance locus in *Arabidopsis thaliana* and its mutation drastically reduces plant tolerance to high Na^+ stress (Liu et al., 2000). The up-regulation observed in the early stage of salt stress in *B. rapa cimosa* is consistent with Qiu et al. (2002) who reported *SOS2* expression to be induced in both root and shoot of *Arabidopsis thaliana* within 3-6h of salt stress. Moreover, our results showed that the expression patterns of calcium sensor component (*SOS3*) is more abundant in the leaf tissue of *B. rapa cimosa* after both 6h and 72h of salt stress (Figure 6). On the other hand, the transcript levels in non-induced *B. di milano* leaves resulted extremely unstable, suggesting that in this case a higher environmental variation affected SOS genes expression. For this reason, even though in salt-treated samples *of B. di Milano* an up-regulation of SOS1 was recorded at 72h of salt exposure and an early (6h) down-regulation of SOS3 emerged. In spite of the attempt to reach the maximum uniformity of growth conditions, still some growth and greenness variation among different individuals plants exposed to the same treatment was observed. Accordingly, the variation in transcription abundance cannot be unambiguously attributed to the effect of salt stress, given that an uncontrolled environmental effect proved to act on the same samples to a comparable extent. Several transcription factors have been involved in the responsive mechanism under abiotic stresses in *B. rapa* (Lee et al., 2008). It is believed that some of them are responsible for the activation or up-regulation of the transcription of stress-inducible genes. In our study, seven abiotic stress-responsive genes belonging to six different transcription factor families have been chosen (Table 5). Of the seven genes chosen, according to Lee et al. (2008), two transcription factors containing bHLH and MYB domains were reported to be down regulated in response to salt in both *Brassica* and *Arabidopsis* species. On the contrary, four chosen genes belonging to the families C2C2-CO-like, Homeobox, and NAC were up-regulated after salt stress; finally, a Cupin family transcription factor was considered to be up regulated in *Brassica* and down-up regulator factor in *Arabidopsis* under salt stress. Our analysis was aimed to test whether the same regulation could take place in the two *B. rapa* cultivars in response to salt stress. However, most transcription factors showed a very unstable expression, with extremely different patterns even between non-treated samples (Figure 7). This was not surprising, considering that a significant environmental effect was also observed in the expression pattern of SOS genes; given that transcription factors responsive to stresses must have a very fast regulation in response to different stimuli to provide the cell the

mechanisms to survive, it is plausible that the same variation that acted on the SOS genes transcription affected to a much higher extent the expression of stress-responsive transcription factors. This modulation resembled, at least for some of them, an on/off mechanism, as for the Cupin family gene TF02, the C2C2-CO-like TF03 and TF04, and the MYB TF06 (Figure 7). Given the extreme sensitivity of their regulation mechanism, a reliable analysis of the expression of these genes will require a very fine adjustment of the experimental conditions. Nevertheless, it was possible to acquire better information for some transcription factors whose expression resulted more stable in control samples; it is the case of the bHLH-containing TF01 and the Homeobox gene TF05, that at least in *B. rapa cimosa* showed a relatively constant expression level in untreated samples. Of them, the expression of the bHLH transcription factor was inconsistent with Lee et al. (2008), as it showed a significant up regulation in response to salt stress (6-fold and 7-fold change in *B. cimosa* at 6h and 72h respectively) (Figure 7). Similarly, the expression of the Homeobox gene (TF05) was induced upon salinization and showed a 6-fold up-regulation after 6h, which decreased to approximately 2-fold after 72h (Figure 7). In this case our data confirms that reported by Lee et al. (2008), which indicated a >5 fold up-regulation of the gene in response to salt stress in both *Brassica* and *Arabidopsis*. Lamentably, the expression of these two genes could not be reliably compared between the two *B. rapa* cultivars, since as observed for SOS genes the *B. rapa var di milano* samples were affected by a higher uncontrolled environmental effect (Figure 7).

5. CONCLUSION

As a general remark emerging from the results of our gene expression analysis, it should be pointed out that the expression of stress-responsive genes, especially when considering transcription factors, is very finely regulated and it varies much more quickly than the physiological parameters, in response to even small stresses or changes in the plant conditions. Our experiment was planned trying to reduce as much as possible the environmental variation: the plants were grown in identical polystyrene trays, with the same peat moss substrate and placed in the same growth chamber; nevertheless, the gene expression data indicated that a significant variability still persisted. Identifying the source of this variation is important for planning future experiments aimed to the analysis of gene expression. A quote of this variation could be attributed to small, uncontrolled disuniformities in the substrate, light exposure or availability of water and nutrients in the different positions of the wells in the tray. However, some variability can also be bound to the initial status of

the seeds: not all of them performed the same way in terms of speed of germination and growth, especially for the cultivar *B. rapa di Milano*.

Another consideration that can be made is that many of the analysed genes showed a relatively low level of expression, resulting in some cases in high threshold cycles (Ct > 30). As Ct increase they get close to the detection limit of the instrument, and the accuracy of the quantification decreases. To have a more robust quantification of these transcripts, the quantity of template cDNA in each well should be increased; this could be achieved by increasing the initial amount of RNA in the reverse transcription step and/or reducing the cDNA dilution for the preparation of the Real-Time template. Moreover, most reliable results are obtained when gene expression is normalized to an housekeeping that is expressed at comparable levels, while in our case EF1alpha showed in many cases a higher expression than the tested genes (Ct < 25). Many housekeeping genes are available for Real-Time experiments in Brassica (Qi et al. 2010); our choice was based on the best stability reported under abiotic stress conditions, but additional tests can be made and the most appropriate housekeeping gene can be chosen based also on its constitutive expression level.

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Table 1. Analysis of variance of shoot FW: ns, non-significant differences; *, significant differences at $P \leq 0.05$; **, significant differences at $P \leq 0.01$; ***, significant differences at $P \leq 0.001$.

Analysis of variance	Shoot FW	Shoot DW
Main Effect		
V	***	***
S	ns	*
T	ns	***
Interaction		
V×S	*	*
V×T	ns	ns
S×T	ns	*
V×S×T	*	*

Table 2. Analysis of variance of gas exchange parameters: ns, non-significant differences; *, significant differences at $P \leq 0.05$; **, significant differences at $P \leq 0.01$; ***, significant differences at $P \leq 0.001$.

Analysis of variance	E	ge	A
Main Effect			
V	ns	ns	***
S	*	ns	***
T	ns	ns	ns
Interaction			
V×S	ns	ns	*
V×T	ns	ns	ns
S×T	ns	ns	ns
V×S×T	**	*	***

Table 3. Analysis of variance of ions accumulations: ns, non-significant differences; *, significant differences at $P \leq 0.05$; **, significant differences at $P \leq 0.01$; ***, significant differences at $P \leq 0.001$.

Analysis of variance	Ca^{+2}	K^{+}	Na^{+}
Main Effect			
V	***	ns	***
S	ns	ns	ns
T	***	ns	***
Interaction			
V×S	ns	ns	ns
V×T	*	**	*
S×T	ns	ns	***
V×S×T	ns	**	ns

Table 4. List of housekeeping gene, salt overly sensitive genes, and primers used in RT-PCR analysis.

Name	Symbol	GeneBank Accession	Primers sequences (5′-3′)
Elongation Factor 1- α	(EF1alpha)	HM565967	FW: TTGAGGCTGGTATCTCGAAGAAC RV: GCTCGGTGGAGTCCATCTTG
Gliceraldehyde-3-Phosphate Dehydrogenase	(GAPDH)	HM565966	FW: CGCCAAGAAGGTGATCATTTC RV: CAGGAGGCGTTCGAGATGAC
Salt-overly-sensitive	SOS1	AT2G01980	FW: GGAAGGAAAGTGCATTGGTG RV: GAGCTTTCAGCGGAAACAAG
	SOS2	AT5G35410	FW: CCATCGCTTTTCATGGTAGA RV: ATAGCTCTGGCTTTGGCATT
	SOS3	AT5G24270	FW: AGATGGTAATCGCGCTTCTT RV: TCAATTTTCCCGTCCTTCTT

Table 5. List of *B. rapa* genes encoding putative transcription factor genes and Real-time PCR primer sequences.

Gene family[a]	SeqD[b]	abbreviation	AGI no.[c]	Transcript name[d]	Nucleotide sequences of primers (5′-3′)
Basic Helix-Loop-Helix (bHLH)	BRAS0001S00019389	TF01	AT2G18300	Bra024474	FW:GATGTGCCATTGTTTCCGCT RV:GAGTTTCCCAGCTTGCCGAT
Cupin super family	BRAS0001S00019512	TF02	AT3G20810	Bra035742	FW:CAAAGGTGGTGGAGCTAGAGT RV:TGCTCCACCAGAAGCTAACC
C2C2-CO-like	BRAS0001S00008429	TF03	AT5G48250	Bra020709	FW:TCCTCACATGCAACTACACGT RV:CTTCACACGCCTCCTCACAT
C2C2-CO-like	BRAS0001S00019462	TF04	AT3G07650	Bra040020	FW:GCGGTTATGCGTTACAAGGA RV:CCCCTTTACACGCCTTCTCA
Homeobox	BRAS0001S00018707	TF05	AT2G46680	Bra004516	FW:TGAACATTGTGGAGCCAGCT RV:CAATTGCTGCTGGATTGGTC
MYB	BRAS0001S00008974	TF06	AT2G46830	Bra004503	FW:GGAAGGACAGGTTTCAAGCCT RV:GTGTTTCCAACCGAATCCGC
NAC	BRAS0001S00010332	TF07	AT4G27410	Bra026353	FW:AGGTTACCACGCGTTCCAAT RV:AGCTGTACCCGAGACTCTGA

Source: Lee et al (2008).

[a]Name of transcription factor family based on classification of *Arabidopsis* homologous gene in TAIR (http://www.arabidopsis.org/)

[b]Unique designation provided by NimbleGen for each probe sequence on *B. rapa* KBGP-24K chip

[c]Arabidopsis homologous gene (

[d]Transcript name from the *B. rapa* Chiifu-401 v1.2 genome sequence

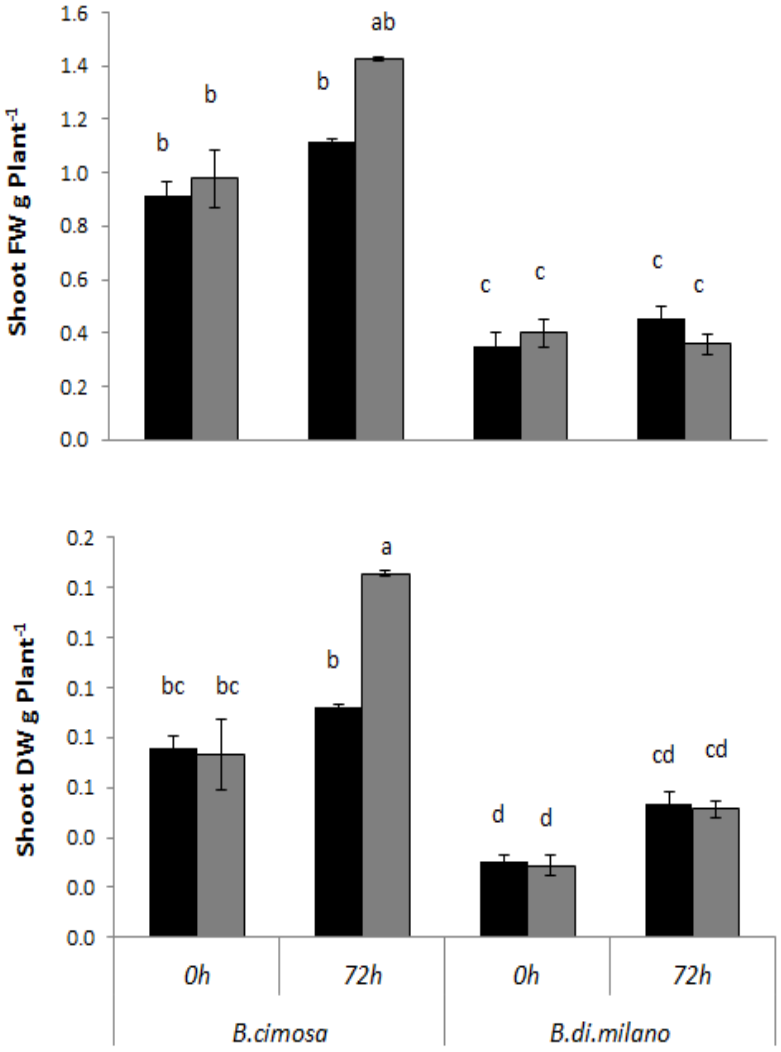

Figure 1. Effect of zero salt (black bar) and 100 mM NaCl (gray bar) on shoot FW of two *Brassica* cultivars at 0h and 72h of salt exposure. Values are the mean ± SE of different replications.

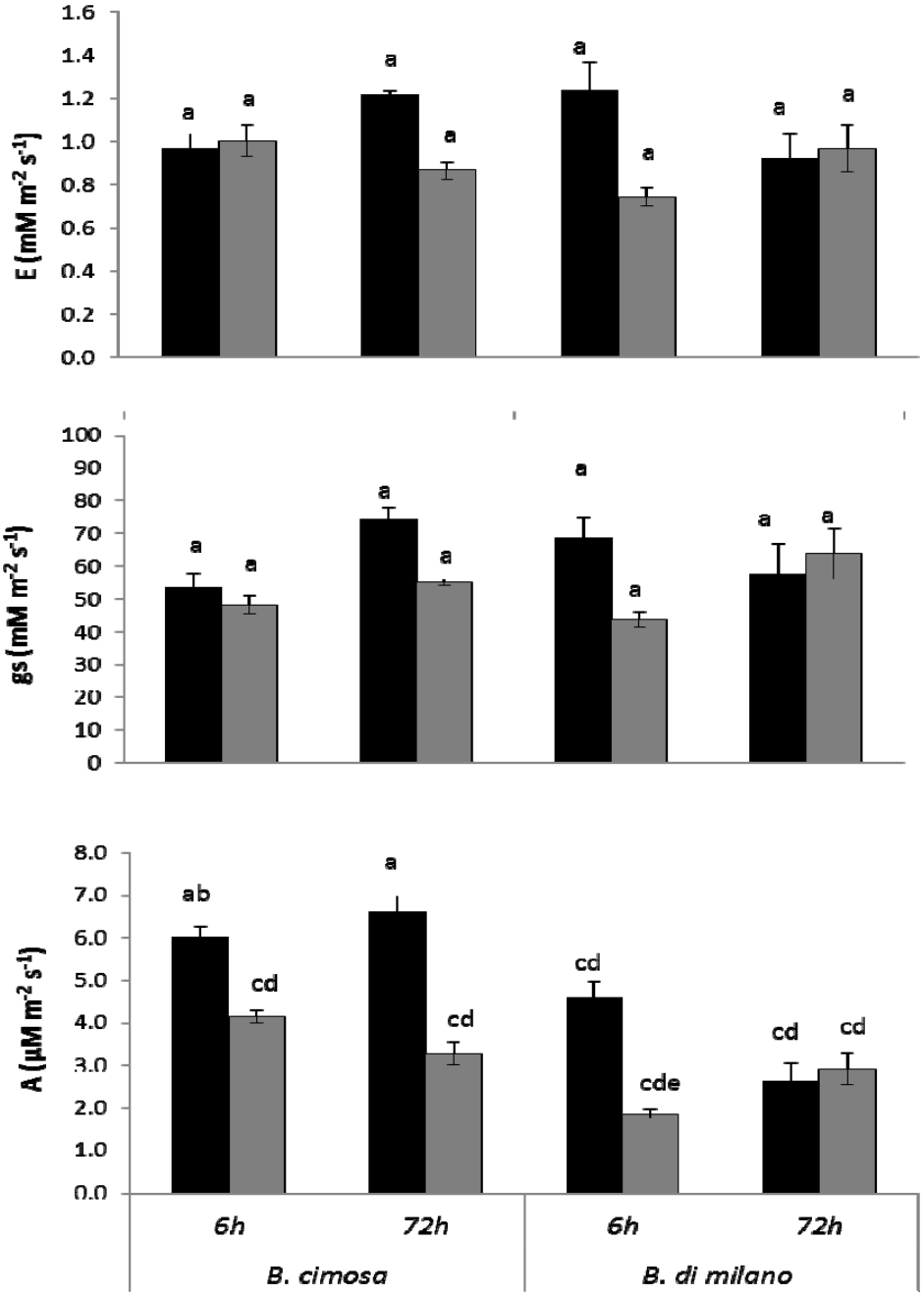

Figure 2. Effect of zero salt (black bar) and 100 mM NaCl (gray bar) on gas exchange parameters of two *Brassica* cultivars at 0h and 72h of salt exposure. Values are the mean ± SE of different replications.

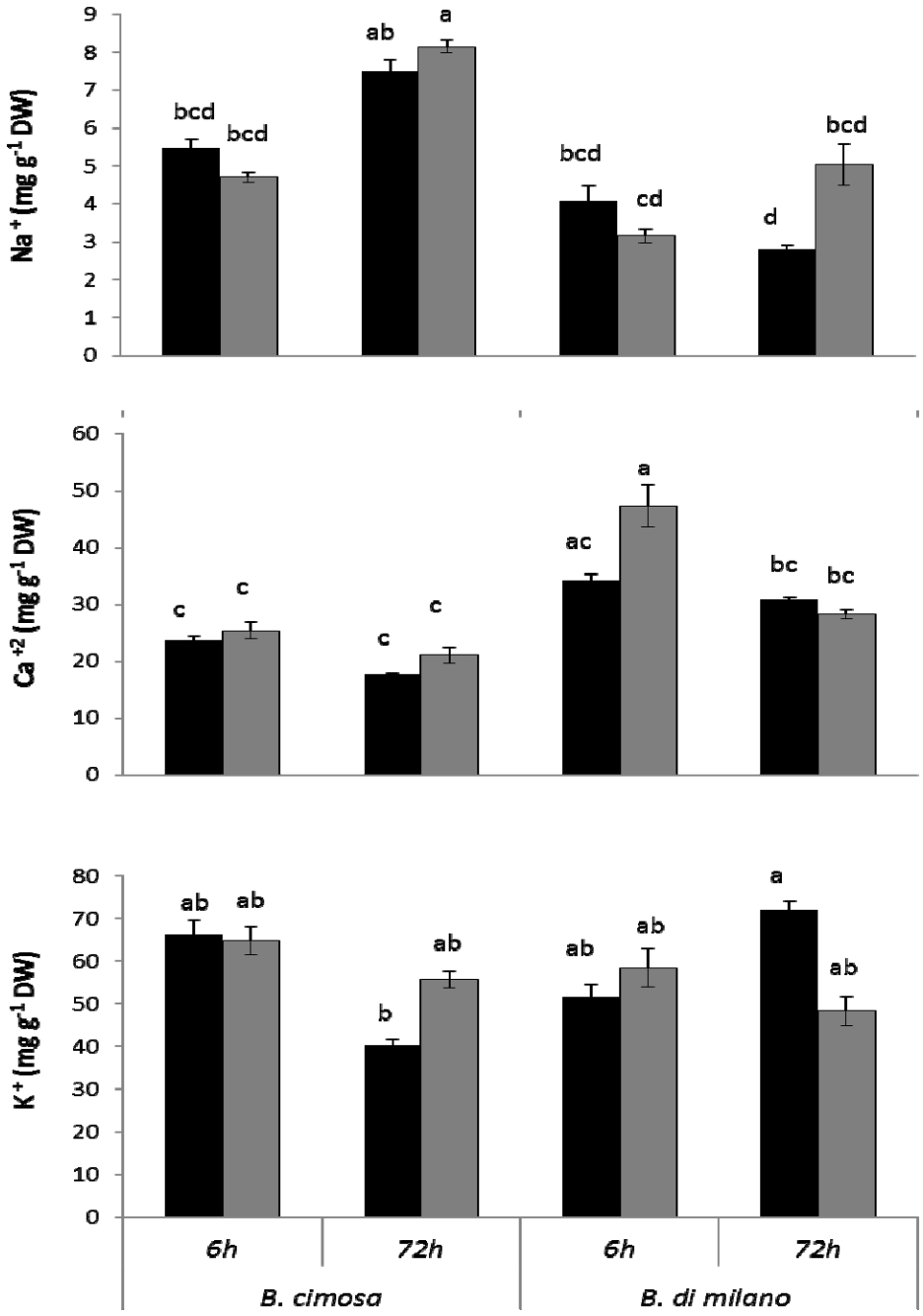

Figure 3. Effect of zero salt (black bar) and 100 mM NaCl (gray bar) on ion accumulation of two *Brassica* cultivars at 0h and 72h of salt exposure. Values are the mean ± SE of different replications.

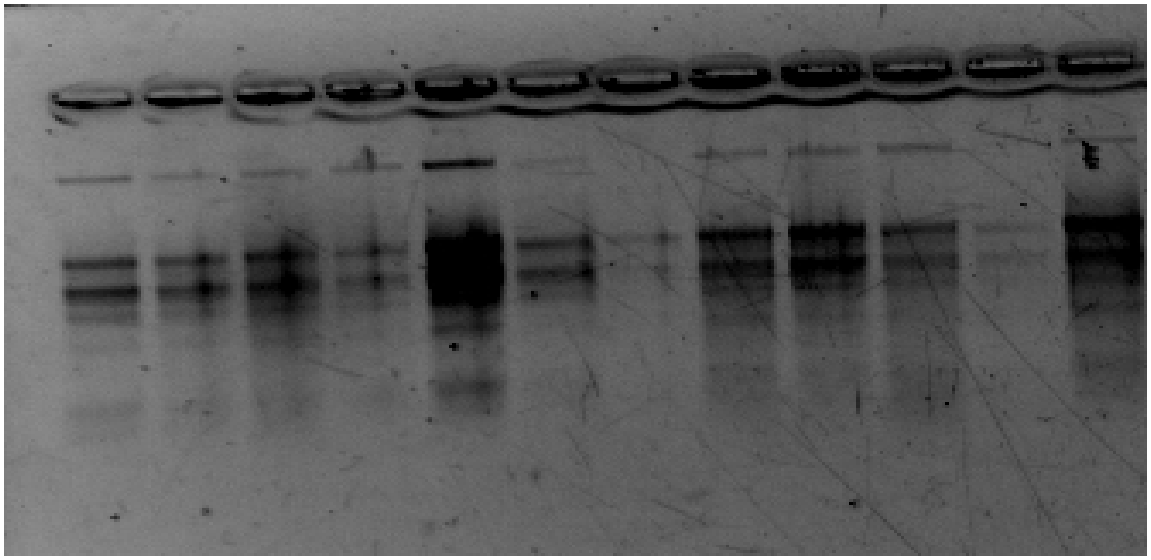

Figure 4. Gel running of total RNA of some samples of *B. rapa cimosa* (the first 6 wells) and *B. di Milano* (the latter 6 wells) leaf of seedling 3-week old under 100 mM NaCl salt stress; the presence of two main bands for ribosomal RNA indicates a good integrity of the extracted RNA. Extraction was repeated for samples showing excessive RNA degradation or insufficient yield.

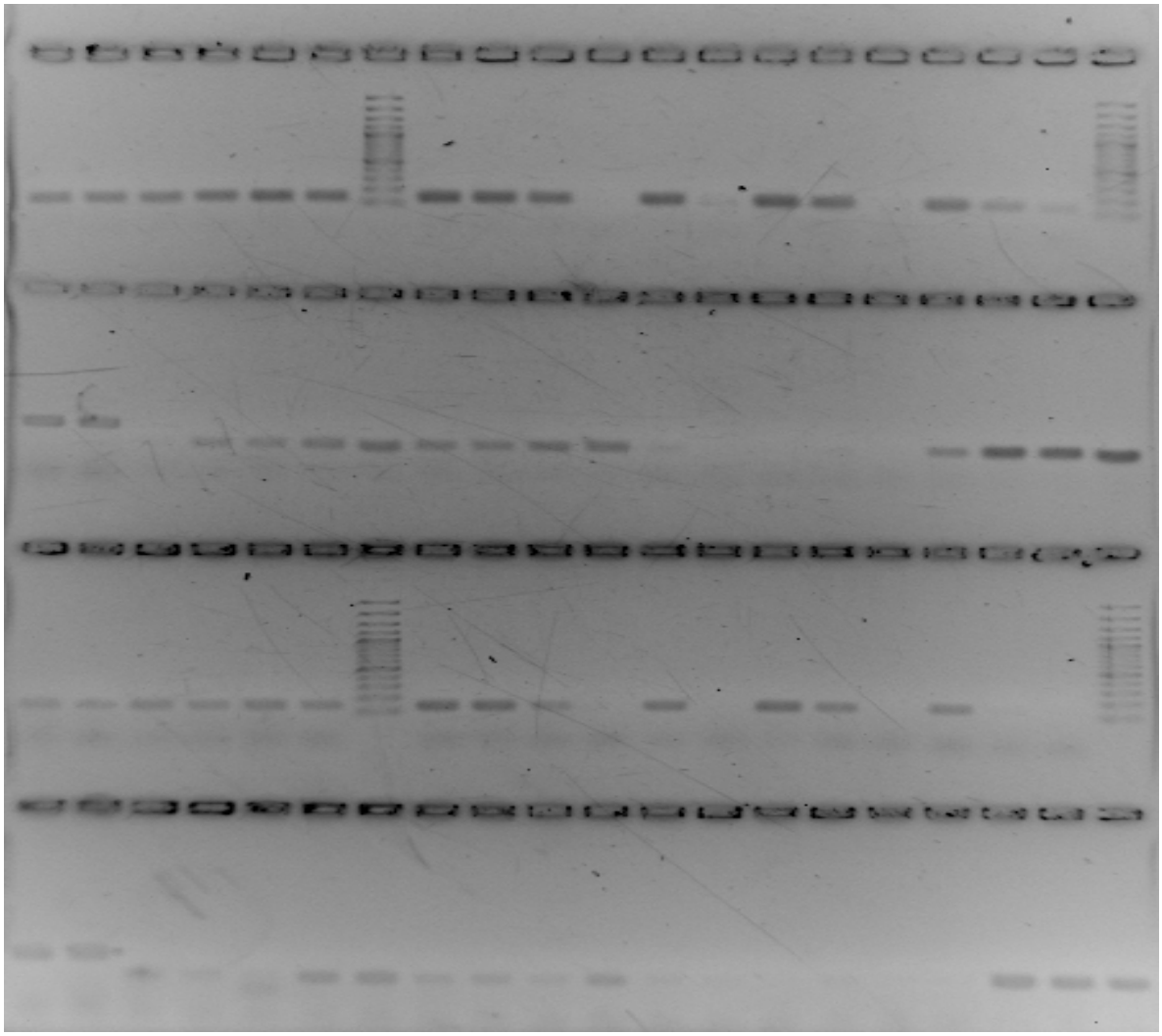

Figure 5. Amplification of two housekeeping genes from cDNA obtained from leaf tissue of B. rapa. PCR products of EF1alpha are reported on the left and GAPDH on the right.

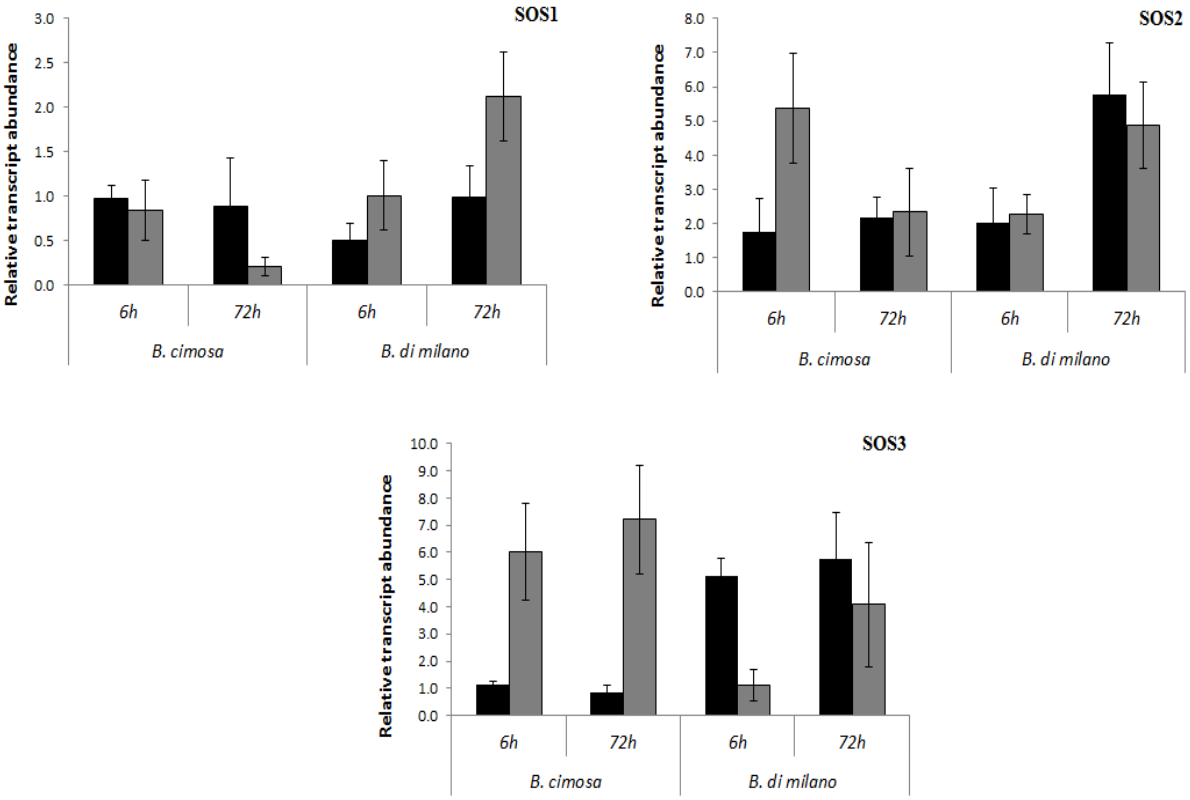

Figure 6. Relative quantity of transcripts for SOS pathway genes in two *Brassica* cultivars at 6h and 72h after salt exposure (black bars: 0 mM; gray bars: 100 mM NaCl). Values are the mean ± SE of 2 technical × 3 biological replicates.

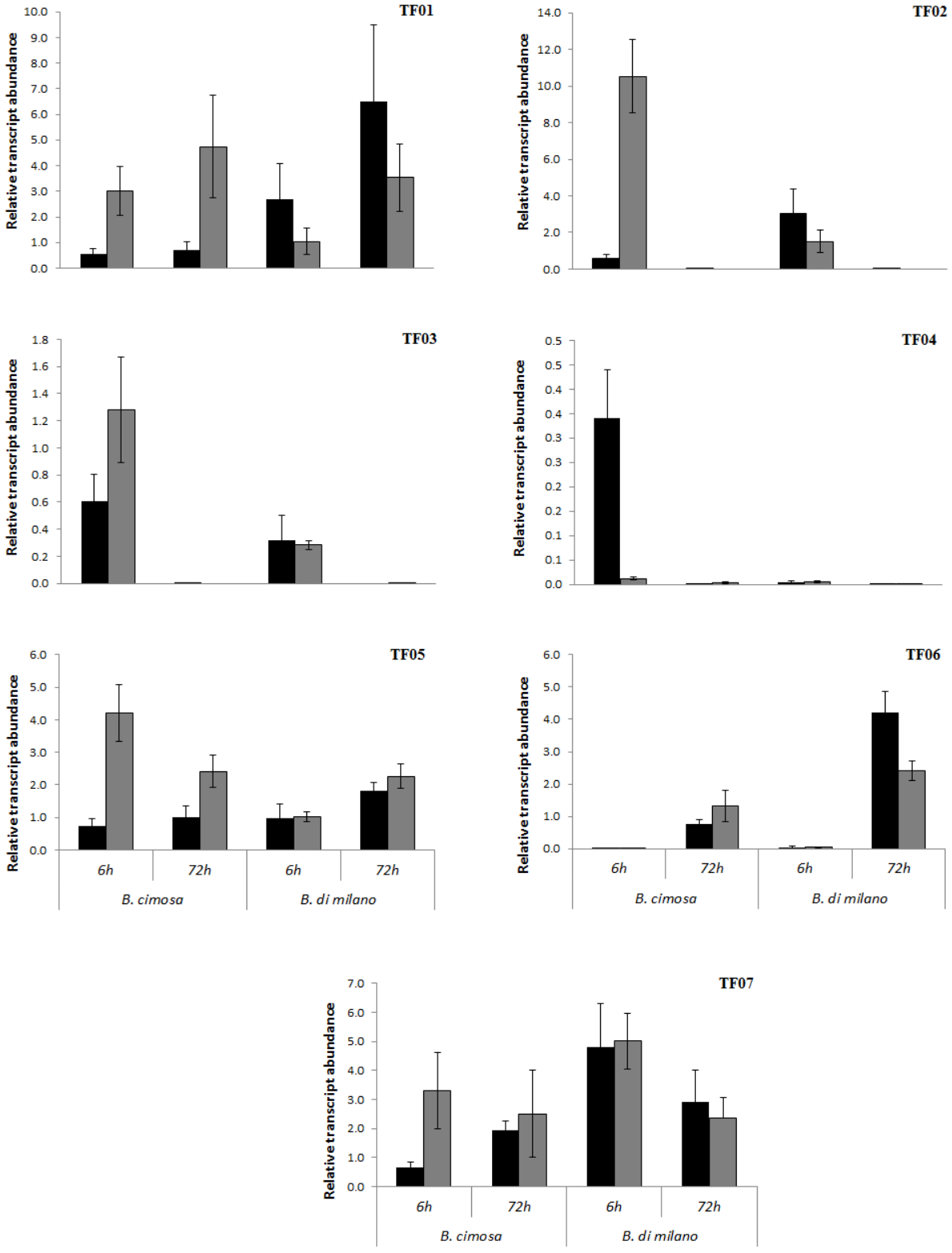

Figure 7. Relative transcript abundance of the seven transcription factors analysed in two *Brassica* cultivars after 6h and 72h of salt exposure (black bars: 0 mM; gray bars: 100 mM NaCl). Values are the mean ± SE of 2 technical × 3 biological replicates.

CONCLUSIONS AND FUTURE PROSPECTS

Soil salinity is one of the most serious constraints to crop productivity, especially in the arid and semiarid regions of the world. High concentrations of soluble salts in the soil environment cause nutrient imbalance, water deficit, and toxicity of salt ions in growing plants. Therefore, plants growing in saline soils have to encounter two types of stresses, osmotic stress and ion toxicity. Salt tolerant plants adapt specific structural and physiological modifications to cope with high salinities. Morpho–anatomical adaptations include the prevention of undue water loss from the plant by the development of thick epidermis and sclerenchyma, well developed bulliform cells for extensive leaf rolling, and increased density of trichomes, and this is vital in water limiting environment under high salinities. Increased moisture retaining capacity is the other adaptive feature which is critical under physiological drought due to salinity stress. Development of excretory structures like vesicular hairs and salt glands is a major structural adaptation and very crucial for salt tolerance. Physiological adaptations include restricted toxic ion uptake at root level. At cell level, succulence is crucial for dumping off toxic ions in relatively inert areas like vacuoles. Toxic ions like Na^+ and Cl^- are important for osmotic adjustment in highly salt tolerant species. Lastly, the most important point is that ion exclusion which is one of the most vital phenomena for high salt tolerance in plants.

However, high salt concentrations normally impair the cellular electron transport and lead to the overproduction of the ROS-$O_2^{\bullet-}$, $^{\bullet}OH$, H_2O_2, and $^1O_2$. Salinity stress results in an excessive generation of these ROS. Stomatal closure upon salt stress may limit the entry of CO_2, which, in turn, may cause the over reduction of photosynthetic electron transport system. ROS play two divergent roles in plants: in low concentrations, they act as signaling molecules for the activation of defense responses under stresses, whereas in high concentrations, they cause exacerbating damage to cellular components. If prolonged over a certain extent, abiotic stresses, through overproduction of ROS, would result in oxidative damage to lipids, proteins, and nucleic acids, in turn causing severe damage to cell viability. The enhanced production of ROS is, however, kept under tight control by versatile and cooperative ROS-scavenging antioxidant mechanisms that modulate intracellular ROS concentration. These mechanisms can be conveniently divided in two groups, nonenzymic antioxidants such as GSH, AsA, tocopherols, carotenoids, etc., and the enzymic antioxidants like CAT, POX, SOD, as well as enzymes of AsA–GSH cycle such as APX, MDHAR,

DHAR, and GR. Antioxidant responses of plants not only depend on the species-inherent strategy but also on the tissue, duration, and severity of the stress period. Molecular and cellular knowledge associated with abiotic stress induced various damages, and metabolic alterations are necessary to improve abiotic stress tolerance in plants. Naturally, abiotic stress-tolerant plants provide helpful tools for such research. Enhancements in the expression of components of antioxidant defense system involved in ROS-scavenging show significant improvements in metabolic status of plants, and this strategy has been used to develop crop plants with enhanced stress tolerance. However, attempts to increase abiotic stress tolerance by overexpressing one of the components of antioxidant defense system have not always been successful because it may not change the capacity of the pathway as a whole. Further, it can disturb the balanced interaction among the components. Various abiotic stressful conditions of the environment severely limit agricultural productivity throughout the world. Therefore, future work employing biotechnological approaches is essential to produce crop plants with in-built capacity of enhanced levels of multiple abiotic stress tolerance by constitutively expressing high levels of antioxidants, for cultivation in stress-prone environments.

The mechanism of high salinity tolerance is just beginning to be understood. The overall progress of research on salinity stress responsive genes and their products reflects their central role in plant growth and development under stress conditions. Much effort is still required to uncover in detail each product of genes induced by salinity stress and their interacting partners to understand the complexity of the high salinity stress signal transduction pathways. Determination of the upstream receptors or sensors that monitor the stimuli, as well as the downstream effectors that regulate the responses, is essential, which will also expedite our understanding of salinity stress signaling mechanisms in plants.

ANNEXES

Annex 1: Covered pages of scientific papers

V. Improved stomatal regulation and ion partitioning boosts salt tolerance in grafted melon. *Functional Plant Biology*. http://dx.doi.org/10.1071/FP12350.

VI. Ionic partitioning and stomatal regulation Dissecting functional elements of the genotypic basis of salt stress adaptation in grafted melon. *Plant Signaling & Behavior*. http://dx.doi.org/10.4161/psb.27334

VII. Salinity thresholds and genotypic variability of Cabbage (*Brassica oleracea* L.) grown under saline stress: physiological adaptation and nutritional value. *Journal of the Sciences of Food and Agriculture*. (submitted).

VIII. Horti lumen: turning on the light in interior farming. Congress proceedings, *Acta Horticulturae* – ISHS (in press).

Annex 2: Published abstract

III. Irrigazione di brassicacee con acque saline: aspetti produttivi, fisiologici e biochimici. (Q30_ 13). In: Acta Italus Hortus. Riassunti dei lavori presentati alle X giornate Scientifiche SOI. Pubblicazione della Societá di Ortoflorofrutticoltura Italiana. Padova (Italy), 25-27 June 2013, Bonghi. C and Sambo. P, p.108.

IV. Salinity response in melon scions as affected by grafting. In: International Symposium on Vegetable Grafting Book of Abstract. Viterbo (Italy), 3-5 October 2011, VITERBO: G. Colla, p. 89.

Annex 3: Poster

III. Stomatal regulation in grafted melon improves salinity tolerance. International Symposium on Vegetable Grafting. University of Tuscia, Viterbo, Italy. 3th -5th October 2011.

IV. Turning on the light in interior farming. International People-Plant Symposium, IPPS 2012-ISHS. Venlo, Netherland. 6th-8th September 2012.

Annex 3: Conference

IV. Workshop "Plant water relations"Faculty of Agricultural, Naples Federico II, in Portici (Naples), Italy. 17th-19th September, 2012.

V. Workshop "Seconda Giornata Ciras"Department of Food Science and Environment, University of Firenze, Italy. 18th -19th April, 2013.

VI. Conference on "X Giornate Scientifiche SOI" Campus di Agripolis-Padova, Italy. 25th-27th June, 2013.

Annex 1

CSIRO PUBLISHING
Functional Plant Biology
http://dx.doi.org/10.1071/FP12350

Improved stomatal regulation and ion partitioning boosts salt tolerance in grafted melon

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Abstract. Grafted plants are often more tolerant to salinity than nongrafted controls. In order to distinguish differential response components in grafted melon (*Cucumis melo* L.), salt stress was imposed on several rootstock–scion combinations in four experiments. The rootstock used was an interspecific squash (*Cucurbita maxima* Duch. × *Cucurbita moschata* Duch.), RS841, combined with two cantaloupe (*C. melo* var. *cantalupensis*) cultivars, namely London and Brennus, against both self-grafted and nongrafted controls. Physiological, morphological and biochemical adaptations to 0, 40 and 80 mM NaCl were monitored. Upon salinity, plant biomass and leaf area were improved by grafting *per se*, since self-grafted plants performed similarly to the heterografted ones. However, improvements in the exclusion of Na^+ and the uptake of K^+ were due only to the rootstock genotype, since ionic composition was similar in self-grafted and nongrafted plants. These results indicate that the favourable effects of grafting on plant growth cannot be ascribed to a more efficient exclusion of Na^+ or enhanced nutrient uptake. On the other hand, growth improvements in both self- and heterografted plants were associated with a more efficient control of stomatal functions (changes in stomatal index and water relations), which may indicate that the grafting incision may alter hormonal signalling between roots and shoots.

Additional keywords: cantaloupe, interspecific rootstock, nutritional imbalance, salt stress, water relations.

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Introduction

Soil salinisation is limiting the future of agriculture in many areas of the world and the improvement of salt tolerance in crops is becoming an imperative of agricultural research. Scientists have therefore approached different aspects of salinity related to both soil and plant issues (Munns 2002). For the latter, much effort over the last decades has been dedicated to understanding the fundamental biology of plant stress adaptation with the ultimate aim of identifying key stress tolerance functions (Orsini *et al.* 2010*a*).

One of the main elements that, so far, has not earned enough interest is understanding the role played by the root system in conferring tolerance. Under salt stress, toxic, osmotic and nutritional factors deplete plant growth. The adoption of tolerant rootstocks in vegetable grafting has been suggested to improve plant performances under stress. Grafting results in more efficient water and nutrient use, increased yield, extended harvest periods and improved fruit quality (Romero *et al.* 1997; Estan *et al.* 2005). Rootstocks can also influence tolerance to extreme temperature and moisture (drought, flooding) and salt stress (Colla *et al.* 2010). In cucurbits, grafting experiences addressed the induction of resistance against disease infestation (Cohen *et al.* 2002), low root-zone temperature (Bulder *et al.* 1990), soil alkalinity (Edelstein *et al.* 2011) and the enhancement of both water and nutrient uptake (Huang *et al.* 2010). Indications on the induction of tolerance to salinity by grafting have been provided in the last decade by several authors (Estan *et al.* 2005; Oztekin *et al.* 2007; Edelstein *et al.* 2011), although this research was often limited to the evaluation of salt-tolerant genotypes, rather than the identification of the main elements reducing the effects of salinity on the shoot. Morphological adaptations to salinity in grafted cucurbits have been widely described, mainly resulting in more vigorous root systems (Romero *et al.* 1997; Zhu *et al.* 2008) and greater root : shoot ratios (Colla *et al.* 2006*a*;

Annex 1 - continued

SHORT COMMUNICATION

Plant Signaling & Behavior 8, e27334; November; © 2013 Landes Bioscience

Ionic partitioning and stomatal regulation

Dissecting functional elements of the genotypic basis of salt stress adaptation in grafted melon

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Keywords: vegetable grafting, *Cucumis melo* L., salt stress, NaCl, ion partitioning, stomata

Vegetable grafting is commonly claimed to improve crop's tolerance to biotic and abiotic stresses, including salinity. Although the use of inter-specific graftings is relatively common, whether the improved salt tolerance should be attributed to the genotypic background rather than the grafting per se is a matter of discussion among scientists. It is clear that most of published research has to date overlooked the issue, with the mutual presence of self-grafted and non-grafted controls resulting to be quite rare within experimental evidences. It was recently demonstrated that the genotype of the rootstock and grafting per se are responsible respectively for the differential ion accumulation and partitioning as well as to the stomatal adaptation to the stress. The present paper contributes to the ongoing discussion with further data on the differences associated to salinity response in a range of grafted melon combinations.

Salinity results in plant downregulation of physiological functions, accumulation of ions in vegetative tissues up to toxic concentrations and, more generally, to the overall depletion of the crop performances.[1] Several researches have confirmed the beneficial role played by the adoption of grafting in counteracting the detrimental effect of salinity.[2-3,4] To date, grafting is a common practice in melon (*Cucumis melo* L.) cultivation, mainly through the adoption of interspecific rootstocks, which are generally selected genotypes of squash (*Cucurbita maxima* Duch x *Cucurbita Moschata* Duch.). Although these rootstocks have proven to improve crop's performances in presence of biotic[5,6] and abiotic[4] stresses, whether the beneficial effects should be attributed to the rootstock genotype rather than grafting per se is still a controversial matter among scientists. In a recent publication,[7] the comprehension of the role of grafting in improving the response to salinity in melon was addressed. The manuscript reported on the effects associated to cultivar, grafting, and salinity over 4 experiments in a range of different environmental conditions. As only grafting and salinity presented significant interactions, results from all experiments were jointly discussed, thus offering innovative elements for the comprehension of the effect of grafting on the plant physiological response to the stress. In the present manuscript, additional results obtained from one of the experiments conducted at Bologna University, Italy (experiment 1#)[7] are discussed, with the aim of further elucidating how the differential stomatal and ionic response observed in self-grafted vs. interspecific grafting may lead to similar performances upon salt stress (0, 40, and 80 mM NaCl, starting from 10 Days After Transplanting, DAT, and lasting 30 d). Similarly to other experiments presented in the manuscript, 2 melon cultivars (namely Brennus, ZKI, Hungary, and London, Nunhems, The Netherlands) were used, altogether with a squash rootstock (Rs841, Monsanto, USA). Every genotype was used either non-grafted, self-grafted, or grafted on the interspecific squash rootstock. However, the peculiarity of this experiment was that also non-grafted and self-grafted Rs841 plants were included in the trial. The ANOVA analysis highlighted that the melon scion genotype did not actually affect the plant's response to salinity, with significant differences observed only between plants either non-grafted, self-grafted, and interspecific graftings. Fresh biomass production was depleted as a consequence to salinity in all grafting combinations, although to a greater extent[7] in non-grafted plants undergoing 80 mM NaCl. Upon salinization, an increase in Na^+:K^+ ratio in roots was observed in all grafting combinations under study (**Fig. 1**), confirming that, in both grafted and non-grafted plants, an increase of Na^+ was to be experienced independently from the genotype of the root system.[4] An explanation of the highest Na^+:K^+ values observed in all salinized root tissue of Rs841 (either non-grafted, self-grafted, or grafted with a melon scion) may be found in the consistent lower Na^+ loading in the epigeous organs: filtration of Na^+ at the grafting union level was observed (**Fig. 1**), although to a limited extent, whereas major differences were observed in stems and leaves. In these organs, although negligible changes could be detected upon salinity in any of the plants with a Rs841 root system, a general increase of the Na^+:K^+ ratio was recorded in all self- and non-grafted melons.

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Annex 2

International Symposium on Vegetable Grafting 3 – 5 Oct. 2011, Viterbo – Italy

[P48] Salinity response in melon scions as affected by grafting

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Grafted plants are often more tolerant to stresses such as drought and salinity than non-grafted controls. The wide literature available usually attributes the increased tolerance to different elements, such as ion exclusion or accumulation within the rootstock, impaired stress signaling, improved water uptake and exchanges, or even the increased vigor attributable to the grafting adaptation itself. Indeed, as far as salt tolerance goes, a rootstock effect, a scion effect and a rootstock x scion effect may be generally observed. In order to dissect the differential elements involved in the salt response in grafted melon, salt stress was imposed on several rootstock/scion combinations in three experiments under different environmental conditions and locations across Europe (Izmir, Turkey; Budapest, Hungary; Bologna, Italy). In all experiments the rootstock used was an interspecific squash hybrid (*Cucurbita maxima* x *Cucurbita moschata*), combined with different Cantaloupe (*Cucumis melo* var. *cantalupensis*) cultivars. Self-grafted and non-grafted controls were included in the trials in order to evidence grafting-related effects. Plants exposed to salinity (0, 40, and 80 mM NaCl), were monitored in terms of physiological (leaf gas exchanges, porometry and overall transpiration), biochemical (ion accumulation and osmolytes), and morphological (root and leaf features) adaptations.

Annex 3

Stomatal regulation in grafted melon improves salinity tolerance

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MAIN OBJECTIVE

Grafted plants are often more tolerant to stresses such as drought and salinity than non-grafted controls. As far as salt tolerance goes, a rootstock effect, a scion effect and a rootstock x scion effect may be generally observed. In order to dissect the differential elements involved in the salt response in grafted melon, salt stress was imposed on several rootstock/scion combinations in three experiments under different environmental conditions and locations across Europe (Bologna, Italy, BOL; Budapest, Hungary, COR; Izmir, Turkey, EGE).

MATERIALS AND METHODS

Four independent experiments (BOL exp 1# and 2#; COR exp 3#; EGE exp 4#) were conducted in order to compare the effect of grafting on the response of melon (*Cucumis melo*) cultivars to salinity. In all experiments, the adopted scions were cv. *Brennus* and *London*, the rootstock was a commercial *Cucurbita maxima* x *Cucurbita moschata* hybrid. Self-grafted and non-grafted controls were used in all combinations. Plants were exposed to salinity (0, 40, and 80 mM NaCl).

Measurements:

Morphological adaptations:
- Plant growth (fresh and dry weight, leaf area, root:shoot ratio)
- Stomatal size, density and index

Physiological adaptations:
- Leaf gas exchanges
- Plant water relations (potentials, and water loss)
- Electrolyte leakage and cell membrane stability

Fig. 1.

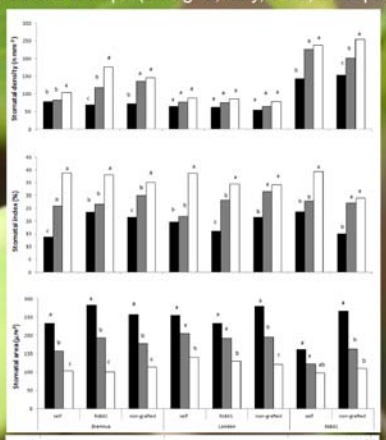


Fig. 2.

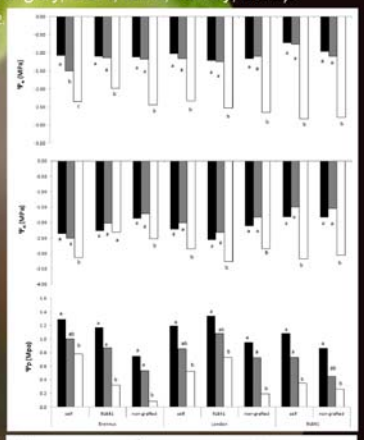


Fig 3.

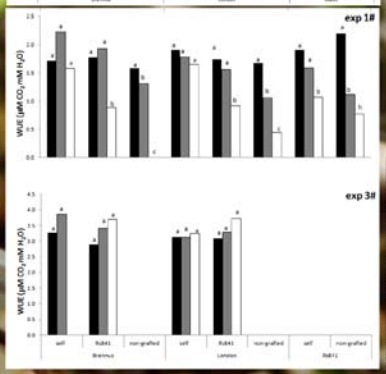
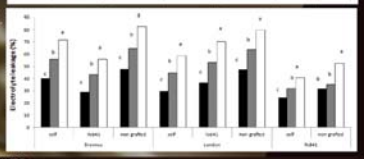


Fig. 4.



RESULTS

Significant differences in the salinity response of grafting combinations of muskmelon were observed:

- In general, plant growth was reduced more in non-grafted plants than in grafted plants, while self-grafted melons performed equally (and sometimes better) than inter-specific grafting (data not shown).
- Stomatal conductance (g_s) was reduced uniformly by salinity in all treatments in exp. 1#, whereas in exp. 3 was reduced only in self grafted plants. Consistently a reduction in leaf transpiration (E) was observed in all salt treated plants. The photosynthetic activity was reduced by salinity in all treatments. Overall plant water loss was reduced in self-grafted Brennus already at 40 mM NaCl, whereas 80 mM NaCl were needed to detect reduction in plants grafted on Rs841 or non-grafted. On the other hand, the greater effect of salinity on water loss in London was experienced by non-grafted plant (data not shown).
- Salt stress increased stomatal density and index in Brennus plant non-grafted or grafted on Rs841 yet under moderate salinity, while such increase was observed in self-grafted Brennus only under 80 mM NaCl. London was not affected by salinity in any grafting combination. Rs841 increased stomatal density and index both in self-grafted and non grafted plants. Stomatal area was reduced in all treatments with the rate of salt added to the solution (Fig. 1).
- Self-grafted Brennus were the only treatment reducing leaf water potential (Ψ_w) yet under 40 mM NaCl. At highest salinity all treatments reduced Ψ_w. The osmotic potential (Ψ_s) was decresed in all treatments (excluding Brennust x Rs841) only upon 80 mM NaCl. Salt reduced quite uniformly pressure potential in all treatments (Fig. 2.)
- Water use efficiency (WUE) derived from gas exchange (A/E) was reduced under salt stress in non-grafted plants, whereas it was maintained in all grafted plants (Fig 3.).
- Electrolyte leakage increased in all plant leaves as a consequence of salinity. Values were indeed generally lower in the Rs841 self- and non-grafted treatments.

CONCLUSIONS

Salinity seriously affected morphological and physiological performance in all treatments, although injuries were generally higher in non-grafted plants. Overall, improved performances under salinity were not strictly associated to the adoption of inter-specific Rs841 rootstock, but indeed satisfactory results were achieved in self-grafted plants. Further investigation should address the comprehension of how the differential stomatal regulation and its consequences on the plant water relations may have affected ion accumulation among plant organs.

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Annex 3 - continued

HORTI lumen

Turning on the light in interior farming.

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INTRODUCTION

Home gardening in cities is expanding as a way for reducing costs and environmental impact of the urban food supply. In metropolitan spaces, plant life is mainly constrained by land availability and access to light. Light Emitting Diodes (LED) represent a valid alternative to cool white neon and led systems for intensive vegetable cultivations.

Their technology is based on the selection of frequency light ranges detectable by plants and the removal of surplus light. Due to their flexible vertical and horizontal structure they can be used in domestic spaces such as kitchens, cabinets, basements or in work spaces, offices, etc. (LED) is an alternative source of light for crop production and may represent a valid alternative to cool white neon, since it increases the energy use efficiency and maintains the advantages of low heat emission.

FIGURE 1. LETTUCE PLANTS GROWN UNDER LED LIGHT

MATERIALS AND METHODS

Seeds of lettuce plants cv. Regina di Maggio were germinated for 10 days under controlled greenhouse conditions. 10-day seedlings were transplanted into new pots and grown in growth chamber with a drip irrigation system under 25-22° C day-night, 60% RH, and 16-h light/8-h dark photoperiod.

The growth chamber was subdivided in two parts, separated by a white screen. One section was illuminated with a led light Horti Lumen (Studio Antonioni, Bologna, Italy) whereas in the other, the light was provided by two cool-fluorescent neon lights (Philips Master TL.D 90 De luxe 950, 60W each for a total power of 120 W and light intensity over the canopy of about 100-110 µmol m^{-2} s^{-1}, Amsterdam, The Netherlands).

Two led lights were used, namely HL1 (equipped with 16 LEDs, having a power requirement of 24 W and light intensity on the canopy of 150-170 µmol m^{-2} s^{-1}), and HL2 (equipped with 32 LEDs, with power requirement of 45 W, and light intensity of 400-450 µmol m^{-2} s^{-1}).

At 20 days after transplanting (DAT), different physiological and colorimetric determinations were performed on leaves such as: leaf transpiration (E), stomatal conductance (g_s) net photosynthesis (A), total leaf water potentials (Y_t) and leaf greenness. At harvest (30 DAT), total leaf area, roots and shoots were weighed

All data set were analysed by analysis of variance (ANOVA), performed by using Co-Stat software. Treatment means were compared using Least Significant Difference (LSD) tests at the level 5% of significance.

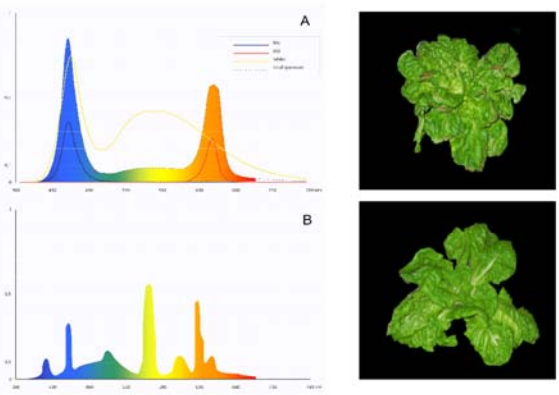

FIGURE 2. SPECTRUM AND LETTUCE PLANTS GROWN ON HORTI LUMEN (A) AND THE CONTROL NEON LIGHT (B)

RESULTS AND DISCUSSION

- *Plants grown under HL1 presented slow growth rate, and the yield was extremely little as compared to control plants. Differently, plants grown under HL2 presented a more compact and moderately rolled up leaf margins shape -as consequence of the absent UV component in the light radiation- compared to control plants grown under neon light.*
- *HL2 shoots were bigger in size (+76%, Fig. 3A), and presented a more developed root system (+88%, Fig. 3B) thus resulting in an overall increase in plant size (+77%, Fig. 3C) as compared to control plants.*
- *Greater leaf transpiration (+50%) associated with higher stomatal conductance (+104%) and doubled photosynthetic rate (+114%) was observed in plants grown under HL2.*
- *Concurrently, HL2 plants presented much higher (+50%) leaf greenness as compared to control plants. Leaf greenness is an indirect measure of leaf chlorophyll content which is directly correlated with plant access to light. Being chlorophyll concentration a critical determinant in defining plant photosynthetic activity, it may be advanced that the higher light available enhanced leaf chlorophyll and therein photosynthetic rates, which caused an increased transpiration flux and therein an acceleration of plant growth, leading to higher yield and a more rapid cycle.*

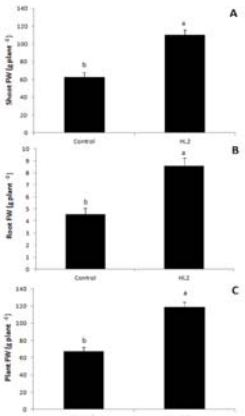

FIGURE 3. MORPHOLOGICAL FEATURES OF LETTUCE PLANTS GROWN UNDER NEON (CONTROL) OR LED (HL2) LIGHTS

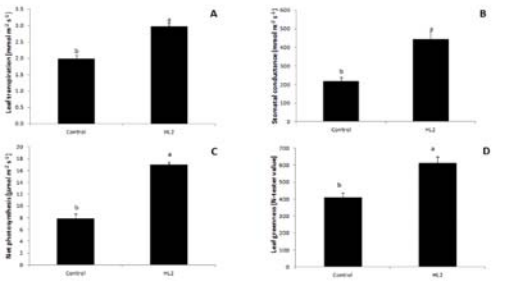

FIGURE 4. PHYSIOLOGICAL FEATURES OF LETTUCE PLANTS GROWN UNDER NEON (CONTROL) OR LED (HL2) LIGHTS

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